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Position-effect variegation in *Drosophila* depends on the dose of the gene encoding the E2F transcriptional activator and cell cycle regulator

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SUMMARY

A dominant mutation due to the insertion of a P-element at 93E on the third chromosome of *Drosophila melanogaster* enhances position-effect variegation. The corresponding gene was cloned by transposon tagging and the sequence of the transcript revealed that it corresponds to the gene encoding the transcriptional activator and cell cycle regulator dE2F. The transposon-tagged allele is homozygous viable, and the insertion of the transposon in an intron correlates with a strong reduction in the amount of transcript. A homozygous lethal null allele was found to behave as a strong enhancer when heterozygous. Overexpression of the gene in transgenic flies has the opposite effect of suppressing variegation. A link is established here, and discussed, between the dose of a transcriptional activator, which controls the cell cycle, and epigenetic silencing of chromosomal domains in *Drosophila*.

Key words: *Drosophila*, position effect variegation, dE2F transcription factor, cell cycle

INTRODUCTION

white$^{mottled\ 4}$ is a chromosomal inversion resulting from a break of the X chromosome within centromeric heterochromatin, and another within euchromatin. In this rearrangement, the euchromatic gene *white* responsible for the red color of the eye, is relocated at the vicinity of heterochromatin and exhibits variegated expression. It is silenced in some cells and not in others, thus resulting in a mosaic phenotype. The phenomenon, reported for many chromosomal rearrangements and affecting many genes, is called position-effect variegation (PEV). The most popular model proposes that the condensed and inactive conformation of heterochromatin spreads over the breakpoint of the rearrangement and occasionnally encompasses and inactivates neighbouring euchromatic genes (Eissenberg, 1989; Tartof et al., 1989; Henikoff, 1990; Reuter and Spierer, 1992). The proportion of cells in which inactivation occurs for a given gene in a given rearrangement is regulated by dominant and recessive genetic modifiers acting in *trans*. Overall, an estimated 100-200 genes either enhance or suppress variegation (Reuter and Wolff, 1981; Tartof et al., 1989; Sinclair et al., 1989; Wustmann et al., 1989). It has been proposed that the products of many of these genes encode chromatin components or modifiers of chromatin components. They are recognized by having a dose-dependent effect on variegation. Molecular studies show that some suppressor mutations indeed identify heterochromatin constituents, with dose-dependent opposite effects (Eissenberg et al., 1990, 1992; Reuter et al., 1990; Garzino et al., 1992; Tschiersch et al., 1994). Enhancer mutations, in contrast, have identified transcriptional activators, or candidates for such a function (Dorn et al., 1993a; Farkas et al., 1994). Whether these factors act directly on chromatin, as 'architectural transcription factors', or by indirect effects through their target genes remains to be determined.

There is an interesting structural and functional overlap between the modifiers of PEV and the regulators of the bithorax complex of homeotic genes. Two classes of dominant *trans*-regulators affect the expression of these homeotic genes, which are clustered in large chromosomal domains. On the one hand, the Polycomb-group of genes are seen as repressors of homeotic genes. They act in a dose-dependent mode on the maintenance of the repressed state of homeotic genes (Moehrle and Paro, 1994). On the other hand, the trithorax-group of genes are general activators of homeotic genes. Structural and functional overlap was found between suppressors of variegation and Polycomb-group genes (Paro and Hogness, 1991; Moehrle and Paro, 1994; Fauvarque and Dura, 1993), and between enhancers of variegation and trithorax-group genes (Tamkun et al., 1992; Dorn et al., 1993a; Farkas et al., 1994; Reuter et al., unpublished data). Therefore, the study of modifiers of position-effect variegation may shed light on general mechanisms of genetic control of activity of chromosomal domains. Other epigenetic silencing phenomena such as telomere and mating-type silencing in yeast, or X-inactivation and parental imprinting in mammals may use similar mechanisms to those used in PEV.

A large effort is underway to identify dominant enhancers

of position-effect variegation in *Drosophila* (Dorn et al., 1993b). We report here the genetic and molecular characterization of one of them, *E(var)3-93E*^{164}, which corresponds to the *Drosophila* cell cycle controlling transcriptional activator dE2F (Ohtani and Nevins, 1994; Dynlacht et al., 1994; Duronio et al., 1995; Duronio and O'Farrell, 1995).

MATERIALS AND METHODS

Genetic analysis

All fly stocks were maintained under standard conditions. A description of chromosomes and mutations can be found in Lindsley and Zimm (1992). The allele of *E(var)3-93E* induced by insertion of the P{pUC*hsneo ry*$^+$} transposon described by Dorn et al. (1993a) is designated *E(var)3-93E*^{164}.

Autosomal transposition of P{pUC*hsneo ry*$^+$} was selected after a cross of *w*^{m4h}; *Su(var)2-1*^{01}*/CyRoi; ry*^{506} females with *w*^{m4h} P{pUC*hsneo ry*$^+$}*/Y; +/+; TM3,ry*^{RK} *Sb e P{ry*$^+$*(Δ2-3)}/ry*^{506} males. The *TM3,ry*^{RK} *Sb e P{ry*$^+$*(Δ2-3)}* chromosome is an efficient source of P transposase (Reuter et al., 1993). After purine treatment (Finnerty et al., 1970) *w*^{m4h}*/Y; Su(var)2-1*^{01}*/+; ry*^{506} *and w*^{m4h}*/Y; CyRoi/+; ry*^{506} males will only survive if the P{pUC*hsneo ry*$^+$} element transposon is present. Exceptional males with an enhanced *white-mottled* phenotype were selected for further analysis and were crossed to *w*^{m4h}; *Cy/T(2;3)ap*^{Xa} *Su(var)2-1*^{01}*/Sb* females. *T(2;3)ap*^{Xa} *Su(var)2-1*^{01}is abbreviated below as *XaSu*. The offspring *w*^{m4h}*/Y; +/XaSu/+* males with an enhancer phenotype (variegated instead of a suppressed red eye phenotype) were backcrossed again to *w*^{m4h}*; Cy/XaSu/Sb* females. The putative enhancer can be localized to the different chromosomes with the help of aneuploid segregants of the *ap*^{Xa} translocation (Reuter and Wolff, 1981). Segregation of P{pUC*hsneo ry*$^+$} was monitored by dot blot hybridization with a pUC18 probe using single flies from each of the 5 different genotypes (*+/XaSu/ry*^{506}, *+/XaSu/Sb*, *Cy/XaSu/ry*^{506}, *Cy/XaSu/Sb* and *Cy/+; Sb/ry*^{506}). Balanced stocks were constructed after crosses with *w*$^+$*; CyO/Sco; ry*^{506} and *w*$^+$*; TM3,ry*^{RK} *Sb e/ry*^{506} females. The enhancer effect of the mutations on *w*^{m4h} variegation were quantified by red eye pigment measurements in a *XaSu* background after a cross of *w*^{m4h}; *Cy/XaSu/Sb* females to *w*^{m4h}*; +/XaSu/ry*^{506} *E(var)3-93E* males. The *+/XaSu/ry*^{506} *E(var)3-93E* offspring males were compared to the *+/XaSu/Sb* and *Cy/XaSu/Sb* control genotypes.

