Deletion Scanning of the Regulatory Sequences of the *Fbp1* Gene of *Drosophila melanogaster* Using *P* Transposase-Induced Deficiencies

Pascale Lapie, Fahd Nasr,¹ Jean-Antoine Lepesant and Jean Deutsch²

Laboratoire de Biologie du Développement, Institut Jacques Monod, CNRS et Université Paris 7, 75251 Paris Cedex 05, France

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ABSTRACT

A procedure permitting deletion scanning of potential *cis*-regulatory sequences within a transgene whose genomic position remains fixed was applied to the study of the upstream sequences of the ecdysteroid-inducible *Fat-body-protein-1* (*Fbp1*) gene. Deficiencies were induced in a *Fbp1:Adh* fusion transgene by means of a secondary *P* transposase mutagenesis. Phenotypic and molecular screens were used to select mutant transposons that retained their original genomic location and carried a deletion affecting the *Fbp1* sequences but not the *Adh* reporter gene. Molecular mapping of the deletion breakpoints was achieved by sequence analysis and expression of the reporter gene was quantified by measurement of ADH activity. This procedure was efficient in detecting *cis*-acting elements, even those with moderate effects on levels of gene expression. For example, we have succeeded in identifying a negative regulatory element. Deletion of this element leads to a 50% increase in the reporter ADH activity. This element binds the transcription factor AEF-1. In addition, we have detected a strong, positively acting element contained within a 32-bp region located immediately upstream of an ecdysone-response element.

THE *Fat-body-protein-1* (*Fbp1*) gene of *Drosophila melanogaster* has long been studied in our laboratory as a model for gene regulation by hormones. Its expression is induced by the steroid hormone ecdysone in a single tissue, the larval fat body, at the end of the third instar. We have recently analyzed its *cis*-acting sequences by transformation with *in vitro* engineered DNA constructs, in which *Fbp1* sequences were fused to a reporter gene (MASCHAT *et al.* 1986; DEUTSCH *et al.* 1989; MASCHAT, DUBERTRET and LEPESANT 1991; LAVAL *et al.* 1993). A short 70-bp *cis*-acting element, located near the transcription start site, was identified as being sufficient to specify gene expression in the larval fat body at the end of the third instar. This element has properties in common with enhancers. It has been characterized *in vivo* as an ecdysone response element (EcRE) and *in vitro* as containing a binding site for the ecdysone receptor complex (LAVAL *et al.* 1993; ANTONIEWSKI, LAVAL and LEPESANT 1993). However, the expression of a reporter gene driven by this enhancer, when it is isolated from sequences further upstream, is drastically reduced compared with that of the endogenous gene. This revealed that important *cis*-acting sequences must lie outside the enhancer segment. Our previous results indicated that when a region of *ca.* 1.5 kb upstream of the transcription start was used in

the gene fusion, a correct level of expression was achieved (LAVAL *et al.* 1993). The aim of the present work was to collect new information on the *cis*-acting sequences located in this region.

The mapping of *cis*-acting sequences through the *in vitro* construction of fusion genes and the study of their expression by *P* element-mediated germ line transformation has a severe drawback for our purpose. The expression of a *P* element-derived transgene is subject to position effects dependent on the site of integration. The quantitative traits of expression are more affected than the qualitative ones (SPRADLING and RUBIN 1983; WILSON, BELLEN and GEHRING 1990). Position effects can mask quantitatively minor regulatory influence of *cis*-acting sequences to be tested (LAURIE-AHLBERG and STAM 1987). To overcome this problem, we chose an alternative strategy, first suggested by DANIELS *et al.* (1985). These authors had shown that *P* transposase creates deletions of internal sequences in a previously inserted *P* transposon. These deficiencies were most frequently generated *in situ*, *i.e.*, without changing the genomic location of the transposon. This observation provides a tool whereby it is possible to circumvent position effects by generating internal deficiencies of variable extent within a targeted transgene. The mutant transposons retain the same genomic location and can thus be directly compared. Such *in vivo*-generated deficiencies can then be analyzed *a posteriori* at the molecular level, instead of being engineered *a priori*. To our knowledge since its initial proposal by DANIELS *et al.*

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¹ Present address: Centre de Génétique Moléculaire, CNRS, 91198 Gif-sur-Yvette, France.

² To whom reprint requests should be sent.

(1985), the use of this procedure has been reported only once, by DELIDAKIS and KAFATOS (1989), who applied it to the identification of sequences involved in chorion gene amplification.

In the present work, the strategy was applied to the study of the quantitative effects of DNA sequences acting on the expression of the *Fbp1* gene. A transgenic line, bearing a transgene in which the 1.5-kb segment of upstream *Fbp1* sequences were fused to the *D. melanogaster* *Adh* coding region, was chosen as the starting material. This reporter gene made it possible to use a simple quantitative enzymatic assay for an accurate measurement of expression. A number of mutants were selected on the basis of both a phenotypic and a molecular screen. Even when the effect on the level of gene expression is small, the procedure of scanning a regulatory region by transposase-induced *in vivo* deficiencies proved to be powerful in detecting *cis*-acting elements. A negative distal element was identified, which contained binding sites for the negative transcription factor AEF-1 previously identified by FALB and MANIATIS (1992). A strong positively acting element contained within a 32-bp segment located immediately upstream of the proximal ecdysone-responsive enhancer was also detected.

