MOLECULAR CHARACTERIZATION OF P ELEMENT INSERTIONS AFFECTING THE *LIGHT* LOCUS OF *DROSOPHILA MELANOGASTER*

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Molecular Characterization of P Element Insertions

Affecting the light Locus of Drosophila melanogaster

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ABSTRACT

The purpose of this work was to investigate hybrid dysgenic mutants and resulting non-revertants associated with the heterochromatic *light* locus in *Drosophila melanogaster* to determine the nature of the P element insertions at the DNA level.

In hybrid dysgenesis transposition and excision of P elements and P-mediated chromosome rearrangements result in germ-line alterations. Excision of P elements is often precise, producing revertants, but can also be imprecise, resulting in non-reverting lines. Previous hybrid dysgenic crosses produced two mutants associated with the *light* locus, hd1 and hd2, that are the basis for this study. Both are the result of 1.1 kb P element insertions. Of the three non-revertants derived from hd2, NRII12, NRII39 and NRI31, hybridization studies showed an increase in band size of 0.5 kb for NRII12, and showed an identical shift of 0.1 kb for NRII39 and NRI31, suggesting that excision was imprecise, and P sequences remained at the site of insertion.

In order to determine the nature of the P element mutations, hd2 clones, previously obtained from a genomic library, were sequenced. The P element insertion site was identified and the sequence and orientation of the element was determined. The P element contained an internal deletion, producing the non-autonomous element 1.1 kb in length.

In order to investigate NRII12, clones from a genomic library were sequenced and revealed that the P element contained another internal deletion as well as the one found in hd2, resulting in a 0.4 kb element. The P element contains only 16 of the 31 base pairs of the terminal inverted repeat essential for transposition, which accounts for its non-reverting character.

To determine the sequence of NRII39 and NRI31, PCR was used to amplify the region associated with the *light* locus containing the P element sequences. Sequencing of the single-stranded PCR products showed that the two non-revertants were identical. Each retained the 8 bp genomic duplication at the site of insertion as well as 30 bp of P element DNA .

In addition, PCR was used to determine the insertion site of the P element of hd1. Using primers derived from the sequenced DNA associated with the *light* locus, it was determined to be inserted 100 bp downstream of the hd2 insertion site.

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INTRODUCTION

P elements are a family of transposable elements found in the fruit fly *Drosophila melanogaster.* The effects of P elements were first observed in 1960, when males from natural populations were crossed with females from established laboratory stocks, and unusual phenomena were seen (Hiraizumi and Crow, 1960). These included temperature-dependent sterility, male recombination, chromosomal aberrations, segregation distortion, elevated rates of mutation, nondisjunction, and altered frequency of female recombination (Kidwell *et al.*, 1977). These traits were not seen in the reciprocal cross, and were almost always restricted to the germline (Engels, 1983). In 1977, the syndrome was termed hybrid dysgenesis (Kidwell *et al.*, 1977). Strains were classified as either M for maternal (lab stocks) or P for paternal (wild stocks), such that the germ cells of the F1 hybrids of an M female and P male cross exhibited the characteristics of hybrid dysgenesis.

Bingham *et al.* demonstrated that the hybrid dysgenesis syndrome was due to multiple mobile elements which they termed P elements (Bingham *et al.*, 1982). P strains were found to contain approximately 30 to 50 elements distributed throughout the genome whereas M strains contained none (Bingham *et al.*, 1982). Furthermore, the P strain eggs have P cytotype that results in repression of transposition (O'Hare and Rubin, 1983). The M strains with M cytotype are not capable of repression. Therefore, when P elements are introduced into M cytotype eggs, the P elements are mobilized, transpose at high frequency to new chromosomal locations and the syndrome of hybrid dysgenesis is observed (Engels, 1979). Both chromosomal and cytoplasmic components are involved in control of induction and stability of mutants induced by hybrid dysgenesis (Kidwell *et al.*, 1977). The

determination of cytotype is still not well understood. It has an unusual pattern of inheritance that depends on several factors such as maternal age, temperature, and the particular strains involved (Kidwell, 1981; Ronsseray, 1984, 1986).

O'Hare and Rubin characterized the complete P element by isolating insertions into a dysgenesis-induced allele at the previously cloned *white* locus (O'Hare and Rubin, 1983). All complete P elements are 2907 bp in length and are identical in sequence with the exception of positions 32 and 33, which are hypervariable (Engels, 1988). The terminal 31 bp inverted repeats are essential for transposition. There is also an 11 bp inverted repeat found 125 bp from each end, a 17 bp inverted repeat found within exon 1 and an overlapping 20 bp direct repeat within exon 3 whose functions are unknown (O'Hare and Rubin, 1983) (Figure 1A).

There are 4 exons within the complete P element (Rio *et al.*, 1986). P element-mediated germline transformation allows a mutated P element to be returned to flies so that the effect of the mutation on P element functions can be determined (Spradling and Rubin, 1982; Rubin and Spradling, 1982). Using this technique, it was shown that all 4 open reading frames are required to encode the 87 kd transposase (Karess and Rubin, 1984) which is necessary for transposition (Spradling and Rubin, 1982; Rubin and Spradling, 1982; Engels, 1984) as well as for precise and imprecise excision events (Rubin *et al.*, 1982).

Only one third of the elements found in a P strain are full length P elements. The remainder are heterogenous in length, ranging from 0.5 to 1.6 kb, and are derived from the complete element by internal deletions (Rubin *et al.*, 1982; O'Hare and Rubin, 1983). These nonautonomous elements are still capable of transposition and excision, but only in the presence of an autonomous element that can provide transposase (Engels, 1988).

FIGURE 1 The structure of the complete 2.9 kb P element. Numbers shown refer to the numbering used for the published complete P element sequence (O'Hare and Rubin, 1983).

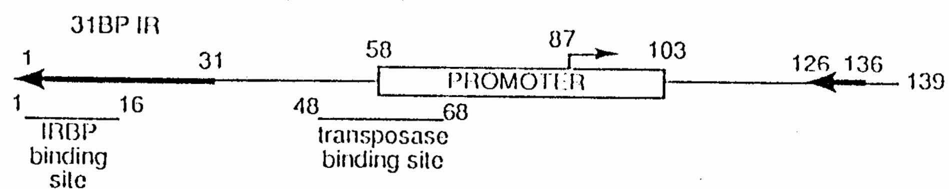
A) Schematic diagram of the 2.9 kb P element including its repeat structures (data from O'Hare and Rubin, 1983).

B) Map of the 139 bp at the 5' end of the P element that are necessary for transposition. Known binding sites for transposase and inverted repeat binding protein (IRBP) are shown along with the inverted repeats (IR), the location of promoter sequences and the start of transcription (data from Kaufman *et al*, 1989).

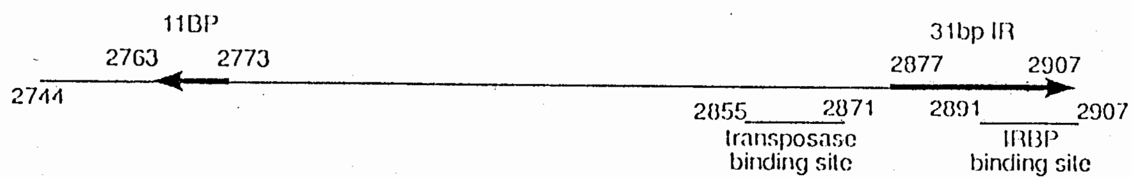
C) Map of the 163 bp at the 3' end of the P element that are necessary for transposition. Known binding sites for IRBP and transposase are shown along with the inverted repeats (data from Kaufman *et al*, 1989).

A

B



C



The mechanism of transposition is still unknown. Transposition is restricted to the germline as a result of differential splicing of the transposase mRNA. The ORF2-ORF3 intron is properly spliced in the germ cells, resulting in the 87 kd transposase (Laski *et al.*, 1986). In somatic cells this splice does not occur, and the resulting mRNA encodes a 66 kd polypeptide (Rio *et al.*, 1986). This protein is now known to be a negative regulator of transposition (Kaufman *et al.*, 1989). Little is known about the mechanisms involved in controlling this alternative splicing. Recently, sequences from 1911 to 2150, which include the ORF2-ORF3 intron, have been shown to confer germline specificity to this splicing event (Laski, 1989).

Certain P element sequences are essential for transposition (Figure 1B). At the 5' end approximately 139 bp of DNA, including the 31 and 11 bp inverted repeats, is required (O'Hare and Rubin, 1983; Rubin and Spradling, 1983). Recently, it has been shown that transposase protects a region from position 48 to 68, interacting with a 10 bp consensus sequence within this region (Kaufman *et al.*, 1989). It is interesting to note that the binding of transposase to this site overlaps sequences essential for P element transcription (Kaufman *et al.*, 1989). This suggests that either the transposase or the smaller 66 kd protein, which both have a hypothetical helix-turn-helix DNA binding motif (Rio *et al.*, 1986), may regulate the level of transcription from the P element promoter (Kaufman *et al.*, 1989).