Excisions of the P{pUC*hsneo ry*$^+$} element were induced by crossing homozygotes for the mutation with a *TM6,Ubx/Sb P{ry*$^+$*(Δ2-3)*}strain. The dysgenic males were then crossed to female balancers *TM3, Sb ry*^{RK}*e/TM6b, Tb e* and exceptional *ry*$^-$ male progeny were used to establish independent lines and tested for precise excision of the transposon on genomic Southern blots.

Effects on PEV were tested by crosses with *w*^{m4h}*; Cy/XaSu/Sb* females. Crosses of these females with Canton S males were used as control.

Molecular biology

Plasmid rescues were obtained by following the protocol of Pirrotta (1986). RNA in situ hybridization on whole mounts were prepared as described by Tautz and Pfeifle (1989) modified by Cléard et al. (1995). Northern blot analyses were performed with the protocol of Cléard et al. (1995), except for the probe, which was a DNA probe and for the hybridization mixture which was the following: 5× Denhardt's, 6× SSPE, 8% dextran sulfate, 36% urea (Merck no. 8487) 100 μg/ml denatured sonicated salmon sperm DNA. Primer extension were done according to the protocol of Mason et al. (1993) with primers noted on the dE2F promoter region (see Fig. 4).

Germline transformation

The plasmid construct to be injected was prepared by inserting an *Eco*RI full length cDNA fragment of 4.2 kb from the plasmid pNB1.4 into the plasmid pHSS7 derived from pHSS6 (Seifert et al., 1986) containing *Not*I sites at each extremity of the polylinker. The *Not*I insertion was then cloned downstream of the *Hsp70* promoter in the *Not*I site of the pNHT4 transformation vector (Schneuwly et al., 1986).

Injections into embryos were done according to the method of Spradling (1986). *ry*$^+$ flies were selected, characterized by in situ hybridization and crossed to the *w*^{m4h}*; XaSu/Sb* strain at 29°C.

RESULTS

A new dominant enhancer of position-effect variegation maps at 93E

Insertional mutations with an enhancer of PEV phenotype have been successfully isolated after mobilization of the P{pUC*hsneo ry*$^+$} transposon (Dorn et al., 1993b). Genomic DNA fragments flanking the insertion site can be isolated via plasmid rescue and afterwards used as an entry point for cloning of the corresponding gene. A new genetic screen has been applied, which ensures efficient mutant isolation by detection of a P{pUC*hsneo ry*$^+$} transposition as well as the dominant enhancer effect in individual offspring males. After remobilization of an X chromosomal P{pUC*hsneo ry*$^+$} insert in parental males, transpositions of the modified P-transposon into autosomes were selected as males homozygous for *ry*^{506} that survived purine treatment. Purine efficiently kills *ry*$^-$ flies (Finnerty et al., 1970) and the surviving males must have received a *ry*$^+$ allele through insertion of P{pUC*hsneo ry*$^+$} into an autosomal site. Because all flies are *w*^{m4h}, either in absence or in presence of the strong suppressor *Su(var)2-1*^{01} (Reuter et al., 1982), an enhancer effect of the insertions can be monitored in the surviving F_1 males by an increase in white

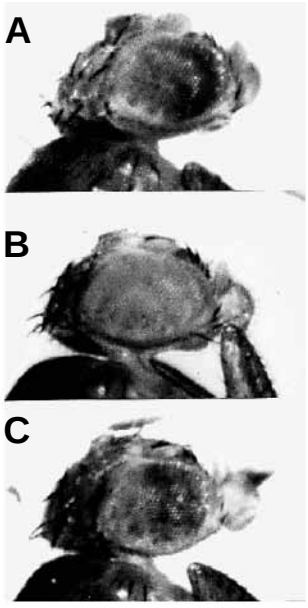


Fig. 1. Effect on position-effect variegation of an insertional mutation at 93E. (A) Head of a male carrying the X-chromosome rearrangement *white-mottled-4h. (w*^{m4h}*/Y;+/Cy;+/Sb).* (B) Head of a male carrying the enhancer mutation in the same background as in A *(w*^{m4h}*/Y;+/Cy;E(var)3-93E*^{164}*/Sb)*. (C) Head of a male revertant for the P-induced enhancer mutation *(w*^{m4h}*/Y;+/Cy;excE(var)3-93E*^{164}*/Sb)*. For details of crosses, see Materials and Methods.

mottled mutant phenotype. In about 250,700 offspring, 32 (1.3×10^{-4}) enhancer mutations have been isolated. The mutations were genetically mapped and localized by in situ hybridization (see Materials and Methods). An enhancer mutation, *E(var)3-93E*^{164}, was further analyzed.

Mutations induced by a transposable element can be readily mapped on larval salivary gland polytene chromosomes by in situ hybridization using transposon sequences as a probe. A single signal was found at 93E on the right arm of the third chromosome (not shown), thus suggesting that there is a single insertion of the P-transposon. This was confirmed by genomic Southern blot hybridization (not shown). Effects of mutations on PEV can be detected by observing their effects on the mosaic phenotype seen on the compound eye of flies with a variegating rearrangement of the *white* gene. Inactivation of *white* results in clones of white ommatidia in a wild-type red background. Fig. 1A illustrates the mottled phenotype of *w*^{m4h}, and Fig. 1B the enhancement of variegation due to the heterozygous mutation *E(var)3-93E*^{164}. The mutation *E(var)3-93E*^{164} was found to be homozygous viable and fertile.

To ascertain that the phenotype is indeed caused by the insertion of the transposon, the element was mobilized to produce phenotypic revertants associated with an excision of the transposon. The P-induced mutant was crossed to a transposase-producing strain (P{*ry*$^{+}$ Δ2-3}), and the progeny analyzed for the loss of the transposon as seen by the loss of the *ry*$^{+}$ gene. Eleven independent *ry*$^{-}$ lines were analyzed by Southern blot hybridizations. Eight were internal deletions in the transposon, and three apparent precise excisions. As illustrated in Fig. 1C, one of the three apparent precise excisions clearly reverts the enhanced variegation phenotype. Molecular analysis reported below confirmed the correspondence between the mutation and the phenotype.

Molecular cloning of the *E(var)3-93E* locus

The modified P element P{pUC*hsneo ry*$^{+}$} allows plasmid rescue of genomic DNA on one side of the transposon. Digestion with *Sal*I or with *Eco*RI followed by ligation allowed us to recover plasmids of 9.1 and 7.1 kb respectively. The plasmids contain 4.4 kb of pUC plasmid sequence and the remaining DNA consists of flanking *Drosophila* genomic sequence. As a control, the rescued plasmids were found to hybridize to the same 93E locus on wild-type polytene chromosomes, thus demonstrating that the genomic sequence originates as expected from that region (not shown). With the genomic fragment as a probe, we screened a genomic library (Maniatis et al., 1978) to isolate sequences extending on both sides of the P-element. The region encompassing the 5′ end of the transcription unit was isolated in a second step by using the 5′ end of a cDNA clone (described below) as a probe. The resulting map of the genomic region and the position of the transposon is depicted in Fig. 2. The 5′-end exon is separated from the main coding region by a 12-kb-long intron.