MATERIALS AND METHODS

Drosophila stocks: The PL1 and PL2 transgenic lines contain a single copy of the *P[ry, Fbp1:Adh]* construct (Figure 1A) located on the third chromosome. This construct was injected in a *ecd^{1a}, st, ry⁵⁰⁶* recipient strain and further transferred into a *Adh^{rn6}, cn* genetic background by appropriate crosses. Both lines are homozygous viable (LAVAL *et al.* 1993). The following balancer strains were used for appropriate crosses (Figure 2): the *Adh^{rn6}, cn; MKRS/TM2, ry, Ubx* stock was a generous gift from M. ROARK. The *CyO, Adh^{rn6}, cn, pr; TM3, ry^{rk}, Sb, Ser/T(2;3) ap^{xa}* stock was constructed in our laboratory. The source of transposase was a *ry⁵⁰⁶, Sb, P[ry, Δ2-3](99B)* chromosome (ROBERTSON *et al.* 1988), kindly provided by W. ENGELS and from which we constructed an *Adh^{rn6}, cn; ry⁵⁰⁶, Sb, P[ry, Δ2-3](99B)/TM6, Ubx* stock.

Mutagenic crosses: Fly stocks were kept and crosses were performed at 20° on a standard corn meal, sugar, yeast and agar medium. In the first cross (F_0), the PL1 and PL2 transgenic lines were mated to the *Sb, P[Δ2-3]* strain (Figure 2). Young male progeny (1–3 days old) were individually mated (F_1 cross) to the balancer strain *Adh^{rn6}, cn; MKRS/TM2, ry, Ubx* during three days at 20°. Young males and low temperature were used to reduce as much as possible the accumulation of multiple excision and transposition events within the same gamete (ROBERTSON *et al.* 1988).

DNA extraction: Small-scale DNA extraction was performed on four males as described by RABINOW and BIRCHLER (1989). Other DNA extractions were prepared on 25 adult flies using the method of JUNAKOVIC, CANEVA and BALLARIO (1984) with minor modifications.

Southern blotting: Electrophoresis was performed according to SAMBROOK, FRITSCH and MANIATIS (1989). Vacuum blotting was performed using a Genofit apparatus on a Nylon membrane (Biodyne B Pall). Southern blot hybrid-

ization was as described by SAMBROOK, FRITSCH and MANIATIS (1989).

Plasmid probes: The pDmH19 plasmid (MASCHAT *et al.* 1990) was used as a *Fbp1*-specific probe. It contains a 1.6-kb *HindIII-Sau3A* DNA fragment including *Fbp1* sequences between coordinates -1386 to +276. The pPS1 plasmid, a generous gift from D. NOUAUD, containing the *HindIII* (+40 to +878) fragment of the *P* element subcloned into a pUC8 vector, was used as a 5'-specific *P*-element probe. The pPL3 plasmid containing a *PstI-SalI* (coordinates -3563 to -2911) (DUTTON and CHOVIK 1991) *rosy* fragment subcloned from the pDm2837.S8.1 plasmid (BENDER, SPIERER and HOGNESS 1983) into a Bluescript vector (Stratagene) was used as a *rosy* specific probe. The pPL4 plasmid containing a 2.7-kb *HindIII-XhoI* *Adh* fragment subcloned from the pHAP plasmid (BONNER *et al.* 1984) into a Bluescript vector (Stratagene) was used as an *Adh* specific probe for Southern blots. Plasmids were ³²P-labeled by nick translation (RIGBY *et al.* 1977) to a specific activity of 1–2 10⁸ cpm/μg.

Oligonucleotide primers: The list and sequences of all primers used are presented in Table 1. Oligonucleotides were synthesized with a DNA synthesizer (Pharmacia) and purified as recommended by the manufacturer.

PCR probes: Seven fragments, from 150–275 bp long, almost completely encompassing the 1.5-kb *Fbp1* sequence (Figure 4) were generated by PCR amplification from the pDm158 plasmid (LAVAL *et al.* 1993) using primers A1 to G2. A Perkin Elmer Cetus thermal cycler was used and the conditions of the reaction were set as follows: (4 min at 94°) × 1; (1 min at 94°, 2 min at annealing temperature, 3 min at 72°) × 25; (1 min at 94°, 2 min at annealing temperature, 15 min at 72°) × 1. The annealing temperature for each reaction was 2–4° below the melting temperature of the primer. The reaction mixture was made up of the following in a final volume of 22 μl: 520 pmol of DNA, each primer at a concentration of 0.36 μM, dNTPs 0.2 mM each, Tris-HCl (pH = 8.8) 65 mM, SO₄(NH₄)₂ 16.6 mM, β-mercaptoethanol 0.1 M, MgCl₂ 1.4 mM, BSA 20 μg/ml, Taq polymerase (Gibco BRL) 0.66 units. PCR amplified fragments were purified by elution from a 6% polyacrylamide gel. The purified fragments were labeled in a PCR reaction, using the same primers as for the previous amplification, as follows: (4 min at 94°) × 1; (1 min at 94°, 2 min at annealing temperature, 3 min at 72°) × 10; (1 min at 94°, 2 min at annealing temperature, 15 min at 72°) × 1. The reaction was carried out in a final volume of 40 μl containing: 0.1 pmole of DNA, each primer at a concentration of 2.5 μM, dCTP, dGTP and dTTP at 0.15 mM respectively, dATP at a final concentration of 10 μM, α³²P dATP (3000 Ci/ml) 10 μl, Tris-HCl (pH = 8.8) 65 mM, SO₄(NH₄)₂ 16.6 mM, β-mercaptoethanol 0.1 M, MgCl₂ 1.4 mM, Taq polymerase (Gibco BRL) 5 units.