At the 3' end of the P element, 163 bp are essential for transposition (Mullins et al., 1989) (Figure 1C). Transposase protects nucleotides 2855 to 2871, interacting with the same 10 bp consensus sequence found at the 5' end, that is only found at these two sites in the P element (Kaufman *et al.*, 1989). Although the 3' and 5' ends have several sequence motifs in common, they are not interchangeable; for example an element with both ends containing 5' sequences, or both containing 3'ends is

incapable of transposition (Mullins *et al.*, 1989). The discovery that the transposase binding sites are asymmetrical in their position relative to the ends of the element is one likely reason why P element ends are not interchangeable (Kaufman *et al.*, 1989). Although each end of the P element must have a specific role in transposition, insertion of the element can occur in both orientations (Engels, 1988).

Transposase also has a high non-specific affinity for DNA (Kaufman *et al.*, 1989). This suggests that additional factors are likely necessary to direct the transposase to its specific binding site (Rio and Rubin, 1988). To isolate host factors that may play a role in transposition, the 31 bp terminal repeats, essential for transposition, were used in footprinting studies with partially fractionated nuclear extracts (Rio and Rubin, 1988). An inverted repeat binding protein was identified that interacts with the outer 16 bp of both terminal repeats (Rio and Rubin, 1988).

O'Hare and Rubin found that 8 bp of chromosomal DNA are duplicated in direct orientation upon insertion of a P element (O'Hare and Rubin, 1983). This is thought to be the result of a staggered cut at the target site by the transposase as a consequence of the transposition mechanism (Calos and Miller, 1980; Weinhert *et al.*, 1983). The duplication is not required for transposition (Mullins, 1989); however, it seems to have a role in precise excision (Engels, 1988).

P elements move in a non-random manner, preferring some regions of the genome over others. For example, the frequency of mutation varies from 10^{-3} at the *singed* locus to 10^{-6} at the *alcohol dehydrogenase* locus (reviewed in Engels, 1983). Within a locus there can also be considerable specificity to the insertion site of the P element. Three *white* mutants sequenced by O'Hare and Rubin have P element insertions at precisely the same nucleotide (O'Hare and Rubin, 1983). Several other "hotspots" for insertion have been found in the *singed* locus, *notch* locus and the

white locus as well (Roiha *et al.*, 1988; Kelley *et al.*, 1987; O'Hare and Rubin, 1983). O'Hare and Rubin compared 18 P element insertion sites and derived an 8 bp consensus sequence (O'Hare and Rubin, 1983), although further studies show that only a weak homology is necessary for insertion (Searles *et al.*, 1986). Other factors such as chromosome structure may also contribute to the specificity of P element insertion. P elements appear to prefer insertion sites in the regulatory regions of genes. Of 14 mutants induced by hybrid dysgenesis at the *Notch* locus, 13 involved P element insertions at or near the transcription start site of the gene (Kelley *et al.*, 1987). P elements have also been shown to cluster in the 5' control regions of other loci such as *rudimentary* (Tsubota *et al.*, 1985), the large subunit of RNA polymerase II, *RpII215* (Voelker *et al.*, 1984), and a small heat shock gene, *Hsp28* (Eissenberg and Elgin, 1987).

P elements can undergo several types of excision. Although the mechanism for excision is still unclear, the process is dependent on transposase. Precise excision of the P element and one copy of the 8 bp genomic duplication restores the original sequence. Internal excisions of varying sizes disrupt the transposase coding sequences, but the resulting element is still capable of transposition as long as the essential terminal sequences, including the 31 bp inverted repeats, are intact. Other imprecise excisions can remove flanking DNA as well as part or all of a P element. Often the breakpoints occur at short (2 to 6 bp) direct repeats (O'Hare and Rubin, 1983; Black *et al.*, 1988; Rio *et al.*, 1986; Tsubota and Schedl, 1986). If the mechanism of precise excision depends on the 8bp direct repeat created by duplication, then imprecise excisions may be special cases where other direct repeats are used instead (Engels, 1988).

Reversions of mutations caused by P element insertion occur at high frequencies of 10^{-2} to 10^{-3} (Rubin *et al.*, 1982). These reversions are usually associated with precise or near-precise excision of the element and one copy of the 8 bp genomic duplication (O'Hare and Rubin, 1983; Searles *et al.*, 1986; Tsubota and Schedl, 1986). Most excisions that appear exact at the level of Southern blotting, are actually due to internal deletions leaving small (<40 bp) fragments of P element sequence behind. In many cases the breakpoints are located in the 31 bp inverted repeats (Searles *et al.*, 1986).

To date, the evidence suggests that P elements have a preference for euchromatic sites over heterochromatic sites (Engels, 1988). This is based on *in situ* hybridizations with P elements (Bingham *et al.*, 1982), a study of dysgenesis induced chromosome rearrangements that showed the breakpoints to almost all be euchromatic (Engels and Preston, 1984), and the cytological positions of many studied insertions such as the xanthene dehydrogenase gene (Spradling and Rubin, 1983). There have been only 2 previously reported cases of P element insertions in heterochromatin, both on the Y chromosome (Engels, 1988). The hybrid dysgenesis-induced mutants that are the basis of this thesis are unusual as they are associated with DNA that is located in the heterochromatic region of chromosome 2L of *Drosophila melanogaster,* and may provide more insight into the nature of heterochromatin and P-element transposition.

Heterochromatin is the term used to describe certain regions of the mitotic chromosomes which remain dense throughout the cell cycle (Heitz, 1928). There are two types; alpha, which never uncoils from its compact form, and beta, which uncoils somewhat to form a diffuse structure (Lakhotia and Jacob, 1974). Unusual properties of heterochromatin such as chromosomal condensation, under-replication

in polytene chromosomes, late replication, infrequent recombination and the phenomenon of position-effect variegation suggest that genes in these regions must differ from euchromatic genes (reviewed in John and Miklos, 1979). Position-effect variegation is the term given to the observation that the expression of euchromatic genes is altered in some cells of a tissue when rearrangements bring the gene within the range of influence of heterochromatin (Hannah, 1951). This phenomenon is not yet fully understood but provides evidence for the importance of the structure of heterochromatin for gene regulation.

Heterochromatin represents 28% of the *Drosophila* genome, yet its function is unknown (Peacock, *et al.*, 1977). For the most part it consists of multiple-copy, short sequence DNA that is transcriptionally inert, interspersed with rDNA and families of middle-repetitve transposable elements (Brutlag, 1980). Heterochromatin has been localized to pericentric, telomeric or intercallary sites on the chromosome, by observation of heteropycnotic regions (chromosomal regions that remain condensed blocks of chromatin throughout the cell cycle) (Hannah, 1951). The Y chromosome is completely heterochromatic, as is the proximal one third to one half of the X chromosome (Hannah, 1951). Chromosomes 2 and 3 are heterochromatic to the right and left of the centromere for one fourth of the chromosome (Hannah, 1951). *Drosophila melanogaster* is known to be able to tolerate deletions of up to 80% of the heterochromatin on one X chromosome with little effect, suggesting that it has no function (Yamamoto and Miklos, 1978). Other studies have suggested that different segments of heterochromatin in a genome may have different roles ranging from chromosome pairing and segregation (Cooper, 1964; Peacock and Miklos, 1973), chromosome rearrangement (Holmquist and Dancis, 1979) and recombination (Miklos and Nankivell, 1976).

A correlation between the location of highly repeated DNA sequences and heterochromatin, shown by *in situ* hybridizations (Jones and Robertson, 1970; Pardue and Gall, 1970; Peacock *et al.*, 1973), supported the original idea that heterochromatin was genetically inert (Gershenson, 1933). However, recent studies have revealed important loci located in heterochromatin, although gene density is much lower than that of euchromatin (Spradling *et al.*, 1975; Hilliker and Holm, 1975; Hilliker, 1980; Pimpinelli *et al.*, 1988; Marchant and Holm, 1988; 1988b) and little is known about their organization, expression and regulation. The known heterochromatic loci were discovered genetically, by analyzing deficiencies from compound chromosomes and their detachment products (Hilliker, 1976). The Y chromosome contains loci that include 7 fertility factors and a nucleolar organizer (Brousseau, 1960). The X chromosome heterochromatin has only 1 locus described to date (Schalet and Lefevre, 1973). Analysis of chromosome 3 revealed 11 genes (Marchant and Holm, 1988; 1988b). Chromosome 4 was originally thought to lack any visible heteropycnotic regions; however, the small size of the chromosome was likely responsible for this conclusion (Hannah, 1951). Subsequent studies suggest that most of chromosome 4 is heterochromatic, including recent microcloning of part of this chromosome which show that clusters of localized repeats and sequences are also found in heterochromatin of the X chromosome (Miklos *et al.*, 1988) On chromosome 2 eighteen loci have been identified including the *light* locus (Hilliker, 1980).