Deduced protein sequence of transcripts from the *E(var)3-93E* locus identifies the *Drosophila* E2F gene

We mapped transcription units in the vicinity of the transposon. Restriction fragments on each side of the insertion site (Fig. 2) were used as probes on northern blots of embryonic RNA. A 2.0 kb *Eco*RI fragment (localized on the genomic region in Fig. 2A) detects three overlapping transcripts of 3.6, 4.2 and 4.7 kb (Fig. 3A). This fragment, located 3.0 kb 3′ of the P transposon and which gave a strong signal on northern blots, was used to select cDNA clones in a 4-8 hour cDNA library (Brown and Kafatos, 1988). About 50,000 colonies were screened and 26 clones were isolated. Eight clones were analysed in detail to establish the complete map of the transcripts depicted in Fig. 2. cDNA sequencing allowed us to detect an open reading frame, which was compared to known sequences in databases, and identified the cDNAs as encoding the *Drosophila* homolog of E2F, dE2F (Ohtani and Nevins, 1994; Dynlacht et al., 1994). The eight clones analyzed can be arranged in three classes, varying in the length of their 3′ untranslated region (not shown), but we could not unambiguously assign these classes to the three sizes of transcripts seen on northern blots.

When comparing the eight cDNAs analyzed, it appeared that four of them have a deletion of 18 nucleotides in the open reading frame. This leads to the deletion of six amino acids (QQLQQQ). Sequencing of our genomic clone from the Maniatis library included DNA coding for these amino acids, without interruption of the open reading frame before or after the stretch. There is hence no evidence that this deletion results from alternative splicing. The sequence resembles *opa* repeats

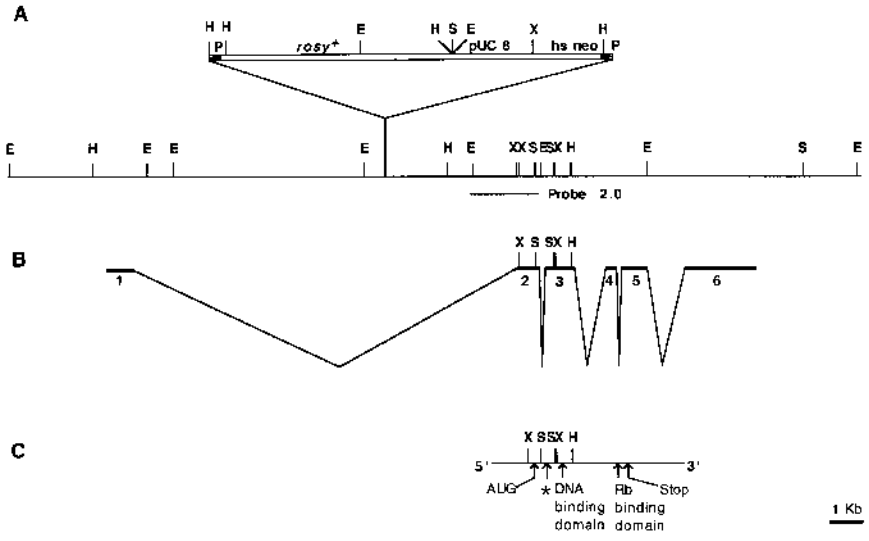
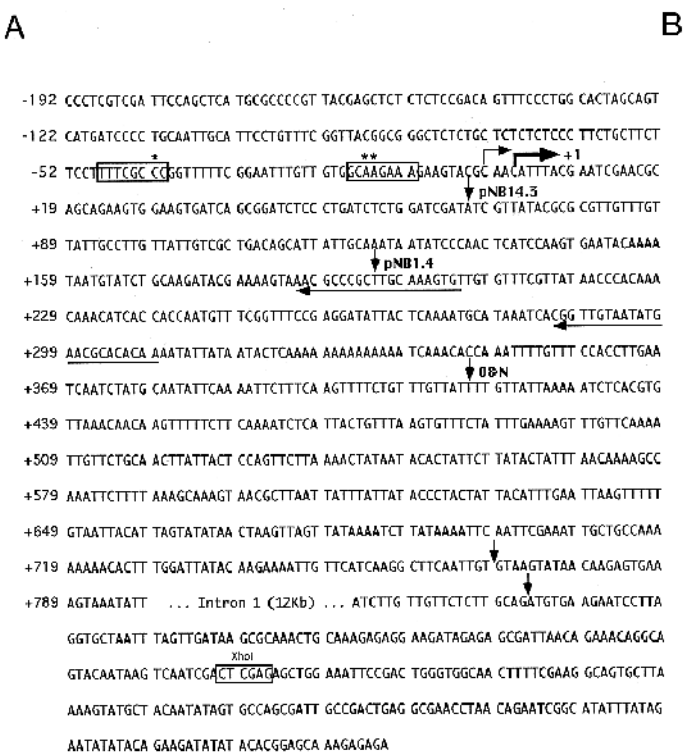


Fig. 2. Genome and transcript organization of the *Drosophila E2F* locus. (A) Genomic map and position of the P{pUC*hsneo ry*+} transposon insertion. Probe 2.0 is the *Eco*RI restriction fragment used as probe on northern blots (see text). E, *Eco*RI; H, *Hin*dIII; X, *Xho*I; S, SalI. (B) Maps of the transcription unit and of the largest cDNA clone. Exons 4, 5, and protein coding part of exon 6 are from Duronio et al. (1995). (C) Features of the deduced protein sequence: the scheme is aligned on the cDNA restriction sites; * marks the position of the polymorphism QQLQQQ (see text); E2F DNA binding domain and Rb binding domain determined by Nevins (1992) and Ohtani and Nevins (1994). The intron/exon junctions have been sequenced showing the absence of additional miniexon within the large first intron by comparison with cDNA sequence.



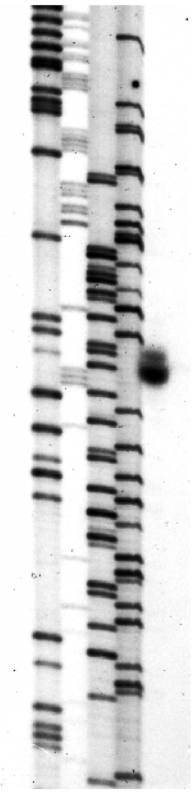


Fig. 3. The promoter region of the *dE2F* locus. (A) Sequence of the promoter region and transcription starts of *dE2F*. The bent arrows indicate the two transcription start sites, the major one being in bold. The two boxes are the potential E2F binding sites with differences from the consensus sequence (TTTCGCGC) noted with an asterisk. pNB14.3 and pNB1.4 are the longest cDNAs analyzed. The backwards arrows show the two oligonucleotides used to perform the primer extension experiment. O&N marks the beginning of the sequence published by Othani and Nevins (1994). The two vertical arrows indicate the start and the end of the first 12 kb intron. (B) Primer extension done with the most 5′ oligonucleotide. Sequencing reactions on the side (order G-A-T-C) were from the same oligonucleotide on a 6% sequencing gel. The other oligonucleotide gave the same result (not shown). The translation starts 86 nucleotides downstream of the sequence presented in the figure.