Mapping the deficiencies: PCR amplification of genomic DNA was used in order to amplify specifically DNA from the deleted transposon of the $\Delta(ry)$ and the $\Delta(ry-Fbp1)$ mutant lines. The positions of primers used are diagrammed in Figure 6. PCR was carried out as follows: (4 min at 94°) × 1; (1 min at 94°, 2 min at annealing temperature, 6 min at 72°) × 25; (1 min at 94°, 2 min at annealing temperature, 18 min at 72°) × 1. The reaction mixture was made up of the following in a final volume of 22 μl: 0.4 10⁻¹⁷ moles of genomic DNA, each primer at a final concentration of 0.36 μM, dNTPs 0.46 mM each, Tris-HCl (pH = 8.8) 65 mM, SO₄(NH₄)₂ 16.6 mM, β-mercaptoethanol 0.1 M, MgCl₂ 2.4 mM, BSA 20 μg/ml, Cetus Taq polymerase 1.5 units. PCR products were sized by comparison with molecular weight markers. Direct sequencing of the PCR products at the deletion breakpoint regions was performed according to the

FIGURE 1.—(A) Structure of the *P[ry, Fbp1:Adh]* transposon. The DNA construct has been described elsewhere (LAVAL *et al.* 1993). The 5' and 3' *P* element ends (dotted boxes) are those derived from the Carnegie vector series (RUBIN and SPRADLING 1983). The *rosy* 7.3-kb *HindIII* fragment encompassing the entire *rosy* gene was used as a transformation marker (black box). The *HindIII*-*HpaII* (−1386 to +80) *Fbp1* fragment (hatched box) was fused to the entire coding and polyadenylation sequence (positions +9 to +2750) of the *Adh* gene (vertical hatched box). Arrows indicate the start sites and orientation of transcription of the *Fbp1:Adh* hybrid gene and of the *ry* gene. (B) DNA sequence of the *HindIII*-*BglII* *Fbp1* upstream region. The *Fbp1* sequence between coordinates −1386 (*HindIII* site) and −770 (*BglII* site) is presented, the +1 nucleotide being the start of transcription of the *Fbp1* gene. Similarity of *Fbp1* sequences with the EcRE consensus defined by CHERBAS, LEE and CHERBAS (1991) [RG(G/T)TCAnTGA(C/A)CY] is shown (coordinate −940 to −952). Each half of the pseudo palindrome is underlined by an arrow and the center base with an open rectangle. The two AEF-1 (FALB and MANIATIS 1992) binding sites are indicated by black dots (coordinates −1121 to −1114 and coordinates −1104 to −1097). The coordinates of the deletion breakpoint of the $\Delta(\text{ry-Fbp1})$ lines H12 (−1105), A1 (−1096) and R5 (−995) are indicated by an arrow head. The *Fbp1* probe (coordinates −1140 to −952) used in gel shift experiments of Figures 9 and 10 is underlined. The *Fbp1* 37-bp fragment (coordinates −1126 to −1090) used in gel shift assays (Figure 9) is boxed.

procedure of SACRAMENTO, BOURHY and TORDO (1991).

Sequencing upstream sequences of the *Fbp1* gene: The pPL1 plasmid was constructed by inserting a 611-bp *HindIII*-*BglII* *Fbp1* fragment (position −1386 to −770) isolated from the pDmH19 plasmid (MASCHAT *et al.* 1990) into the *HindIII* and *Bam*HI sites of the Bluescript vector (Stratagene). The template was denatured using NaOH 0.2 N, and was then sequenced on both strands with the dideoxy-chain termination reaction method of SANGER, NICKLEN and COULSON (1977) using a T7 polymerase sequencing kit from Pharmacia.