The *light* locus is located in proximal heterochromatin of chromosome 2L in *Drosophila melanogaster* (Hilliker and Holm, 1975), and is known to undergo position-effect variegation if placed near euchromatin (Schulz, 1936; Hessler, 1958). It is an essential gene with unknown function for which nonlethal hypomorphic

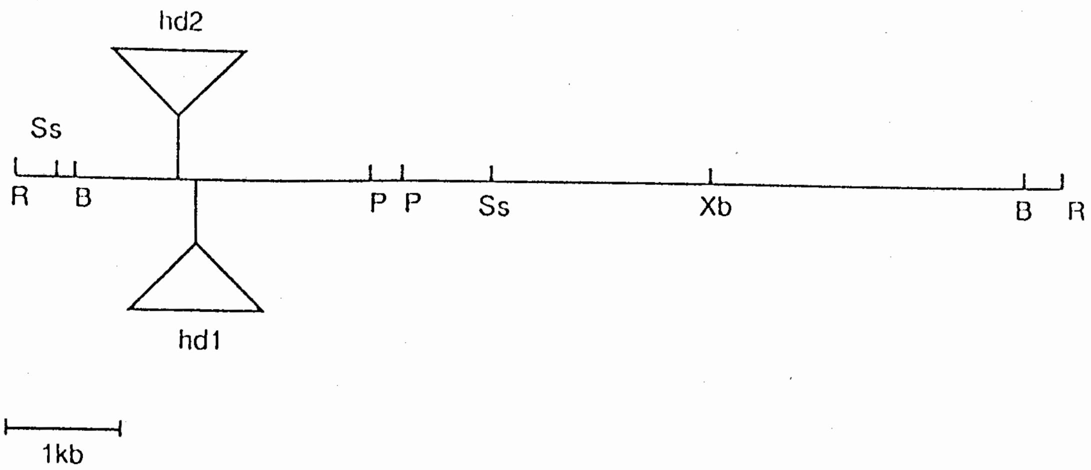
mutations result in reduced pigment levels in several tissues including the eyes and Malpigian tubules (Hilliker, 1976). Devlin *et al.* have cloned a putative *light* clone by P element tagging (Searles *et al.*, 1982; Devlin *et al.*, submitted). The cloned region is unusual for heterochromatin in that it consists of single-copy DNA surrounded by middle-repetitive sequences (Devlin *et al.*, submitted). *In situ* hybridizations revealed that unlike other heterochromatic sequences, the cloned sequences may not be under-replicated (Devlin *et al.*, submitted). The hybridization signal is diffuse, suggesting that this region of heterochromatin may have an unusual organization (Devlin *et al.*, submitted).

To obtain the heterochromatic clone, putatively associated with the *light* locus, two *light* mutations, hd1 and hd2, were induced by hybrid dysgenesis, and hd2 was chosen to attempt to clone the locus by P element tagging. Southern blots showed that both these mutants contained 1.1kb P element insertions (Figure 2). In addition to these mutants, three nonreverting lines arose from hd2 (NRII12, NRII39 and NRI31), presumably from some type of excision event. The purpose of this work was to further characterize hd2 and the three nonrevertants derived from this mutant, as well as to examine the P element insertion site of the other hybrid dysgenic mutant, hd1.

FIGURE 2 Restriction map of the 9 kb Eco R1 clone putatively associated with the *light* locus. The P element insertion sites of hybrid dysgenesis-induced mutants hd1 and hd2 are shown (R. Devlin *et al*, submitted).

Restriction sites:

B = BamHI; R = EcoRI; P = PstI; Ss = SstI: Xb = XbaI


hd2
Ss
R
B
P
P
Ss
Xb
B
R
hd1
1kb

MATERIALS AND METHODS

MATERIALS

Restriction enzymes were obtained from Pharmacia and BRL. Klenow, ligase, and calf intestinal alkaline phosphatase were obtained from Pharmacia. All radioactive nucleotides were from ICN.

STOCKS OF DROSOPHILA MELANOGASTER

hd2, hd1, NRII12, NRII39 and NRI31 strains of *Drosophila melanogaster* used in this analysis were supplied by R. Devlin. The mutations were balanced over an In(2LR)SM1,Cy chromosome marked with an EMS-induced *light* mutation (lt^{AH}) induced by A. Hilliker (Devlin *et al.*, submitted; Hilliker and Holm, 1975). The balancer chromosome contains a duplication for the *light* locus, with one wild-type copy; the other copy contains a 150 bp insertion, a 1 kb deletion and an altered restriction pattern within the EcoRI sites of the putative *light* region.

ISOLATION OF GENOMIC DNA

DNA was isolated by a rapid extraction method developed by R. Devlin (personal communication). 300 to 600 mg of flies were homogenized in a Wheaton 7 ml homogenizer using phenol saturated with TE (10mM Tris, 1mM EDTA) and extraction buffer (100 mM Tris pH 8.5, 50 mM NaCl, 25 mM EDTA, 1% sarkosyl). After centrifugation, the supernatant was recovered and extracted again with phenol, followed by phenol:chloroform:isoamyl alcohol (50:50:1). After 15 minute digestion with RNase A (200 μg/ml) at 37°C, the solution was extracted with phenol, then

phenol:chloroform:isoamyl alcohol as above, and chloroform:isoamyl alcohol:ethanol (50:50:1). Two volumes of 95% ethanol and 0.1 volume of 3M sodium acetate were used to precipitate the DNA, followed by a 70% ethanol wash. The DNA pellet was dissolved in double distilled water and stored at 4°C. This yielded approximately 200 to 300 ng of DNA per mg of flies.

RESTRICTION DIGESTS

Restriction enzymes and appropriate buffers were obtained from Pharmacia and BRL and the reaction conditions outlined by the suppliers were followed. In genomic DNA digests, 5 µg of DNA was digested with an excess of enzyme for 7 hours to ensure complete digestion.

AGAROSE GEL ELECTROPHORESIS

Agarose gels were made from agarose dissolved in 1X TBE (89mM Tris, 89mM borate and 2.5mM EDTA) and ethidium bromide was added to a final concentration of 0.2 µg/µl. The gels differed in their agarose concentration depending on the size of the DNA fragments to be separated (as calculated in Maniatis, 1982). Genomic digests were run on 0.5% gels, whereas most plasmid digests were generally run on 0.7% gels. All gels were run in 1X TBE buffer. After electrophoresis, gels were photographed on a short wave ultraviolet transilluminator, using Kodak film.

RECOVERY OF DNA FROM AGAROSE GELS

DNA was recovered from agarose gels by electro-elution. A small gel slice containing the DNA band of interest was placed into dialysis tubing. Enough 1X TBE was added to cover the gel slice, the tubing was sealed at the ends with dialysis

clips and immersed in the electrophoresis tank, and the DNA was electroeluted into the buffer. When complete, the polarity of the electrodes was reversed for 30 seconds to remove DNA from the side of the tubing. The buffer was collected, spun in the ultra-microcentrifuge for 10 minutes to pellet any agarose, and the supernatant was collected, precipitated on dry ice for 15 minutes with 0.1 volume 3M sodium acetate and two volumes of 95% ethanol. DNA pellets were washed with 70% ethanol, dried and resuspended in 1X TE.

LABELLING OF PROBES

In order to label DNA probes, 0.5 μg of cloned DNA was labelled by nick translation (Rigby *et al.*, 1977), using α-^{32}P dATP and/or α-^{32}P dCTP (10 μCi/μl stock). The reaction was carried out for 2 hours at 15°C. Unincorporated nucleotides were removed from the labelled DNA by Sephadex G-25 chromatography. The specific activity of labelled DNA was measured by Cherenkov counting in a scintillation counter. Probes prepared in this manner had a specific activity of approximately 2×10^7 cpm/μg.

SOUTHERN BLOTS

Southern blots were carried out according to Maniatis (Maniatis *et al.*, 1982). Genomic DNA was run on a .5% agarose gel (5 μg/lane) for 12 hours at 30 V. Before transfer, the gel was treated for 15 minutes in 0.25 HCl, then denatured in 1.5M NaCl, 0.5M NaOH, with two 15 minute washes, and neutralized in 1M ammonium acetate, 0.02M NaOH, by two 30 minute washes. Transfer of the DNA to the nitrocellulose (Smith and Summers, 1980) was carried out overnight, and DNA was then baked onto the filter under vacuum at 80°C for 2 hours.

HYBRIDIZATION OF DNA

The nitrocellulose filters were prehybridized for at least one hour at 60°C in 5X SSPE and 0.3% SDS (1X SSPE is 0.18M NaCl, 10mM sodium phosphate, 1mM EDTA pH 7.4)(Maniatis *et al.*, 1982). Denhardt's solution and 10 mg of boiled salmon sperm DNA to bind nonspecifically to the nitrocellulose, were also added to the prehybridization (Maniatis *et al.*, 1982).

Hybridizations were carried out in 5X SSPE, 0.3% SDS containing the boiled probe (approximately $1x10^7$ cpm). Hybridizations were done at 60°C and left overnight. Three post-hybridization washes (10 minutes, 10 minutes, and 15 minutes) were done with 0.2X SSPE, 0.2% SDS. The filters were then exposed to pre-flashed Kodak XAR-5 or XK-1 film for 12-24 hours, with a screen, at -70°C.

TRANSFORMATION OF DNA

E. coli JM 83 cells were made competent according to the $CaCl_2$ procedure (Mandel and Higa, 1970). Plasmid DNA was introduced into the competent cells by incubating on ice for 1 hour before being heat shocked at 42°C for 2 min (Hanahan, 1983). The cells were plated directly on NZYM plates (Maniatis *et al.*, 1982) with ampicillin added to 100 ug/ml.