(Wharton et al., 1985). We assume that it is a polymorphism in the population used for making the cDNA library.

E2F is known to be autoregulated in mammals and E2F binding sites have been described in the promoter region of the mammalian E2F gene (Johnson et al., 1994; Neuman et al., 1994). We have mapped the 5′ end of the transcription unit on the genomic DNA by primer extension. The result is shown in Fig. 3, which also presents genomic sequence upstream of the two starts of transcription. It should be noted that this promoter has no obvious TATA box. Two potential E2F binding sites were found within 50 base pairs upstream of the start sites.

The mutation affects transcript levels

Next we looked for effects of the insertional mutation on transcripts of the locus. Fig. 4 depicts northern blot hybridizations of RNA extracted at selected developmental stages using the cDNA as a probe. Early embryos (0-4 hours) contain three different sized transcripts, but only the two larger ones are detected in later stages. In a separate experiment, RNA from 0-2 hours was blotted. Only the shorter transcript is visible, thus establishing its maternal origin.

As the mutation is homozygous viable, RNA was extracted from homozygous mutant embryos to examine possible alterations of *dE2F* expression. Fig. 4 shows a severe reduction of all RNA species in the mutant, compared to wild type. The distribution of transcripts was examined in whole-mount homozygous mutant embryos (not shown). Transcripts were detectable, but at a much lower level. The pattern was similar to that seen in wild-type embryos (see below).

R. Duronio and P. O'Farrell have provided us with a null

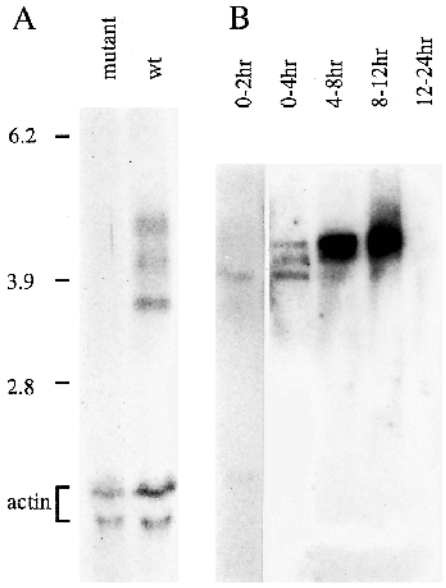


Fig. 4. Northern blot analysis of the transcripts of the *dE2F* gene. (A) 20 μg of total RNA (overnight collection) from mutant (164) or wild-type embryos hybridized with probe 2.0 (see Fig. 2). The size of the transcripts is 3.6, 4.2, 4.7 kb. The blot was also probed with actin (Fyrberg et al., 1980) as a control of the amounts of RNA transferred per lane. Size markers are indicated on the side. Transcripts are difficult to see in the mutant in this experiment and exposure, but were weakly detectable in others and in whole-mount mutant embryos. (B) Developmental northern blot. 20 μg of five embryonic stages were used except for lane 0-2hr and 12-24hr where only 10 μg of RNA were loaded. The blots were probed with an *Eco*RI fragment containing the full length cDNA of dE2F (pNB1.4). The absence of the large transcripts in 0-2 hours embryos has been confirmed on other blots (not shown).

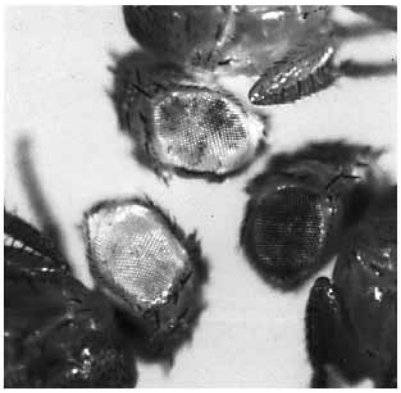


Fig. 5. PEV depends on the dose of *dE2F*. Two wild-type doses of the *dE2F* gene (Canton S control) in a w^{m4h} background *(w^{m4h}/Y;+/Cy;+/Sb)* (top). One wild-type dose of *dE2F* (heterozygous for the null mutation $dE2F^{91}$; Duronio et al., 1995) in a w^{m4h} background *(w^{m4h}/Y;+/Cy;$dE2F^{91}$/Sb)* (bottom left), and two wild-type doses of *dE2F* and one copy of a heat inducible transgene in the same background *(w^{m4h}/Y;+/Cy;P{pNHT4-E2F ry^{+}}/Sb)* (bottom right). Flies were all raised in mild heat shock condition (29°C).

and homozygous lethal allele of the *Drosophila dE2F* that they have determined to be a nonsense mutation at amino acid 31 ($dE2F^{91}$, Duronio et al., 1995). We have found this allele to be a strong dominant enhancer, actually stronger than our P-induced mutation, thus confirming allelism between the *dE2F* gene and the enhancer of variegation phenotype (Fig. 5).

These two experiments, namely reduction of transcript level in the insertional mutant and the enhancer effect of the null mutant, show that the dominant enhancer effect is due to reduced activity of *dE2F*.

The *dE2F* mutation enhances *yellow* variegation

To assess whether the dose of *dE2F* also affects other variegating rearrangements, the null mutation was placed in *trans* with two minichromosomes bearing a variegating $yellow^{+}$ gene (*Dp(1;f)1187* and *Dp(1;f)8-23*, Karpen and Spradling, 1990; Tower et al., 1993). The color phenotype was scored on the middle bristles of the triple row at the anterior wing margin. For both minichromosomes, the percentage of yellow bristle was significantly higher in heterozygous flies compared to the siblings bearing two wild-type copies of *dE2F*. With *Dp (1;f)1187*, 23% (mean) of the bristles were yellow in flies with a mutant copy of *dE2F*, in contrast to 12% in control flies (two copies of *dE2F*). A *t*-test confirms that these two values are significantly different at the 99% confidence level. Using the *Dp(1;f)8-23* rearrangement, heterozygous mutant flies showed 33% of yellow bristles versus 20% in the control. A *t*-test confirms that these two values are significantly different at the 95% confidence level. We conclude that a reduced dose of *dE2F* acts as a dominant enhancer of the variegation of different genes in different variegating chromosomal rearrangements.