Rearing and staging of larvae: Precisely staged larvae were used for the assay of ADH activity, because the expression of the *Fbp1* gene is known to vary rapidly during the third instar, and rearing conditions are known to influence the physiological state of the larvae. Synchronously developing third instar larvae were obtained by collecting eggs laid overnight. The same number of eggs was collected in each culture tube (75 eggs/tube). Larvae were reared at 20° on M medium: 100 g/l dried yeast, 120 g/l sucrose, 17.5 g/l agar, 12 g/l K_2HPO_4 , 9 g/l KH_2PO_4 , 0.88 g/l methylhydroxy-4-benzoate, 0.8% propionic acid, 0.05% bromophenol blue. Multiple criteria were used in order to stage the larvae with precision: morphological criteria such as

body shortening and eversion of anterior spiracles; behavioral criteria such as arrest of wandering and adherence to the walls of the tube; physiological criteria such as the clearing of food from the gut. The addition of the dye bromophenol blue to the culture medium facilitates the use of this last criterion, which has been shown to correlate with the onset of pupariation (MARONI and STAMEY 1983). Only larvae fitting the complete set of criteria were used. These larvae correspond to stage 2 according to BAINBRIDGE and BOWNES (1981) and we estimate that they were within 30 min of the onset of pupariation.

Spectrophotometric assay of ADH activity: ADH enzyme activity was measured using the method of MERÇOT and HIGUET (1987). Briefly, crude extracts were prepared from 10 third instar larvae in 1-ml Tris-HCl buffer (pH = 8.6) 0.1 M. For each measurement, 200 μl of this extract was added to 900 μl of a reaction mix containing 800- μl propanol-2 10% as a substrate, and 100 μl of NAD^+ 20 mM as a cofactor, in Tris-HCl buffer (pH = 8.6) 0.1 M. Change in absorbance at 340 nm due to the reduction of NAD^+ was read every 5 sec for 40 sec using a Perkin Elmer Lambda 1 spectrophotometer in a cuvette precisely maintained at 25°. A unit of ADH enzyme activity was defined as $\Delta A_{340} = 0.001/\text{min}$ (equivalent to $1.61 \cdot 10^{-4}$ moles NADH produced

TABLE 1
Nucleotide sequence of primers used in PCR experiments

No.	5' Coordinate	Sequence (5'-3')
1	<i>Adh</i> +842	CCAGACCAATGCCTCCCAGA
2	<i>Fbp1</i> -1157	CCTGGCATGCAACGGAAGTG
3	<i>ry</i> +3727	CCAGGATCATTTACGCCATGGAACATTG
4	<i>P</i> +3	TGATGAAATAACATAAGGTGGTCCCGTCG
5	<i>P</i> +414	GGCTATACCAAGTGGGAGTAC
6	<i>ry</i> +4125	TCGCGCCGAAATCGGAAGTC
7	<i>ry</i> +1558	GGGTAGCCCGGAAATAGGA
8	<i>ry</i> +185	ATTGCCACGACCAGCTTGG
9	<i>ry</i> -1574	AACCGACAACCTCATGTGCCCGCCGAT
10	<i>ry</i> -2638	GGTAGTTTACAACACAAGGGATTTGACAA
11	<i>ry</i> -3049	ACTGTTACGAAAGCACGGGA
A1	<i>Fbp1</i> -1331	GCCATACCACTCTCTTGTTCCTT
A2	<i>Fbp1</i> -1157	CCTGGCATGCAACGGAAGTG
B1	<i>Fbp1</i> -1140	CGCAGCACAGACAACCAACC
B2	<i>Fbp1</i> -867	CATGTGAATACACTTAGAGTGC
C1	<i>Fbp1</i> -846	TGGAATTTTAGATAGGCACCCC
C2	<i>Fbp1</i> -639	TCGGTGAACGCATGACTTGT
D1	<i>Fbp1</i> -634	GCTGATTTCTTGACATAAACAAA
D2	<i>Fbp1</i> -406	GCAGTTCGCATTTGTATTGTAGC
E1	<i>Fbp1</i> -403	TTTGCAAGATGTTTGCTCCGAA
E2	<i>Fbp1</i> -260	CGTTTCTATTTTGTGGGTCACAAG
F1	<i>Fbp1</i> -259	AACCGGAGTTACGTACCC
F2	<i>Fbp1</i> -106	GCTGGTGCGAAAATATGTTT
G1	<i>Fbp1</i> -154	GCGGTTTTTTTAGGAGCTTC
G2	<i>Fbp1</i> +68	GAGAACTGAAGGATTGGAG
H1	<i>Fbp1</i> -1126	CCAACCAACAAAAAAAATGGC
H2	<i>Fbp1</i> -1090	GTCGCTAAAAACAACGGTA
I1	<i>Fbp1</i> -952	GCGTGAGTGAGTGATTGGCGATTG

For *ry* primers, the 5' coordinate is indicated relative to the *EcoRI* site as zero (COTÉ *et al.* 1986). For *Fbp1* primers the 5' coordinate is indicated, +1 being the start of transcription (MASCHAT *et al.* 1990). For the *Adh* primer the 5' coordinate is indicated according to the sequence published by KREITMAN (1983). We used the sequence of the *P* element published by O'HARE and RUBIN (1983) as the reference for the 5' coordinate of the *P* primers. The sequence of the oligonucleotide primer no. 5 (Figure 6) from the *P*-element sequence was communicated by W. ENGELS.

per minute). For each extract the activity of three samples was measured. For each mutant line three extracts were assayed. A control measurement was carried out for each extract omitting the cofactor NAD⁺. In all cases this control was negative. Total protein was measured by the method of Lowry using the BIORAD protein assay kit, and the data were expressed as units of ADH activity per µg of total protein. Each series of assays comprised a positive control using an extract from the original nonmutated transgenic line, and a negative control using an extract of an *Adh*^{null} isogenic line devoid of any transposon.