ISOLATION OF PLASMID DNA

Plasmid DNA was isolated from bacterial cells in alkaline lysis preps using the procedure from Maniatis (Maniatis *et al.*, 1982). Cells were treated with 1% SDS and 0.2N NaOH, followed by 3M KAc (Birnboim and Doly, 1979). After centrifugation, the plasmid DNA was recovered in the supernatant, extracted twice

with phenol:chloroform:isoamyl alcohol (1:1) and precipitated with 95% ethanol. DNA pellets were washed in 70% ethanol, dried, and resuspended in 1X TE. The DNA solution was then treated with RNase A (20 μg/ml) for 20 minutes at 37°C.

SEQUENCING OF DNA

To prepare template for sequencing, plasmid DNA was isolated from one 10 ml culture and gel purified to obtain supercoiled DNA, as described above, yielding approximately 5 μg DNA for each set of sequencing reactions.

Cloned DNA was sequenced by the dideoxy method (Sanger *et al.*, 1977). The gel purified pUC plasmids had to first be denatured by the addition of 2N NaOH, and precipitated with 5M ammonium acetate and 95% ethanol to allow for single strand sequencing (Hattori and Sakaki, 1986). Collapsed plasmids were pelleted and stored at -20°C until use.

All reagents for the sequencing reactions were supplied by Pharmacia. Sequencing reactions were carried out according to the Klenow procedure described by Hattori and Sakaki (Hattori and Sakaki, 1986). The collapsed plasmid was resuspended in distilled water, with either the forward and reverse primer for pUC19 (at a concentration of 0.2μM) and Klenow buffer. Primers were annealed to the template by incubating at 65°C for 15 minutes, followed by cooling for 15 minutes at room temperature. One unit of Klenow enzyme and 2μl (20μCi) of ^{35}S dATP were added to the annealed primer/template DNA, and divided equally into the four tubes each containing 3 μl of G,A,T or C deoxy/dideoxynucleotide mix. Reactions were incubated at 42°C for 20 minutes. A chase solution (1 μl of 1mM dNTPs) was added to each reaction tube and reactions were incubated for another 20 minutes at 42°C before being stopped by the addition of stop solution (80% deionized

formamide, 50mM Tris borate pH8.3, 1mM EDTA, 0.1% xylene cyanol and 1% bromophenol blue). ^{35}S dATP was used in the reactions instead of ^{32}P dATP because of advantages such as less exposure to radioactivity, longer half-life, and sharper bands (Williams *et al.*, 1986).

Samples were heated to 85°C for 3 minutes, then kept on ice until 3 μl of each sample were loaded on a sequencing gel. Sequencing reactions were run on 6% acrylamide, 8M urea gels at 1550 V for approximately 3.5 hours. Early gels were washed for 30 minutes in 10% acetic acid and 12% methanol, in order to fix the urea and prevent quelching of the ^{35}S ATP signal. Gels were backed on filter paper and dried under vacuum at 80°C for 30 minutes, before being placed directly on Kodak XAR film for 18 hours. Difficulties in maintaining an intact gel throughout the soaking procedure however, outweighed any advantage. Later gels were dried directly without soaking, without any significant change in clarity. The signal obtained from the unsoaked gels was fainter than that of the soaked gels, and a longer exposure of 24 to 48 hours was necessary.

SUBCLONING

All clones and subclones were cloned into pUC19 (Messing *et al.*, 1985). Vector was first cut with the appropriate restriction enzyme or enzymes. The enzyme was inactivated by extraction with phenol/chloroform followed by ethanol precipitation. To improve upon the number of positive clones obtained, the vector was dephosphorylated at the 5' ends using calf intestinal alkaline phosphatase (Maniatis *et al.*, 1982). If two enzymes were used for directional cloning, the vector was gel purified to separate the vector from the small fragment of vector polylinker. To prepare insert DNA, the fragment of interest was gel purified as well. Ligation

reactions were set up using a 3:1 molar ratio of insert to vector. For ligation of DNA with blunt ends, a 10:1 molar ratio of insert to vector was used. Ligations were carried out according to Maniatis (Maniatis, *et al.*, 1982). Half of the ligation mix was used to transform competent *E. coli* JM 83 cells as previously described. The transformation mix was then plated on NZYM plates containing ampicillin (100 ug/ml) IPTG (160 ug/ml) and XGAL (40 ug/ml). The presence of functional B galactosidase (B gal) was used to distinguish recombinant clones from religated vector (Messing *et al.*, 1985).

PCR OLIGONUCLEOTIDE PURIFICATION

Oligonucleotides for PCR reactions (20 bp in length) were synthesized by Tom Atkinson, Department of Medical Genetics, UBC. These were purified by using Millipore Sep-Pack C10 cartridges. These cartridges were used with water as a polar solvent, that allowed the DNA to be retained on the cartridge while washing away salts and other high polarity contaminants. Washing with 20% acetonitrile (less polar) then allowed the cartridge eluant to be collected. The resulting oligonucleotide solution was dried using a Savant Speed Vac Concentrator. Pellets were frozen at -20°C until use.

PCR AMPLIFICATION

Polymerase Chain Reaction (PCR) is an enzymatic method for *in vitro* amplification of specific DNA fragments outlined in detail in many articles (White *et al.*, 1989; Williams, 1989; Sakai *et al.*, 1989; Kwok and Higuchi, 1989) Reagents used in the reactions were supplied by Perkin Elemer Cetus (GeneAmp DNA Amplification Reagent Kit) and Taq polymerase was supplied by Perkin Elmer Cetus

or BRL. Amplification reactions were set up with a total volume of 25 μl according to the protocol by Perkin Elmer Cetus Corp. One ng of genomic DNA was combined with 1.3 μl of each primer (10mM stock), along with deoxynucleotides (200 μM each dNTP), 10X reaction buffer (100nM Tris-HCl pH8.3, 500mMKCl, 15mM $MgCl_2$, .1% gelatin) and 1 unit of Taq polymerase. W-1 detergent was also added to a final concentration of .05% with Taq polymerase supplied from BRL in order to stabilize the enzyme. A layer of mineral oil was added on top of the reactions to prevent evaporation during the amplification.

An Ericomp Programmable Cyclic Reactor was used to carry out the amplification cycles. Annealing of primers to genomic DNA was carried out at 50°C. Extension of primers was carried out at 72°C for 2 minutes to allow for complete extension of the desired region. 25 cycles of melting, annealing and extending resulted in approximately 3 μg of the desired fragment.

SINGLE STRANDED PCR

Polymerase chain reactions with asymmetric amounts of primers produces single stranded DNA products (Gyllensten, 1989; Gyllensten and Erlich, 1989). To prepare a template for a single stranded reaction, the double stranded PCR product was gel purified on a low melting point TA gel as outlined in the Cetus protocol (1X TA buffer is 0.04M Tris-acetate and 0.002M EDTA.). The DNA band was cut out of the gel, diluted in double distilled water 100 fold, and used in the single stranded reactions. Reactions for the single stranded amplification were carried out according to the Cetus protocol. The reactions were set up as described above, with one primer diluted 100 fold. Temperatures and times for the cycles are the same as those

outlined for the double stranded amplification. The single stranded reactions yielded approximately 2.5 μg of single stranded DNA for sequencing.

SEQUENCING OF SINGLE STRANDED PCR PRODUCTS

The single stranded PCR product was cleaned in Centricon 30 microconcentrators to isolate the DNA from the rest of the components in the amplification reaction before sequencing. Three 20 minute centrifugation washes with 1 ml of double distilled water were carried out at 5000 rpm using a fixed rotor according to the protocol supplied with Amicon centricon 30 microconcentrators). After a fourth centrifugation at 5000 rpm for 40 minutes, 50 μl of DNA solution was recovered from the filter by inverting the unit and spinning at 1000 rpm for 2 minutes. Sequencing reactions were carried out using the TaqTrack Sequencing System of Promega with several modifications to the protocol. Annealing reactions consisted of approximately 1 μg of single stranded template, 16 ng of primer, and 5X reaction buffer (250mM Tris-HCl pH 9.0, 50mM $MgCl_2$). This was incubated for 15 minutes at 65°C followed by 15 minutes at room temperature. ^{35}S dATP was added as well as 1 unit of Taq polymerase (plus W-1 detergent to a final concentration of 0.05% if BRL Taq polymerase was used in order to stabilize the enzyme). The extension and labelling step was carried out at 47°C for 5 minutes. The mix was divided among the four reaction tubes and termination reactions were carried out at 70°C for 10 minutes. Reactions were stopped with stop solution (10mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) and run on sequencing gels as described previously. The PCR products being sequenced were only 250 bp long, therefore it was only necessary to run the gels for 90 minutes.

Gels were dried as previously described and placed directly on XAR film at room temperature for 24 to 48 hours.