Overexpression of *dE2F* suppresses position-effect variegation

A clear-cut demonstration that the transcripts indeed encode the functional protein can be provided by introducing the cDNA into the genome by genetic transformation. It is expected that this overexpression will have the opposite effect of gene dose reduction in the mutant. cDNAs containing the complete ORF were cloned in a P-transposon-derived transformation vector (pNHT4; Schneuwly et al., 1986) that allowed us to place the cDNA under the control of the hsp70 heat shock promoter. We used cDNAs either with the short polymorphic insertion or without. They both gave the same effect on PEV. After injection into a ry^{-} background, transformant lines were selected by the ry^{+} marker of the transformation vector. The effect on variegation was determined by crossing transformants in the *white-mottled* background. Transformant flies and wild-type controls were grown at 29°C, a mild heat shock condition. Fig. 5 shows that the presence of one copy of the *hs-dE2F* transgene (in a background of two wild-type resident copies of *dE2F*) produces a clear suppressor effect. Flies grown at lower temperature (25°C) do not show this effect. Robin Wharton and Maki Asano provided us with their transgenic lines tagged with the miniwhite gene as a marker (unpublished data). Though the miniwhite background rendered the experiment more difficult, we have verified that these lines also suppress position effect variegation (not shown).

Though we have not embarked on a detailed comparison, it seems, at a qualitative level, that the suppressor phenotype is the same whether or not the transgene contains the six amino acid polymorphic stretch. We conclude that overexpression of *dE2F* has the opposite effect to the reduction of gene dosage. $E(var)3\text{-}93E^{164}$ is homozygous viable, but null mutants are homozygous lethals (Duronio et al., 1995). Under mild heat shock conditions (29°C), both of these constructs save the lethality of the null allele $dE2F^{91}$ (data not shown). Rescue of a null allele demonstrate clearly that the transgene is expressed and functional.

The transcripts are ubiquitous in early embryonic development

The distribution of transcripts was examined in whole mount embryos. The results are illustrated in Fig. 6 for ten selected developmental stages. They extend previous work of Hao et al. (1995) and Duronio et al. (1995). The transcripts are ubiquitous in preblastoderm embryos. Incomplete cellular blastoderm embryos (stage 4) show a pattern reminiscent of the one reported by Hao et al. (1995) for dDP1, the factor that makes a heterodimer with dE2F in its active form (Dynlacht et al., 1994) and for *string* and *twine* (Alphey et al., 1992), two genes implicated in cell cycle progression. Staining is preponderant under the peripheral nuclei. At cellular blastoderm (stage 5) the zygotic transcripts appear in three broad bands. Ventral expression seems stronger. By the beginning of gastrulation (stage 6) a very transient banded pattern appears, showing at least 10 bands, having similar spacing to that of segment polarity genes. The ventral furrow is strongly stained. Slightly later (stage 7), the bands have already disappeared leaving most of the labeling in the ventral furrow (mesoderm). At stage 8, transcripts accumulate in mesoderm tissues, and at stages 9-10, the embryo shows staining principally in the neuroblasts and mesoderm. At stages 10-11, the neuroblast staining stands out. At stage 13, anterior and posterior midgut staining appears. By stage 16 the midgut and foregut are also stained.

DISCUSSION

$E(var)3\text{-}93E^{164}$ is a dominant mutation that enhances position-effect variegation. When one dose is lost or when expression

is reduced, heterochromatic position-effect silencing is enhanced. This dominant enhancer effect makes *E(var)3-93E*^{164} a member of the group of haplo-insufficient enhancers of PEV. Overexpression with a transgene under the control of a heat shock promoter has an opposite suppressor effect on variegation. Hence, within the enhancers of PEV, this locus is a haplo-enhancer gene with a triplo-suppressor effect. Triplo-effect is not used here in a strict sense as there might be differences between the overexpression of a cDNA transgene and a duplication of the whole locus. *E(var)3-93E*^{164} is the first molecularly characterized gene with this opposite effect on PEV. Though the classical reporter for PEV is *white-mottled*, we have also determined a parallel enhancer effect for two other rearrangements variegating for the gene *yellow*, making it unlikely that there is a specific effect of the enhancer mutation on the transcription of the *white* gene.

E(var)3-93E^{164} encodes the *Drosophila* E2F transcriptional activator and cell cycle regulator. This is a surprising finding as the dose dependence argues in favor of an architectural effect on chromatin, although dE2F could also be the rate-limiting regulator of an architectural factor. However, previously characterized mutations enhancing PEV have identified other transcriptional activators such as the GAGA-factor (also known as trithorax-like, a regulator of homeotic genes (Farkas et al., 1994)), the *E(var)3-93D* gene, which is also a regulator of homeotic genes as well as a modifier of the activity of *su(Hw)*, and encodes a protein involved in the insulation of chromosomal domains (Dorn et al., 1993a; Gerasimova et al., 1995), and possibly *modulo* (Garzino et al., 1992). Recently, *zeste* was reported as a recessive enhancer of PEV (Judd, 1995). *zeste* encodes a DNA binding protein with two previously known roles: transcriptional activator affecting chromatin configuration at a distance, and an effector of chromosome pairing-dependent effects (reviewed by Pirrotta, 1991). In contrast, suppressor mutations have identified the heterochromatin-associated proteins HP1 (Eissenberg et al., 1992) and Su(var)3-7 (Reuter et al., 1990; Cléard, 1993; Cléard et al., 1995).

In mammals, members of the E2F family of proteins form heterodimers with the DP family of proteins in different combinations, and activate genes involved in DNA metabolism, hence promoting the transition from G_1 to S phase. These dimers can be sequestered by the retinoblastoma tumor suppressor protein (pRb) or other pRb-related proteins (p107, p130). Phosphorylation of the pRb proteins by cyclin-dependent kinases in the course of the G_1 phase releases the E2F-DP dimers. Absence of E2F blocks the cell cycle at the G_1/S transition (reviewed by La Thangue, 1994). In contrast, overexpression of E2F can lead to oncogenic transformation (Beijersbergen et al., 1994; Ginsberg et al., 1994; Singh et al., 1994), or apoptosis (Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994). As in mammalian cells, active dE2F is complexed with dDP in *Drosophila* (Dynlacht et al., 1994). It binds to the promoter of a dE2F-dependent gene, DNA polymerase alpha (Ohtani and Nevins, 1994), and is essential for the G_1 to S progression (Duronio et al., 1995). Interaction of dE2F with pRb arrests cells in G_1 phase, and dE2F binds to cyclin A/cdk2 and can be modulated by viral oncoproteins (Hao et al., 1995). These data, and our findings, show that the *Drosophila* dE2F protein parallels the function of mammalian E2F.