Histochemical staining: Larvae raised and staged as described in the above section were individually dissected at 4° in PBS (pH = 7.5) and stained following a procedure modified from ROARK *et al.* (1990). Dissected tissues were fixed in 4% paraformaldehyde (freshly prepared) for 10 min, rinsed once rapidly and then three times for 10 min in TBS (10 mM Tris-HCl pH 8.6, 80 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂), stained for 30 min in the dark at room temperature in 150 µl of staining solution (140 mM Tris-HCl pH 8.6, 3.7 mM NAD⁺, 1.1 mM phenazine methosulfate, 1.1 mM nitroblue tetrazolium, 5% isopropanol). The staining reaction was stopped by adding a few drops of acetic acid 45% for 5 min. Tissues were then rinsed three times in PBS and mounted in glycerol.

Gel shift assays: A 190-bp fragment containing the AEF-1 site from the adult enhancer of the *D. melanogaster Adh* gene was purified from a plasmid kindly provided by T.

ABEL and T. MANIATIS. The *Fbp1* probe (coordinates -1140 to -952) and the *Fbp1* competitor fragment (coordinates -1026 to -1090) were generated by PCR (see above) using primer couples B1 and I1 and H1 and H2, respectively (see Table 1). The bsϕ35 plasmid bearing the full length AEF-1 cDNA cloned in a Bluescript vector was kindly provided by T. ABEL and T. MANIATIS. AEF-1 transcripts were produced *in vitro* from the linearized bsϕ35 plasmid using a RNA transcription kit (Stratagene) and translated in a rabbit reticulocyte lysate (Promega). The translation products were directly used in gel shift assays performed as described by ANTONIEWSKI, LAVAL and LEPESANT (1993). Quantitative analysis of retarded complexes was performed by scanning gels with a Phosphor Imager apparatus (Molecular Dynamics).

RESULTS

The starting transposon: The present experiments were designed to generate a series of deleted transposons that remained at the same chromosomal location (*i.e.*, *in situ* deletions), from a transgenic line harboring a single copy of an *Fbp1:Adh* transgene. For this purpose, the *P[ry, Fbp1:Adh]* construct diagrammed in Figure 1A was chosen. In transgenic lines harboring one copy of this construct, the *Fbp1:Adh* fusion trans-

FIGURE 2.—Design of the experiment: mutagenic crosses (right panel) and molecular screen (left panel). (*) indicates that the *P[ry, Fbp1:Adh]* transposon has been modified.

gene follows the same expression pattern as that of the endogenous *Fbp1* gene: it is expressed in the larval fat body at the end of the third instar only, it is also induced by 20-hydroxyecdysone, and the *Fbp1:Adh* fusion transcript is accumulated at approximately the same level as the transcript from the endogenous *Fbp1* gene (DEUTSCH *et al.* 1989; LAVAL *et al.* 1993). To position accurately the deletion breakpoints, the nucleotide sequence of the *Fbp1* upstream regions, previously determined downstream of the -770 coordinate (MASCHAT *et al.* 1990), was completed up to the *HindIII* site (coordinate -1386) present in the original transgene (Figure 1B).

Design of the experiment: The procedure used to generate the deleted mutants is outlined in Figure 2. The transgenic line bearing the *P[ry, Fbp1:Adh]* transposon was crossed with a strain carrying *P[ry, Δ2-3](99B)*, providing a transient source of *P* transposase (ROBERTSON *et al.* 1988).

Mutants were selected by a combination of phenotypic (Figure 2, right panel) and molecular screens (Figure 2, left panel). The phenotypic screen consisted in selecting for the loss of at least parts of the target transposon, as demonstrated by the reversion of (*ry*⁺)

to (*ry*⁻) phenotype. From individual (*ry*⁻) revertant males, independent mutant lines were constructed, using appropriate markers and balancers. Flies of each generation were tested by Southern blot analysis in order to identify those in which *Fbp1* sequences had been deleted while *Adh* sequences and transposon location had been preserved. To minimize the number of blots and crosses, an initial screen used pooled flies: groups of four (*ry*⁻) male F₁ progeny, derived from independent crosses, were extracted and analyzed together. This enabled us to discard mutants in which the *Adh* gene was lost or deleted.

DNAs from the balanced progeny of the selected lines were submitted to a second Southern blot analysis, in order to select mutants in which the *Adh* sequences were preserved and the *Fbp1* sequences deleted (see Figure 3B). A third Southern blot analysis was performed on the selected homozygous mutant lines using various enzymatic digestions in order to confirm the molecular analysis of *Adh* and *Fbp1* sequences and test for the genomic location of the transposon (Figure 3C).