RESULTS

SOUTHERN ANALYSIS OF hd2 AND NON-REVERTANTS

In order to determine the molecular nature of the mutant hd2 and the three nonreverting lines, NRII12, NRII39 and NRI31, the genomic DNA was examined by Southern blot (Figure 3). Genomic DNA was digested with BamHI and PstI to yield the fragment containing the region of unique DNA putatively associated with the heterochromatic *light* locus (Figure 2). The blot was probed with the unique BamHI - PstI fragment from wild type flies. The 6 kb and 2.7 kb bands are from the balancer chromosome with a *light* duplication. The position of the wild type band (2.4 kb) is shown. In hd2, this band has shifted due to a 1.1 kb increase in size. Further restriction analysis of the hd2 clones by R. Devlin showed that this shift was due to a 1.1 kb P element insertion. In NRII12, the wild type band has shifted due to a 0.5 kb increase in size relative to wild type. In both NRII39 and NRI31, an identical slight shift of approximately 0.1 kb has occurred. It could not be determined at the level of the Southern blot whether NRII39 and NRI31 still contained some, if any, inserted P element sequences.

The Southern blots were repeated, using a different restriction enzyme to digest the DNA, to confirm the size of band shifts. SstI was the enzyme used as sites for this enzyme flanked both the BamHI and PstI sites and still produced a fragment composed of unique DNA. Resulting band shifts using the same probe as above, were identical to those from Figure 3 (data not shown).

To completely determine the nature of the hd2 mutant and non-revertants beyond the level of the Southern blot, it was undertaken to sequence across the

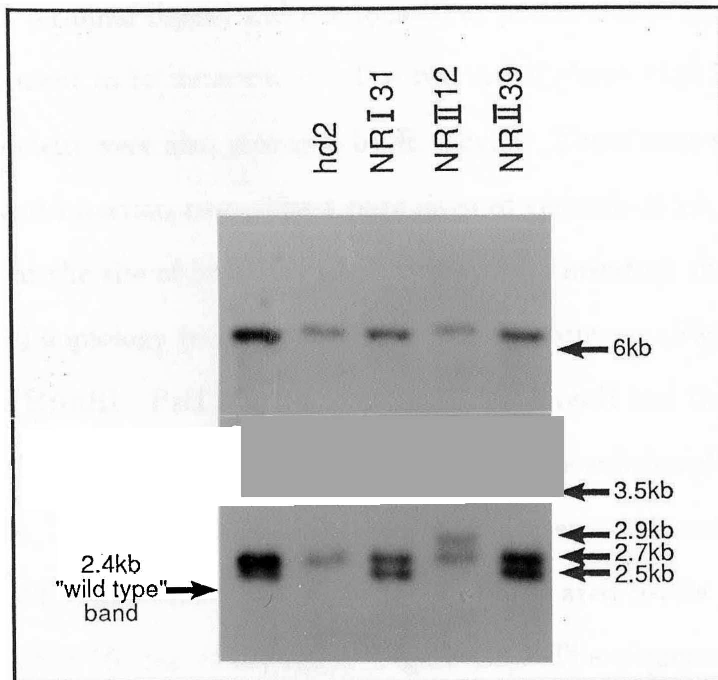
FIGURE 3 Southern blot analysis of hybrid dysgenesis-induced mutation hd2 and 3 non-reverting lines derived from hd2; NRII12, NRII39, and NRI31. Southern blots of genomic DNA digested with BamHI and PstI, producing a fragment containing the unique region DNA, were probed with the single copy region of DNA from wild-type flies. The 6kb and 2.7 kb bands are from the balancer chromosome with a duplicated *light* locus. The 2.4 kb band is the wild-type band (R. Devlin *et al*, submitted).

hd2 1100 bp shift in wild-type band due to 1100 bp element insertion.

NRI31 100 bp shift in wild-type band due to P element insertion.

NRII12 500 bp shift in wild-type band due to P element insertion.

NRII39 100 bp shift in wild-type band due to P element insertion.


hd2
NRI31
NRII12
NRII39
6kb
3.5kb
2.9kb
2.7kb
2.5kb
2.4kb
"wild type"
band

insertion site of each to determine the P element sequences present and determine if genomic rearrangements, if any, were present.

ANALYSIS OF hd2

The BamHI - PstI fragment from hd2 containing the P element insertion, cloned into pUC19, was provided by R. Devlin along with a partial restriction map of this clone (Figure 4A). The positioning of AvaII sites within the P element (one within each terminal repeat and one located at position 450) allowed the orientation of the P element to be determined. The two AvaII clones that include the two ends of the P element were also provided by R. Devlin. These were sequenced to confirm the P element insertion site. The 8 base pairs of genomic DNA were found to be duplicated at the site of insertion as expected. The insertion site sequence has only partial (3/8) homology to the insertion consensus sequence (Figure 4B).

The BamHI - PstI clone was digested with AvaII and the 600 bp and 475 bp fragments that contain the P element sequences were subcloned by blunt end ligation into pUC19. The 600 bp AvaII fragment was further subcloned using the internal XbaI site. The subclones were sequenced and compared to the published P element sequence pπ25.1 (Rubin *et al.*, 1982) (Figure 4D). The sequence was identical except for the nucleotide at hypervariable position 33, where a T replaces the usual A. There is an internal deletion of 1.8 kb which results in the non-autonomous 1.1 kb element (Figure 4C). The deletion is positioned from nucleotide 892 or 893 to nucleotide 2684 or 2685 respectively. The exact position of the breakpoint cannot be determined due to the particular nucleotides involved.

FIGURE 4 Analysis of hybrid dysgenesis-induced mutant hd2.

A) Restriction map of the unique BamHI - PstI fragment of hd2 and the 1.1 kb P element insertion.

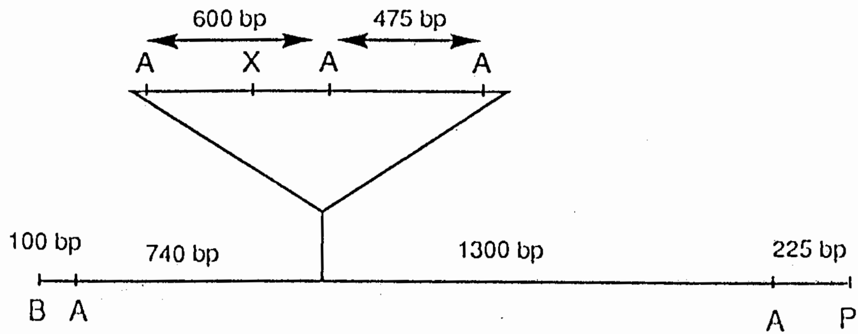
Restriction sites:

A = AvaII; B = BamHI; P = PstI; X = XhoI

B) Comparison of the 8 bp weak consensus sequence for P element insertion, and the hd2 insertion site. The 8 bp genomic duplications are underlined. Sequences between the duplications (CATG...CATG) are from the ends of the P element (data from O'Hare and Rubin, 1983).

C) Schematic diagram of the structure of the 1.1 kb P element of hd2 and its orientation. Internal deletion of P element sequences from position 892/893 to 2685/2686 is shown. Solid regions flanking the P element represent the 8 bp genomic duplication. Hatched regions are intact 31 bp inverted repeats necessary for transposition.

A



B

consensus	GGCCAGACCATG....CATGGGCCAGAC
hd2	•ATG•C•T• •••• .... •••••ATG•C•T

C

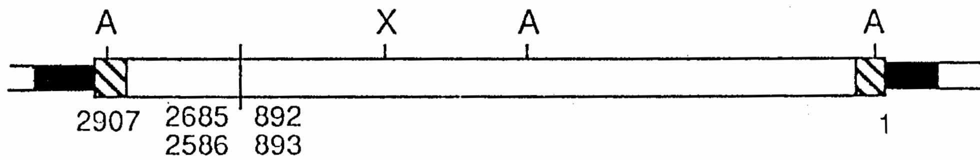


FIGURE 4

D) Sequence and comparison of the 1.1 kb hd2 P element to the complete 2.9 kb P element pπ25.1 (data from O'Hare and Rubin, 1983). Note the single nucleotide change at position 33. The position of the internal deletion of hd 2 is marked by arrows.