In the widely accepted 'structural' model of heterochromatic PEV, enhancers of variegation are constituents, or modifiers of constituents, of euchromatin that resist epigenetic silencing or promote derepression. They can also be negative regulators of heterochromatin constituents. We propose two hypotheses to

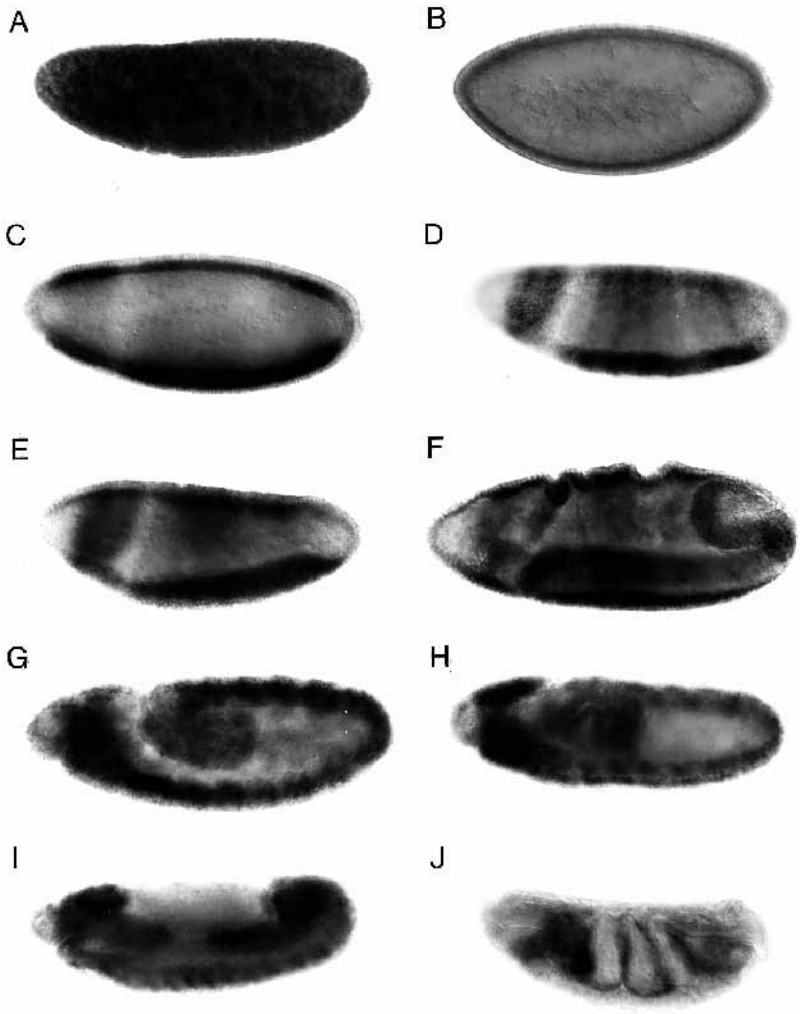


Fig. 6. Pattern of expression of the *dE2F* transcripts in embryos. RNA in situ hybridisation was performed on whole mounts as described by Cléard et al. (1995) with the longest cDNA as a probe. (A) Stage 1 embryo. The transcripts are ubiquitous. (B) Incompleted cellular blastoderm (stage 4). Staining is preponderant under the peripheral nuclei. (C) Cellular blastoderm (stage 5). The zygotic transcripts start in three broad bands. (D) Beginning of gastrulation (stage 6). A very transient banded pattern appears showing at least 10 bands having similar spacing to that of segment polarity genes. The ventral furrow is strongly stained. (E) Gastrulation (stage 7). The bands have already disappeared leaving most of the labeling in the ventral furrow (mesoderm). (F) Stage 8 embryo. Transcripts accumulate in mesoderm tissues. (G) Stage 9-10. Staining principally in the neuroblasts and mesoderm. (H) Stage 10-11. Neuroblasts staining stands out. (I) Stage 13. Anterior and posterior midgut staining appears. (J) Stage 16. The midgut and foregut are also stained.

explain how the dose of dE2F affects position-effect variegation. First, dE2F might be a positive transcriptional activator of another enhancer gene, or a negative regulator of a suppressor gene. Decreased amounts of E2F would decrease the expression of the other enhancer or increase the expression of the suppressor, producing the haplo-enhancer effect. In contrast, increased amounts of dE2F would increase the levels of the other enhancer or decrease the level of a suppressor, leading to the triplo-suppressor effect. It should be noted here, that though E2F was characterized as an activator of transcription during the S phase, a negative effect is also plausible via the complex between E2F and the hypophosphorylated form of pRb (Weintraub et al., 1995). The association of E2F and pRb could also prevent DNA condensation. In mammalian cells, the pRb protein indeed seems to localise not only over euchromatin but also as large nuclear granules located at the border between euchromatin and heterochromatin, suggesting that the protein may influence DNA condensation (Szekely et al., 1991). dE2F could also prevent the spreading of the heterochromatic conformation by its association with its many target sites on euchromatin. In such a model, there must be E2F binding sites between the variegation-inducing heterochromatin and the variegating gene. Among other known enhancers of PEV, the *trithorax-like* locus does indeed encode a protein, the GAGA factor, with binding sites both at its euchromatic targets sites and in heterochromatin (Raff et al., 1994).The product of *E(var)3-93D*, probably also a transcription factor, is associated with many sites in euchromatic arms of polytene chromosomes (Dorn et al. 1993a).

Our second hypothesis proposes that the dose of dE2F modulates PEV through its function as a cell cycle regulator. In this respect, it is interesting to note that involvement of mitosis control in variegation was suggested by the finding that mutation of the PP1 phosphatase gene suppresses variegation and leads to abnormal mitosis (Baksa et al., 1993) and that mutations in PCNA, an auxiliary factor in DNA replication, are recessive suppressors of PEV (Henderson et al., 1994). In humans, PP1 binds to the retinoblastoma protein, the same protein that releases E2F upon phosphorylation (Durfee et al., 1993). A lower dose of PP1 in flies could prevent some dephosphorylation of pRB (a protein yet to be isolated in *Drosophila*), resulting in an increased level of free dE2F, and hence suppressing PEV. In this second hypothesis, it remains to be determined how a perturbation of the G1/S transition (or in the length of the S phase) can affect PEV. During DNA replication is likely to be the critical time for establishing, erasing, or maintaining epigenetic programming. If this is true for the effect we see, then a delay in the G1/S transition or a modification of the S phase could have an enhancer effect on the establishment of silencing as detected by heterochromatic position effects. This could be explained, for example, if a longer S phase provides more time for building up the silenced state of large chromosomal domains. An old, and possibly related, observation is that heterochromatic regions are late replicating (Lima de Faria and Jarworska, 1968).

In conclusion, we emphasize that this possible link between epigenetic silencing and cell cycle was recently suggested in the analogous phenomena of mating type locus silencing and telomeric position effect in yeast. Indeed, telomeric silencing is variegated and spreads from the telomere (Renauld et al., 1993). Miller and Nasmith (1984) have shown that progression through the S phase of the cell cycle is required for establishing a repressed state of the silent mating type locus. Recently, Laman and collaborators have looked for mutations suppressing the silencer-defective mutation *rap1* (Laman et al., 1995), an approach analogous to our search for enhancers of *white-mottled* variegation. Three suppressors were cloned and all encode components necessary for normal cell cycle progression. This analogy reinforces the idea that telomeric position effect in yeast and PEV are related. That mutations perturbing cell-cycle progression enhance silencing in both systems supports the hypothesis that establishment of epigenetic silencing occurs in a window of the cell cycle, which is critical either by its duration and/or its timing.