Selection of the starting transgenic line: *P* transposase can induce precise or imprecise excisions at

FIGURE 3.—Southern blot screen of the mutant lines. (A) A simplified restriction map of the *P[ry, Fbp1:Adh]* transposon. *PstI* (P) and *PvuII* (U) sites are indicated on the map. (B) Second Southern blot screen: *PstI* (P) digestion. DNAs extracted from the original nonmutated transgenic line (control) and two (*ry*-) lines (J9 and N1) were digested with the *PstI* restriction endonuclease and submitted to Southern blot analysis. The *Adh* probe reveals a 7-kb fragment from the endogenous gene, and a 2.8-kb one containing the entire coding sequence of the *Adh* reporter gene. The *Fbp1* probe reveals a unique 1.4-kb fragment, from both the *Fbp1* endogenous and transgenic sequences. (*) indicates the *Fbp1* deleted fragment in the N1 line. (The 2.8-kb bands are a remnant of the previous hybridization for detecting *Adh* sequences). (C) Third Southern blot screen for genomic location: *PvuII* (U) digestion. DNAs of the original nonmutated transgenic line (control) and the (*ry*-) J9 and N1 lines were analyzed by Southern blot using *PvuII* digestions. The *Fbp1* probe reveals a 2.5-kb invariant fragment from the endogenous *Fbp1* gene and a 5-kb hybrid *ry-Fbp1-Adh* fragment in the control line. This transgenic fragment is modified (*) in the J9 and the N1 lines. The *Adh* probe reveals a strong 3.6-kb band and a weak 7-kb invariant band from the endogenous *Adh* gene. Two bands are also revealed for each line: a hybrid fragment of variable length *ry-Fbp1-Adh* and a 3-kb hybrid diagnostic band containing *Adh* and flanking genomic sequences. This latter band is identical in the three lanes, thus confirming the preservation of the *Adh* reporter gene and giving first evidence of the preservation of the genomic location of the mutated transposons. The 5'-specific *P*-element probe reveals a 2.1-kb band in all lanes, confirming that the transposon-genomic junction on the other side is preserved. (The faint signals are due to remnants of the previous hybridizations.) This overall Southern blot analysis allowed us to classify the J9 line as a $\Delta(ry)$ mutant line and the N1 line as a $\Delta(ry-Fbp1)$ mutant line (see text).

extremely variable rates on the same transposon inserted at different locations in the genome (ROBERTSON *et al.* 1988; DELIDAKIS and KAFATOS 1989; BERG and SPRADLING 1991). Therefore, we tested two independent transgenic lines for the occurrence of excision events induced by the *P* transposase, as measured by the frequency of phenotypic (*ry*⁻) progeny. The progeny from 34 individual crosses involving the first line and 36 individual crosses for the second one were scored, and the actual gametic frequencies of excision events was calculated. These frequencies turned out to be 0.70 in both lines (see Appendix 1). We therefore decided to continue the mutagenesis experiments on only one of these lines.

Recovery and molecular analysis of the mutants: $\Delta(ry-Fbp1)$ mutants: Seventeen mutants were selected

from a total of 256 individually mated males after the three steps of molecular screening. They fitted our complete set of criteria inasmuch as they possessed a single and nonrelocated transposon that was deleted in the *ry* and *Fbp1* sequences, but not in the *Adh* reporter ones. These mutants will be hereafter designated as $\Delta(ry-Fbp1)$.

A set of seven fragments, *ca.* 150–250 bp long covering the whole *Fbp1* upstream sequence (Figure 4A) was used to probe the second and third Southern blots in order to determine which primer was the nearest to the *Fbp1* breakpoint. This primer was used together with one of the primers designed in the *rosy* or the 5' *P* end sequences (see MATERIALS AND METHODS and Figure 6) for specific amplification of a *ry-Fbp1* fragment encompassing the junction sequences

from the mutant transposon. The breakpoints were precisely determined through direct sequencing of the amplified fragment (see MATERIALS AND METHODS). Results are summarized in Figure 4.

All mutants were different from one another with unique breakpoints in both *Fbp1* and *rosy* sequences. The overall size of the deletions varied from 2–8.2 kb. Breakpoints were distributed all along the *Fbp1* sequence and the transcribed *rosy* sequences.

This analysis led to the determination of the sequence of 17 junctions from *P* transposase induced deletions (Figure 5). The results agree with the previous finding that short direct repeats of 2–6 bp are frequently observed at the site of deletion breakpoints (ENGELS 1989). The sequences presented a blunt junction in only two mutants. In 10 others, the two sequences shared one to four nucleotides. In addition, five cases of insertion of one to four nucleotides were observed at the deletion breakpoint, similarly to previous reports (O'HARE and RUBIN 1983; SEARLES *et al.* 1986; O'HARE *et al.* 1992; TAKASU-ISHIKAWA, YOSHIHARA and HOTTA 1992). In two of them where a 4-bp long insertion allowed sequence comparison, the inserted nucleotides were identical to nearby sequences (Figure 5), in agreement with the slippage and recopying mechanism proposed by O'HARE and RUBIN (1983), but not with the hairpin model proposed by TAKASU-ISHIKAWA, YOSHIHARA and HOTTA (1992).