D

hd2

1 33 50
CATGATGAAATAACATAAGGTGGTCCCGTCGATAGCCGAAGCTTACCGAA

51 100
GTATACACTTAAATTCAGTGCACGTTTGCTTGTTGAGAGGAAAGGTTGTG

101 150
TGCGGACGAATTTTTTTTTGAAAACATTAACCCTTACGTGGAATAAAAAA

151 200
AAATGAAATATTGCAAATTTTGCTGCAAAGCTGTGACTGGAGTAAAATTA

201 250
ATTCACGTGCCGAAGTGTGCTATTAAGAGAAAATTGTGGGAGCAGAGCCT

251 300
TGGGTGCAGCCTTGGTGAAAACTCCCAAATTTGTGATGCCCACTTTAATG

301 350
ATTCGCAGTYGGAAGGCTGCACCTGCAAAAGGTCAGACATTTAAAAGGAGG

351 400
CGACTCAACGCAGATGCCGTACCTAGTAAAGTGATAGAGCCTGAACCAGA

401 450
AAAGATAAAAGAAGGCTATACCAGTGGGAGTACACAAACAGAGTAAGTTT

451 500
GAATAGTAAAAAAAATCATTTATGTAAACAATAACGTGACTGTGCGTTAG

501 550
GTCCTGTTCATTGTTTAATGAAAATAAGAGCTTGAGGGAAAAAATTCGTA

551 600
CTTTGGAGTACGAAATGCGTCGTTTAGAGCAGCAGCTGAGGGAGTCTCAA

601 650
CAGTTGGAGGAGTCTCTACGCAAAATCTTCACGGACACGCAGATACGGAT

651 700
ACTGAAGAATGGTGGACAAAGAGCTACGTTCAATTCCGACGACATTTCTA

701 750
CAGCTATTTGTCTCCACACCGCAGGCCCTCGAGCGTATAACCATCTGTAC

751 800
AAAAAAGGATTTCCTTTGCCCAGTCGTACGACTTTGTACAGATGGTTATC

801 850
AGATGTGGACATAAAAAGAGGATGTTTGGATGTGGTCATAGACCTAATGC

851 892 2685 2692
ACAGTGATGGAGTTGATGACGCCGACAAGCTTTGCTGTACTCGCAAATTAT
893 2686

2693 2742
TAAAAATAAAACTTTAAAAATAATTTCGTCTAATTAATATTATGAGTTAA

2743 2792
TTCAAACCCCACGGACATGCTAAGGGTTAATCAACAATCATATCGCTGTC

2793 2842
TCACTCAGACTCAATACGACACTCAGAATACTATTCCTTTCACTCGCACT

2843 2892
TATTGCAAGCATACGTTAAGTGGATGTCTCTTGCCGACGGGACCACCTTA

2893 2907
TGTTATTTCATCATG

ANALYSIS OF NRII12

A λ library had previously been made using EcoRI digested, size-selected genomic DNA from NRII12 flies (Rob Linning, personal communication). Four positive clones, identified from a double screen with a P element and the unique region of the putative *light* locus, were isolated and provided for further study by Rob Linning. The 9.5 kb inserts were isolated from the phage DNA and further restriction analysis showed that all clones were identical (Figure 5A). To isolate the BamHI - PstI fragment of interest, the 4.05 kb SstI band was subcloned, and the 2.7 BamHI - PstI fragment was further subcloned (Figure 5B). The BamHI - PstI fragment was digested with AvaII to yield 4 fragments, that were each subcloned for further study. Although the order of the AvaII subclones was unknown, the two small subclones (100 and 225 bp) were thought to be identical to those found at either end of the hd2 BamHI - PstI fragment restriction map (Figure 4A). The 740 bp AvaII fragment was sequenced at its ends, and it was determined to be identical to the hd2 740 bp AvaII clone, containing the distal end of the P element. To determine the rest of the P element sequences,the 1.75 bp AvaII clone was further subcloned using a unique HaeIII site located as shown in Figure 5B. The sequence of the 400 bp fragment containing the P element is shown in Figure 5D. The P element of NRII12 is only 400 bp in length due to the internal deletion of hd2 (892/893 to 2684/2685) as well as an additional internal deletion from position 17 to 750 (Figure 5C). This second deletion removes half of the 5' 31 bp inverted repeat as well as additional 5' sequences essential for transposition.

ANALYSIS OF NRII39 AND NRI31

PCR was used to amplify the region containing the putative P element insertion. Sequence from the two hd2 clones that flank the hd2 P element insertion were used to derive sequences for the synthesis of oligonucleotides (Figure 6A). The primers were designed so that they were 20 bp in length, annealed to opposite strands of the template, had 50% GC content and did not form secondary structures or anneal to one another. Two bands were amplified in the PCR reactions; the 210 bp band from the balancer chromosome, and a 250 bp band of interest. The 250 bp band was gel purified and each single strand was amplified using PCR (an example of the amplified band is shown in Figure 8B). Sequencing revealed that the genomic DNA flanking the insertion site was not rearranged and the 8 bp genomic duplication was still present (Figure 6B). The insertion is composed of the terminal 10 bp of the 5' 31 bp inverted repeat, followed by a short region of nucleotides not derived from the P element, and then the terminal 6 bp of the 3' 31 bp inverted repeat. The P element insert has a total length of 31 bp and is not able to revert due to the lack of terminal sequences essential for transposition.

hd1 ANALYSIS

From Southern blot data, the hd1 insertion site was determined to be approximately 130 bp downstream from the hd2 insertion site (Figure 2)(R. Devlin, *et al.*, submitted). Sequencing of this region identified a potential 8 bp sequence with 6 out of 8 bp homologous to the P element insertion sequence (O'Hare and Rubin, 1983). Primers were then designed flanking the potential insertion site (Figure 7A). A primer was also designed complementary to a 20 bp region within the 31 bp repeat of the P element that would bind to each end of the element.

FIGURE 5 Analysis of non-revertant NRII12.

A) Restriction map of the 9.5 kb EcoRI clone isolated from a genomic O library, showing the position of the P element insertion.

Restriction sites:

B = BamHI; R = EcoRI; P = PstI; Ss = SstI

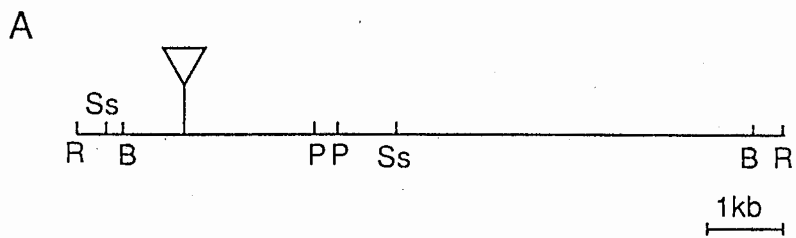
B) Restriction map of the 2.7 kb BamHI - PstI subclone containing the P element insertion.

Restriction sites:

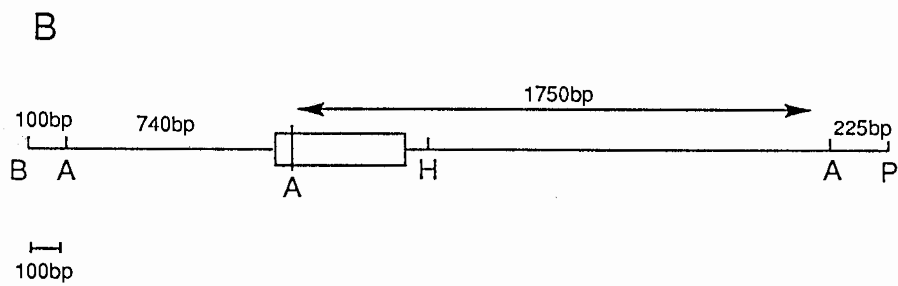
A = AvaII; B = BamHI; H= HaeIII; P = PstI

C) Schematic diagram of the NRII12 P element, showing the internal deletion found in hd2 (892/893 to 2685/2686) as well as the second deletion from 17 to 750 which includes half of the 5' 31 bp inverted repeat, accounting for its non-reverting nature.

A



B



C

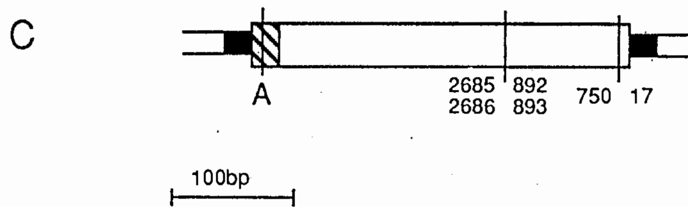


FIGURE 5

D) Sequence of the P element insertion of NRII12. The positions of the two internal deletions are marked with arrows.

D

17 750

NRⅡ12
1 CATGATGAAATAACATACAAAAAAGGATTTCCTTTGCCCAGTCGTACGAC 782

783 TTTGTACAGATGGTTATCAGATGTGGACATAAAAAGAGGATGTTTGGATG 832

833 TGGTCATAGACCTAATGGACAGTGATGGAGTTGATGACGCCGACAAGCTT 882

883 892 2685 TGCGTACTCGCAAATTATTAAAAATAAAACTTTAAAAATAATTTCGTCTA 2724
893 2686

2725 ATTAATATTATGAGTTAATTCAAACCCCACGGACATGCTAAGGGTTAATC 2774

2755 AACAATCATATCGCTGTCTCACTCAGACTCAATACGACACTCAGAATACT 2824

2825 ATTCCTTTCACTCGCACTTATTGCAAGCATACGTTAAGTGGATGTCTCTT 2874

2875 GCCGACGGGACCACCTTATGTTATTTCATCATG 2907

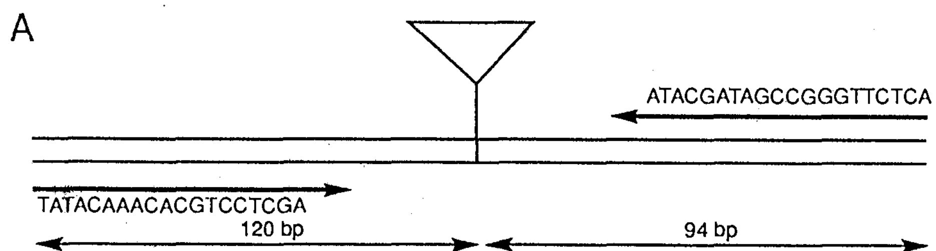
Each genomic primer was then used in PCR reactions paired with the P element primer in order to amplify the site of insertion, as shown in Figure 7. The amplification of the left-hand end of the hd1 insertion resulted in a 245 bp band, slightly smaller than expected based on the potential insertion site. The amplification of the right-hand end of the insertion produced a 170 bp band, slightly larger than was expected (the PCR products are shown in Figure 8A). The 245 bp band was sequenced, which confirmed that the insertion of the hd1 P element was actually only 100 bp downstream from the hd2 insertion site (Figure 7B). The consensus sequence for insertion is weak, with only 1 bp out of 8 in common.