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REFERENCES

Alphey, L., Jimenez, J., White-Cooper, H., Dawson, I., Nurse, P. and Glover, D.M. (1992). *twine*, a cdc25 homolog that functions in the male and female germline of *Drosophila*. *Cell*, **69**, 977-988.

Baksa, K., Morawietz, H., Dombradi, V., Axton, M., Taubert, H., Szabo, G., Torok, I., Udvardy, A., Gyurkovics, H., Szoor, B., Glover, D., Reuter, G and Gausz, J. (1993). Mutations in the protein phosphatase 1 gene at 87B can differentially affect suppression of position-effect variegation and mitosis in *Drosophila melanogaster*. *Genetics* 135, 117-125.

Beijersbergen, R.L., Kerkhoven, R.M., Zhu, L., Carlee, L., Voorhoeve, P.M. and Bernards, R. (1994). E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. *Genes Dev.*, **8**, 2680-2690.

Brown, N.H. and Kafatos, F.C. (1988). Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* **203**, 425-437.

Cléard, F. (1993) *PhD thesis* 2621, University of Geneva.

Cléard, F., Matsarskaia, M. and Spierer, P. (1995). The modifier of position-effect variegation Suvar(3)7 of *Drosophila*: there are two alternative transcripts and seven scattered zinc fingers, each preceded by a tryptophan box. *Nucl. Acids Res.* **23**, 796-802.

Dorn, R., Krauss, V., Reuter, G. and Saumweber, H. (1993a). The enhancer of position-effect variegation of *Drosophila*, E(var)3-93D, codes for a chromatin protein containing a conserved domain common to several transcriptional regulators. *Proc. Natl Acad. Sci. USA* **90**, 11376-11380.

Dorn, R., Szidonya, J., Korge, G., Sehnert, M., Taubert, H., Archoukieh, E., Tschiersch, B., Morawietz, H., Wustmann, G., Hoffmann, G. and Reuter, G. (1993b). P transposon-induced dominant enhancer mutations of position-effect variegation in *Drosophila melanogaster*. *Genetics* **133**, 279-290.

Durfee, T., Becherer, K., Chen, P.L., Yeh, S.H., Yang, Y., Kilburn, A.E., Lee, W.H. and Elledge, S.J. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.*, **7**, 555-569.

Duronio, R.J., O'Farrell, P.H., Xie, J.E., Brook, A. and Dyson, N. (1995). The transcription factor E2F is required for S phase during *Drosophila* embryogenesis. *Genes Dev.* **9**, 1445-1455.

Duronio, R.J. and O'Farrell, P.H. (1995). Developmental control of the G1 to S transition in *Drosophila*: cyclin E is a limiting downstream target of E2F. *Genes Dev.* **9**, 1456-1468.

Dynlacht, B.D., Brook, A., Dembski, M., Yenush, L. and Dyson, N. (1994). DNA-binding and trans-activation properties of *Drosophila* E2F and DP proteins. *Proc. Natl Acad. Sci. USA* **91**, 6359-6363.

Eissenberg, J.C. (1989). Position effect variegation in *Drosophila*: towards a genetics of chromatin assembly. *BioEssays* **11**, 14-17.

Eissenberg, J.C., James, T.C., Foster-Hartnett, D.M., Hartnett, T., Ngan, V. and Elgin, S.C. (1990). Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila* melanogaster. *Proc. Natl Acad. Sci.USA* **87**, 9923-9927.

Eissenberg, J.C., Morris, G.D., Reuter, G. and Hartnett, T. (1992). The heterochromatin-associated protein HP-1 is an essential protein in

Drosophila with dosage-dependent effects on position-effect variegation. *Genetics* **131**, 345-352.

Farkas, G., Gausz, J., Galloni, M., Reuter, G., Gyurkovics, H. and Karch, F. (1994). The Trithorax-like gene encodes the *Drosophila* GAGA factor. *Nature* **371**, 806-808.

Fauvarque, M.O. and Dura, J.M. (1993). polyhomeotic regulatory sequences induce developmental regulatory dependent variegation and targeted P-element insertions in *Drosophila*. *Genes Dev.* **7**, 1508-1520.

Finnerty V., Baillie D.L., Chovnik A. (1970). A chemical system for mass collection of virgin females or males. *Drosophila Inf. Service*, **45**, 190.

Fyrberg, E.A., Kindle, K.L., Davidson, N. and Kindle, K.L. (1980). The actin genes of *Drosophila*: a dispersed multigene family. *Cell* **19**, 365-378.

Garzino, V., Pereira, A., Laurenti, P., Graba, Y., Levis, R.W., Le Parco, Y. and Pradel, J. (1992). Cell lineage-specific expression of modulo, a dose-dependent modifier of variegation in *Drosophila*. *EMBO J.* **11**, 4471-4479.

Gerasimova, T. I., Gdula, D.A., Gerasimov, D. V., Simonova, O. and Corces V. G. (1995). A *Drosophila* protein that imparts directionality on a chromatin insulator is an enhancer of position-effect variegtion. *Cell* **82**, 587-597.

Ginsberg, D., Vairo, G., Chittenden, T., Xiao, Z.X., Xu, G., Wydner, K.L., DeCaprio, J.A., Lawrence, J.B. and Livingston, D.M. (1994). E2F-4, a new member of the E2F transcription factor family, interacts with p107. *Genes Dev.* **8**, 2665-2679.

Hao X. F., A.L., Bandara L. R., Lam E. W.-F., Glover D. and La Thangue N. (1995). Functional conservation of the cell-cycle regulating transcription factor DRTF1/E2F and its pathway of control in *Drosophila melanogaster*. *J. Cell Sci.* **108**, 2945-2954.

Henderson, D.S., Banga, S.S., Grigliatti, T.A. and Boyd, J.B. (1994). Mutagen sensitivity and suppression of position-effect variegation result from mutations in mus209, the *Drosophila* gene encoding PCNA. *EMBO J.*,**13**, 1450-1459.

Henikoff, S. (1990). Position-effect variegation after 60 years. *Trends Genet.* **6**, 422-426.

Johnson, D.G., Ohtani, K. and Nevins, J.R. (1994). Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. *Genes Dev.* **8**, 1514-1525.

Judd, B.H. (1995). Mutations of *zeste* that mediate transvection are recessive enhancers of position-effect variegation in *Drosophila melanogaster*. *Genetics* **141**, 245-253.

Karpen, G.H. and Spradling, A.C. (1991). Reduced DNA polytenization of a minichromosome region undergoing position-effect variegation in *Drosophila*. *Cell* **63**, 97-107.

La Thangue, N.B. (1994). DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. *Trends Biochem. Sci.* **19**, 108-114.

Laman, H., Balderes, D. and Shore, D. (1995). Disturbance of normal cell cycle progression enhances the establishment of transcriptional silencing in *Saccharomyces cerevisiae*. *Mol.Cell. Biol.* **15**, 3608-3617.