$\Delta(ry)$ mutants: From the same mutagenesis and using the same screening procedure, a set of six independent $\Delta(ry)$ mutant lines, in which *rosy* sequences and not *Fbp1* ones were deleted, were randomly collected. These lines were studied in order to test the effect of deleting only *rosy* sequences on the expression of the *Fbp1:Adh* transgene. The molecular structure of these $\Delta(ry)$ mutants was analyzed through the same PCR procedure as described above, although sequencing was not performed on the amplified fragments. The length of the amplified fragments could be determined with a 10% accuracy. The deficiencies extended from 450 bp to about 3.5 kb, and were randomly located all along the *rosy* sequence (Figure 6).

$\Delta(Fbp1)$ mutants: The screening procedure described above, based on the primary selection of phenotypic (ry^-) individuals, yielded $\Delta(ry-Fbp1)$ mutants in which the deficiencies originated from the *rosy* sequences and overlapped at their *Fbp1* end. The very high frequency of (ry^-) revertants (0.70 per gamete) suggested that internal deletions could occur among the (ry^+) progeny. It thus appeared possible to select through a similar Southern blot screen $\Delta(Fbp1)$ mutants in which only some internal *Fbp1* sequences were deleted; 245 independent male progeny from F_1 crosses were tested, but none of them possessed a

single transposon deleted in the 1.5-kb *Fbp1* sequences only, without any hit within the *rosy* or *Adh* sequences. Yet this screen led to the selection of two new mutants of interest. The U4 mutant was not affected in *Fbp1* but bore a 450-bp deletion in the upstream nontranscribed *rosy* sequences, which did not impair the (ry^+) eye phenotype (Figure 6). It was further studied as a $\Delta(ry)$ mutant. One mutant carried one apparently unmodified element transposed at a new genomic site and responsible for the (ry^+) phenotype, and a second at the original site deleted in the *ry* and *Fbp1* sequences but not in the *Adh* ones. These two elements were segregated from each other by classical crosses, thus yielding a *bona fide* $\Delta(ry-Fbp1)$ mutant (C21) (Figure 4).

***Adh* reporter gene expression in the mutant lines:**

Tissue specificity of *Adh* expression: Since all mutants were generated in an *Adh^{null}* genetic background, the ADH activity represented an assay of the expression of the *Adh* reporter gene. This activity was detected by histochemical staining using staged late third instar larvae. In all mutant lines, the expression of the *Fbp1:Adh* transgene was restricted to the larval fat body (data not shown), indicating that the tissue specificity of the endogenous *Fbp1* gene (LEPESANT, KEJZLAROVÀ-LEPESANT and GAREN 1978; LEPESANT *et al.* 1982) was preserved.

The ADH quantitative assay: A faithful quantitative assay of the expression of the reporter gene in the mutant lines was a prime requirement for an accurate assessment of the effect of the deletions. As *Fbp1* expression varies rapidly during the third instar, the alcohol dehydrogenase (ADH) reporter activity was measured from carefully staged late third instar larvae (see MATERIALS AND METHODS). The ADH specific activities of a total of 15 extracts of the control line from five series of experiment performed over a period of several months did not present any significant statistical difference. This indicated that our procedures for larval staging, protein extraction and ADH assay were reliable and reproducible.

ADH activity in $\Delta(ry)$ mutants: Among seven different $\Delta(ry)$ mutants, four did not show any significant difference in ADH activity from the original control line, and three presented a statistically significant 25–30% increase (Figure 7). From the molecular analysis of these $\Delta(ry)$ mutants, this increase cannot be correlated with the length or the location of the deletion within the *ry* sequences (see Figure 6). Thus, the effect of deleting various amounts of *rosy* sequences without deleting *Fbp1* ones was either nil or slight.

ADH activity in $\Delta(ry-Fbp1)$ mutants: In contrast, as illustrated in Figure 8, the ADH activity of $\Delta(ry-Fbp1)$ mutants varied according to the extent of the deletion within the *Fbp1* sequences. Several noteworthy features can be pointed out:

FIGURE 4.—Molecular analysis of the $\Delta(ry-Fbp1)$ mutants. The $\Delta(ry-Fbp1)$ mutants were analyzed through a combination of Southern blot, PCR amplification and sequencing approaches (see text). (A) Determination of the deletion breakpoints within the *Fbp1* sequences. PCR fragments used as probes are indicated as hatched bars under the simplified restriction map of the *Fbp1* sequences of the *P[ry, Fbp1:Adh]*

FIGURE 5.—Nucleotide sequence at the junction deficiency breakpoint. *ry* sequences are drawn as dark lines, and *Fbp1* sequences as hatched lines. Designation of the $\Delta(ry-Fbp1)$ mutant lines is indicated on the left. In class 2, nucleotides in common to both sequences are drawn in boldface; those nucleotides have been arbitrarily attributed to the *Fbp1* sequences. Coordinates at the breakpoint of the last *ry* nucleotide, referring to the *EcoRI* site as zero (COTÉ *et al.* 1986; KEITH *et al.* 1987; LEE *et al.* 1987; CURTIS *et al.* 1989), and the first *Fbp1* nucleotide (coordinate +1 being the start of transcription) are indicated. In the third class inserted nucleotides are drawn in boldface and underlined.