FIGURE 6 Analysis of non-revertants NRII39 and NRI31.

A) Schematic diagram of the region immediately surrounding the P element insertion site of hd2. Positions and sequences of primers used in PCR amplification are shown.

B) Sequence obtained from single stranded PCR product showing the flanking genomic duplication, and the insertion, including P element sequences from both ends of the element as well as non-P element sequence. Numbers shown are from the published P element sequence (O'Hare and Rubin, 1983).

A



B

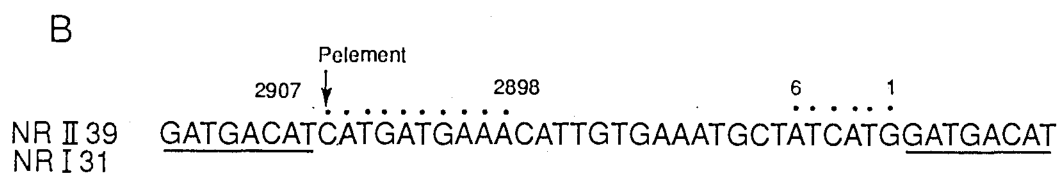
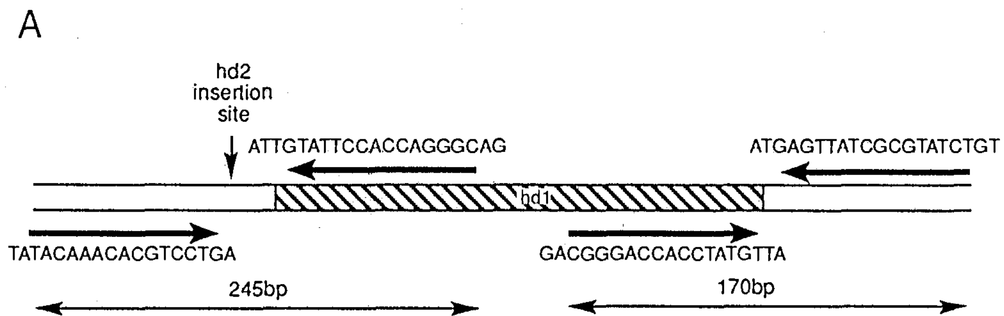


FIGURE 7 Analysis of hd1 P element insertion site.

A) Schematic diagram of the hd1 P element insertion site. Positioning and sequence of the primers used for PCR to amplify the regions of insertion are shown, along with the size of the PCR products.

B) Location of the hd1 P element insertion with repect to the hd2 insertion site, determined from the size of the PCR products and sequencing of the 245 bp PCR product (data not shown).

A



B

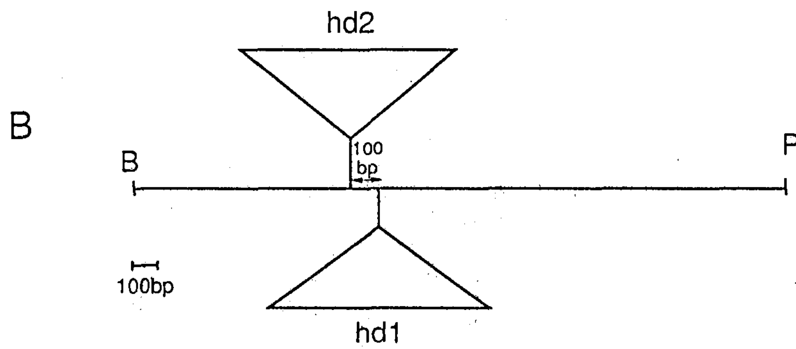
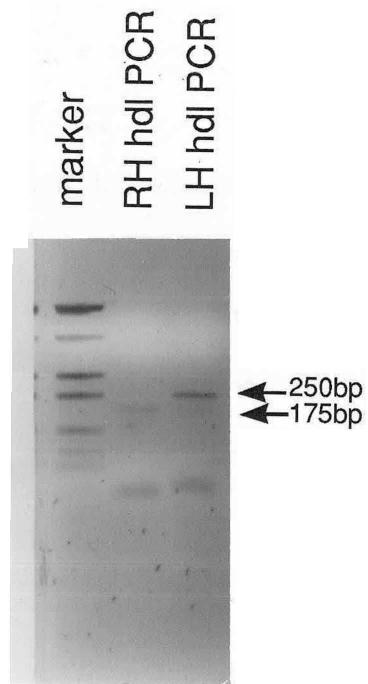
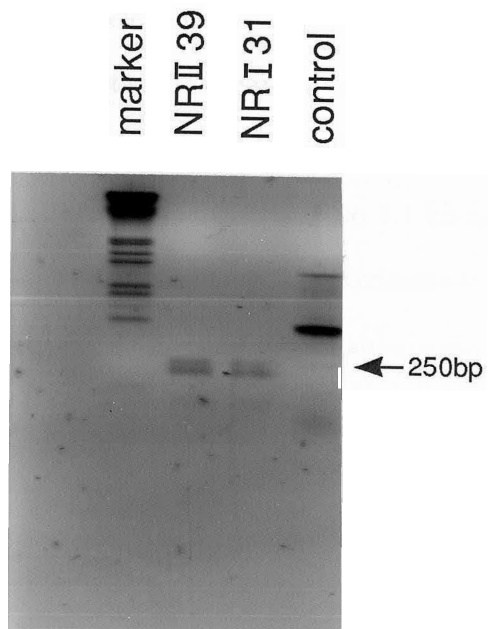


Figure 8 Examples of PCR products, using protocol as previously described.

A). Gel photo of PCR products of DNA flanking the hd1 insertion site. Amplification using the right-hand flanking primer and the P element primer produced the 175 bp band. Amplification using the left-hand flanking primer and the P element primer produced the 250 bp band as shown. The position of primers with respect to the genomic DNA has been previously shown in Figure 7.

B). Gel photo of PCR products of NRII39 genomic DNA using the primers shown in Figure 7, producing a 250 bp band.


marker
RH hdl PCR
LH hdl PCR
250bp
175bp


marker
NRII 39
NR I 31
control
250bp

DISCUSSION

MOLECULAR NATURE OF hd2

The Southern blot band shift data illustrates the presence of the 1.1 kb P element insertion within DNA associated with the *light* locus of hd2. Sequencing of clones of this region showed that the P element was a deletion derivative of the complete 2.9 kb P element. The hd2 element was non-autonomous due to the internal deletion removing most of the coding region for transposase, but still capable of transposition if provided with a source of transposase, as the 139 bp at the 5' end and the 163 bp at the 3' end of the element essential for transposition are still present.

There have been many defective P elements, derived from the complete P element by deletion, reported and analyzed. One particular P element deletion derivative found in abundance, has been termed a KP element (Black *et al.,* 1987; Jackson *et al.*, 1988). The KP element is 1.1 kb in length, derived from the complete P element with an internal deletion removing nucleotides 808 to 2560. There is one exception to the P element homology with a replacement of an adenine with a thymine at position 32. The hd2 P element is also 1.1 kb in length, but is not a KP element. The hd2 internal deletion is found approximately 100 bp away, from 892 to 2685 (or 893 to 2686). hd2 also has a nucleotide substitution in the hypervariable region with a thymine replacing an adenine, but at position 33 rather than 32.

The hd2 P element is very similar to a non-autonomous P element located in the *white* locus, named $w^{\#6}$ (O'Hare and Rubin, 1983). This 1.1 kb element has an internal deletion from 894 to 2685 (only a one nucleotide difference from the hd2

deletion) as well as a thymine to adenine substitution at position 33 (O'Hare and Rubin, 1983).

The breakpoints for internal deletions are often found in short (2 to 6 bp) direct repeats (Black *et al.*, 1987; O'Hare and Rubin, 1983; Rio *et al.*, 1986; Tsubota *et al.*, 1986). Nearly precise P element deletions have been observed with breakpoints in a direct repeat of 6 out of 7 bp contained within the 31 bp repeat (Rio *et al.*, 1986). The hd2 deletion breakpoints (892/893 to 2585/2586) are found within a 3 out of 4 bp direct repeat, which is further support of the theory that recognition of the direct repeat may be involved in excision (Engels, 1988).