Lima de Faria, A. and Jarworska, H. (1968). Late DNA synthesis in Heterochromatin. *Nature* 217, 138-142.

Lindsley, D.L., and Zimm, G.G.(1992) *The Genome of* Drosophila melanogaster. Academic Press, Inc.

Maniatis, T., Hardison, R.C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G.K. and Efstratiadis, A. (1978). The isolation of structural genes from libraries of eucaryotic DNA. *Cell* **15**, 687-701.

Mason P.J., Enver T., Wilkingson D., and Williams J. G.(1993). Assay Of Gene Transcription In Vivo. In *Gene Transcription, A Practical Approach* (ed. B. D. Hames and S. J. Higgins), pp. 5-61. Oxford: IRL press.

Miller, A.M. and Nasmyth, K.A. (1984). Role of DNA replication in the repression of silent mating type loci in yeast. *Nature* **312**, 247-251.

Moehrle, A. and Paro, R. (1994). Spreading the silence: epigenetic transcriptional regulation during *Drosophila* development. *Dev. Genet.* **15**, 478-484.

Neuman, E., Flemington, E.K., Sellers, W.R. and Kaelin, W., Jr. (1994). Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. *Mol. Cell. Biol.* **14**, 6607-6615.

Nevins, J.R. (1992). E2F: a link between the Rb tumor supressor protein and viral oncoproteins. *Science* **258**, 424-429.

Ohtani, K. and Nevins, J.R. (1994). Functional properties of a *Drosophila* homolog of the E2F1 gene. *Mol. Cell. Biol.* **14**, 1603-1612.

Paro, R. and Hogness, D.S. (1991). The Polycomb protein shares a homologous domain with a heterochromatin- associated protein of *Drosophila*. *Proc. Natl Acad. Sci. USA* **88**, 263-267.

Pirrotta, V. (1986). Cloning *Drosophila* genes. In *Drosophila, a practical approach* (ed. D. B. Roberts), pp. 83-109, Oxford: IRL press.

Pirrotta, V. (1991). The genetics and molecular biology of *zeste* in *Drosophila* melanogaster. *Adv. Genet.* **29**, 301-348.

Qin, X.Q., Livingston, D.M., Kaelin, W., Jr. and Adams, P.D. (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Natl Acad. Sci. USA* **91**, 10918-10922.

Raff, J.W., Kellum, R. and Alberts, B. (1994). The *Drosophila* GAGA transcription factor is associated with specific regions of heterochromatin throughout the cell cycle. *EMBO J.* **13**, 5977-5983.

Renauld, H., Aparicio, O.M., Zierath, P.D., Billington, B.L., Chhablani, S.K. and Gottschling, D.E. (1993). Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. *Genes Dev.* **7**, 1133-1145.

Reuter, G. and Wolff, I. (1981). Isolation of a dominant suppressor mutations for position-effect variegation in *Drosophila melanogaster*. *Mol. Gen. Genet.* **182**, 516.

Reuter, G., Dorn R. and Hoffmann, H.J. (1982). Butyrate sensitive suppressor of position-effect variegation mutations in *Drosophila melanogaster*. *Mol. Gen. Genet.* 188, 480-485.

Reuter, G., Giarre, M., Farah, J., Gausz, J., Spierer, A. and Spierer, P. (1990). Dependence of position-effect variegation in *Drosophila* on dose of a gene encoding an unusual zinc-finger protein. *Nature* **344**, 219-223.

Reuter, G. and Spierer, P. (1992). Position effect variegation and chromatin proteins. *BioEssays* **14**, 605-612.

Reuter, G., Hofmann G., Dorn R., Samweber H. (1993). Construction and caracterisation of a TM3 balancer carrying P[(ry+)D2-3] as a stable transposase source. *Drosophila Inf. Service*, **72**, 78-79.

Schneuwly, S., Kuriowa, A., Baumgartner, P. and Gehring, W.J. (1986). Structural organization and sequence of the homeotic gene Antennapedia of *Drosophila* melanogaster. *EMBO J.* **4**, 733-739.

Seifert, H.S., Chen, E.Y., So, M. and Heffron, F. (1986). Shuttle mutagenesis: a method of transposon mutagenesis for Saccharomyces cerevisiae. *Proc. Natl Acad. Sci. USA* **83**, 735-739.

Shan, B. and Lee, W.H. (1994). Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. *Mol. Cell. Biol.* **14**, 8166-8173.

Sinclair, D.A., Lloyd, V.K. and Grigliatti, T.A. (1989). Characterisation of mutations that enhance position-effect variegation in *Drosophila melanogaster*. *Mol. Gen. Genet.* 216, 328-333.

Singh, P., Wong, S.H. and Hong, W. (1994). Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. *EMBO J.* **13**, 3329-3338.

Spradling, A.C. (1986). *P element-mediated transformation, in Drosophila, a practical approach* (ed. D. B. Roberts), pp 175-196, Oxford: IRL press.

Szekely, L., Uzvolgyi, E., Jiang, W.-Q., Durko, M., Wiman, K. G., Klein, G. and Sumegi J. (1991). Subcellular localisation of the retinoblastoma protein. *Cell Growth Differ.* **2**, 287-295.

Tamkun, J.W., Deuring, R., Scott, M.P., Kissinger, M., Pattatucci, A.M., Kaufman, T.C. and Kennison, J.A. (1992). brahma: a regulator of drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SW12. *Cell* **68**, 561-572.

Tower, J. Karpen, G.H., Craig, N. and Spradling, A.C. (1993). Preferential transposition of *Drosophila* P elements to nearby chromosomal sites. *Genetics* **133**, 347-359.

Tartof, K.D., Bishop, C., Jones, M., Hobbs, C.A. and Locke, J. (1989). Towards an understanding of position effect variegation. *Dev. Genet.* **10**, 162-176.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**, 81-85.

Tschiersch, B., Hofmann, A., Krauss, V., Dorn, R., Korge, G. and Reuter, G. (1994). The protein encoded by the *Drosophila* position-effect variegation suppressor gene Su(var)3-9 combines domains of antagonistic regulators of homeotic gene complexes. *EMBO J.* **13**, 3822-3831.

Weintraub, S.J., Chow, K.N., Luo, R.X., Zhang, S.H., He, S. and Dean, D.C. (1995). Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature* **375**, 812-815.

Wharton, K.A., Yedvobnick, B., Finnerty, V.G. and Artanavanis-Tsakonas, S. (1985). opa: a novel family of transcribed repeats shared by the Notch locus and other developmentally regulated loci in D. melanogaster. *Cell* 40, 55-62.

Wu, X. and Levine, A.J. (1994). p53 and E2F-1 cooperate to mediate apoptosis. *Proc. Natl Acad. Sci. USA* **91**, 3602-3606.

Wustmann, G., Szidonya, J., Taubert, H. and Reuter, G. (1989). The genetics of position-effect variegation modifying loci in *Drosophila melanogaster*. *Mol. Gen. Genet.* **217**, 520-527.

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