The insertion site specificity of P elements is probably determined, at least in part, by the target sequence that is duplicated upon insertion of the element (O'Hare and Rubin, 1983). The hd2 target sequence matches the consensus in only 3 nucleotides out of the 8. However, this is not unusual, as other insertion sites also show weak homology (Searles *et al.*, 1986), and some known hotspots for insertion share only 4 bp in common with the consensus (Roiha *et al.*, 1988). The hd2 insertion sequence is further evidence that other factors must play a role in determining target specificity.

hd2 NON-REVERTING LINES

NRII12

The Southern blot data suggested that the non-revertant NRII12 appeared to contain an insertion approximately 500 bp in length, indicating that the hd2 P element had undergone another internal deletion, producing the smaller, non-reverting element. Sequence of the region showed that along with the 892/893 to 2685/2686 deletion of hd2, a second large deletion removes nucleotides 17 to 750,

producing the approximately 400 bp element. The non-reverting nature is due to the removal of sequences essential for transposition that make the element unable to mobilize. Residual P elements sequenced from the *RpII215* locus are the same size as NRII12 (400 bp) and are still be capable of transposition if transposase is supplied (Searles *et al.*, 1986). The NRII12 P element however, is incapable of transposition as the deletions remove some of the sequences at the 5' end of the element that are necessary for transposition (from position 17 to 138), including the binding site for transposase. It is interesting to note that the first 16 bp of the 31 bp repeat which binds IRBP are still present.

The breakpoints for the deletion in NRII12 are entirely within the P element. This result is common, but is not always the case. An excision event at the *rudimentary* locus involved a breakpoint in the flanking DNA (Tsubota *et al.*, 1983). Although there is a tendency for deletion breakpoints to occur within direct repeats (Black *et al.*, 1987; O'Hare and Rubin, 1983; Rio *et al.*, 1986; Tsubota *et al.*, 1986), the breakpoints of the internal deletions found within non-revertant NRII12 (17 to 750) do not occur within any direct repeat structure. Therefore, the mechanism of excision must not depend on the presence of the repeats alone.

NRII39 AND NRI31

The band shift data from the Southern blots for non-reverting lines NRII39 and NRI31 showed that both lines appeared to have an identical slight increase in band size (of <100 bp) from wild type, suggesting that imprecise excision of the P element had occurred, leaving approximately 100 bp or less of P element sequence behind. This type of imprecise excision has been observed by Southern blot analysis and described for other loci such as *rudimentary* (Tsubota *et al.*, 1986) and *RpII215*

(Voelker *et al.*, 1984). PCR successfully amplified the region of interest and sequence analysis proved the 2 lines to be identical, having only a small portion of each of the 31 bp repeats present. NRII39 and NRI31 lack most of the known essential sequences for transposition, resulting in their non-reverting nature.

Defective P elements are usually derived from complete P elements simply by one or more internal deletions, as in the case of NRII12. NRII39 and NRI31 have P element sequences from each end of the element separated by a 10 bp sequence of unknown origin. Examples have been published of defective P elements containing deletion of P sequences along with insertion of 1 to 21 nucleotides not of P element origin (O'Hare and Rubin, 1983; Searles *et al.,* 1986; and Tsubota and Schedl, 1986). There is no similarity between the inserted nucleotides of NRII39 and NRI31 and those of other reported defective elements. The origin or purpose of the additional nucleotides found between the P element sequences is still unknown.

NRII39 and NRI31 therefore appear to be examples of imprecise excision of the P element, that left 30 bp of sequence, of P element and non-P element origin, at the site of insertion. Several studies on different loci show that most P element excisions are imprecise rather than precise (Searles *et al.*, 1986; Salz *et al.*,1987; Tsubota and Schedl, 1986; Rio *et al.*, 1986). Excisions that appear to be precise at the level of Southern blotting are often nearly precise excisions leaving behind small fragments of the element (Salz *et al.*, 1987; Tsubota and Schedl, 1986). The occurence of restoration of wild-type phenotype despite the imprecision of excision has been used to suggest whether the mutation is within regulatory or coding regions of a particular gene (Tsubota and Schedl, 1987). For example, if a P element excises imprecisely, but wild-type phenotype is restored despite the remaining P element sequences (1 to 50 bp), this can be indicative of insertion within a regulatory region

where the residual nucleotides may not greatly interfere with the expression of the gene (Tsubota *et al.*, 1985). This does not always apply, as residual P element sequences within an essential region of a regulatory region such as the promoter itself, are not likely to allow for restoration of wild-type expression. On the other hand, imprecise excision events within a coding region, leaving a residual fragment of P element 1 to 50 bp in length, usually still produce a mutant phenotype (Zachar and Bingham, 1982). Reversion in these cases only results from precise excision of P insertions from within coding sequences. The fact that the non-revertants NRII39 and NRI31 both contain insertions of only 30 bp but remain *light* mutants, suggests that the heterochromatic region containing the insertion is in some way important for the expression of the *light* gene, either as an essential regulatory region or as a coding region.

The 8 bp that are duplicated upon insertion of the P element are still present in NRII39 and NRI31, which is to be expected, as the breakpoints for these deletions are within the 31 bp terminal repeats. Often the excision process produces genomic rearrangements such as inversions, insertions and deletions (Engels, 1988). Sequencing of the DNA around the hd2 insertion site shows that there are no differences in genomic DNA between the mutant hd2 and the non-revertants NRII39, and NRI31. Therefore, the mechanism of excision appears to leaves the genomic DNA unaffected except for the expected 8 bp duplication and insertion.

ANALYSIS OF THE hd1 INSERTION SITE

Southern blot studies by Devlin *et al.* (Devlin *et al.*, submitted), showed that the hd1 insertion site was located approximately 130 bp downstream from hd2 (Devlin *et al.*, submitted). Sequence of this region revealed a sequence 134 bp

downstream from hd2 that had 6 out of 8 bp homology to the consensus sequence and was a likely candidate for insertion. However, PCR amplification of the ends of the P element insertion and sequence from one of the amplified regions showed the insertion site to be only 100 bp downstream from the hd2 site. Here, the target sequence has only 1 bp in common with the consensus sequence, but is used as the preferred site over the site with greater consensus. This is another example supporting the idea that other features of the chromosome not visibly evident by sequencing must be involved in site selection of insertion.

The confirmation that there are two sites of insertion (hd1 and hd2) for P elements in the region associated with the *light* locus within 100 bp is not unusual. The clustering of P elements at particular points in the genome has been observed at several loci such as *rudimentary* (Tsubota *et al.*, 1985), *RpII215* (Searles *et al.*, 1986), and *singed* (Roiha *et al.*, 1988). This clustering may be explained by the nature of P elements to prefer a location near other P elements for some reason (Engels, 1988). Alternatively, it has been suggested that P elements cluster near the transciption start of genes, especially genes with germline activity (Kelley, 1987). The two P element insertion sites of hd1 and hd2, closely clustered in a region of unique sequence within heterochromatin, may actually indicate that the region is associated with the 5' flanking region of a locus, putatively the *light* locus (Kelley *et al.*, 1987).

In conclusion, hd2 has been sequenced and the insertion site and structure of the P element have been determined. The non-revertant NRII12 has been characterized as an example of a deletion derivative of the complete P element. The two other non-reverting lines, NRII39 and NRI31, have been shown to be identical.

They are derived from the hd2 P element by deletion as well as insertion of 10 bp of exogenous origin. All 3 non-revertants are lacking essential P element sequences necessary for transposition, resulting in their non-reverting nature. In addition, the P element insertion site of the other hybrid dysgenic mutant, hd1, was determined to be 100 bp downstream from the hd2 insertion site.

PROPOSALS FOR FURTHER RESEARCH

1. Determine the P element sequences present in hd1. A genomic library could be constructed for hd1, and the putative *light* clone identified by using the P element and unique sequence Bam - Pst clone as probes. Further subcloning and sequencing of positive clones would reveal the P element sequences present and the orientation of the element. If genomic DNA is limiting, PCR could be applied, using a primer internal to the 31 bp repeat of the P element and each of the genomic flanking primers already constructed, to amplify DNA containing the P element. Further single-stranded PCR and sequencing would determine the P element sequences present and the orientation of the element.

2. To further investigate the nature of P element activity in this region, other putative *light* mutants that have been isolated by R. Devlin could be investigated by using PCR to amplify the region. Analysis of these other mutants to determine the nature of the lesions at the DNA level may also identify the extent of the essential sequences of *light* and lead to a better understanding of this heterochromatic region.

3. Other lines isolated show a high frequency of somatic mosaicism for *light.* Somatic excision of the P element is rare due to the tissue-specific splicing event of transposase. Further analysis of the nature of the P element that allows this mosaicism (*eg.* the P element could contain a deletion removing the ORF2-ORF3 intron) would provide further insight into the control mechanism of P element transposition.

4. A molecular characterization of the *light* locus has been undertaken by B. Wakimoto and R. Devlin (personal communication) to determine a possible coding

region and identify *light* cDNA. Transformation studies of the putative *light* locus into *light* mutants would confirm the identity and position of the gene.

5. The nature of heterochromatic genes and of P elements within heterochromatin could be further analysed by examining other heterochromatic loci have been identified on chromosome 3 (Marchant and Holm, 1988, 1988b). A number of P-element induced mutations have been isolated and could be cloned by transposon tagging.

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