A mutant with a deletion breakpoint located at -1105 presented a significant 50% increase in ADH activity. Further deletion up to -821 did not bring any change in the expression of the reporter gene. Within this group of mutants, the ADH activity was significantly higher than that observed in the seven $\Delta(ry)$ mutants tested (see Appendix 2), indicating that this increase is not due to the deletion of *rosy* sequences. This was supported by the fact that two couples of mutants A1, H12 and N3, S11, presenting

close deletion breakpoints in *Fbp1* sequences but widely separate breakpoints in *rosy* sequences showed similar ADH activity. Two conclusions can be drawn from these data:

First, a negative control sequence has to be located near or upstream of the -1105 coordinate. This negative control sequence has been studied in more detail (see next section). Second, similar ADH activity was observed in mutant lines in which sequences from -1105 to -821 were either preserved or deleted. An

transposon: [A (-1331 to -1157), B (-1140 to -867), C (-846 to -639), D (-634 to -406), E (-403 to -260), F (-259 to -106), G (-154 to +68)]. The location of the primers used for amplification and labeling of the probes are indicated by small half-arrows. The *Fbp1* sequences preserved in each $\Delta(ry-Fbp1)$ mutant are indicated by solid lines with the coordinate of the last *Fbp1* nucleotide at the end. The overall extent in nucleotides of the deficiencies is indicated within brackets. The extent of the M16 deficiency was determined by Southern probing and not by sequencing, the thin bar representing the uncertainty of this determination. Lines tagged with a star were not analyzed for the activity of the ADH reporter gene because of reduced viability or fertility. (B) Deletion breakpoints within the *rosy* sequences. The last *ry* nucleotide at the site of the deletion is represented by a bar on a simplified map of the *P[ry, Fbp1:Adh]* transposon. The *ry* sequences are drawn as thick bars for the exons and thin lines for the introns. The arrow and (0) mark the location of the *EcoRI* site, chosen as the origin of the coordinates within the *ry* sequence (COTÉ *et al.* 1986). In each $\Delta(ry-Fbp1)$ mutant line the deficiency is unidirectional, extending from the *ry* site to the *Fbp1* sequences.

FIGURE 6.—Molecular analysis of the $\Delta(ry)$ mutant lines. The $\Delta(ry)$ mutants were analyzed through a combination of Southern blot and PCR amplification (see text). The location and orientation of the various oligonucleotide primers used for PCR amplification of transposon-specific fragments are drawn by small half-arrows under a simplified map of the transposon. The small solid black box represents the 5' *rosy* *PstI-SalI* fragment (coordinates -3563 to -2911) (DUTTON and CHOVIK 1991) subcloned in the pPL3 plasmid used as 5' junction probe. All $\Delta(ry)$ mutant lines analyzed hybridized with this probe. Dark lines represent DNA sequences preserved in the different lines. At the left above each line is the designation of each mutant. The extent of each deficiency, deduced from the length of PCR-amplified transposon-specific fragments, is given within brackets. The region in which the deficiency is located is drawn as a hatched line. The extent of the J4 deficiency is not represented because it was not determined. Southern blotting analysis indicated that this deletion should be located between the *SstI* -980 site and the *PvuII* $+3543$ site.

FIGURE 7.—Expression of the *Adh* reporter gene in the $\Delta(ry)$ mutant lines. ADH activity for each line is expressed as the ratio to the ADH activity of the original transgenic control line, the solid bars representing the confidence intervals at $P = 0.05$ (see Appendix 2).

EcRE-like element is included within this segment at position -946 (see Figure 1B). One half of the pseudo-palindrome is well conserved, while the other half is more divergent, a situation that has been encountered in other EcREs shown to be functional *in vivo* (CHERBAS, LEE and CHERBAS 1991). This EcRE-like site therefore does not seem to play a major role in the regulation of the level of expression of the gene.

Within the next region (from -822 to -194) the ADH activity decreased gradually in parallel with the increasing length of the deletion within the *Fbp1*

upstream sequences (Figure 8). Deleting up to -194 yielded a twofold reduction as compared with the original transgenic line activity, and a threefold reduction as compared with the mutant deleted to -821 . This clearly indicates the presence of activating sequences in the -821 to -194 region. It was not possible to correlate precisely the decrease of the activity with the loss of one or several discrete elements within this region. This could be due to the analysis of too small a number of mutants, and/or to the clustering of important *cis*-acting sequences within a short region. It is worth noting that *in vivo* footprinting experiments (to be reported elsewhere) showed specific protection of two segments comprised within this region (A. DAHAN and J.-A. LEPESANT, unpublished data). One of these segments contains an EcRE-like element at position -194 (Figure 8).

Finally, the mere deletion of a small 32-bp segment between coordinates -194 and -162 led to a drastic decrease in ADH activity. The ADH activity measured in the mutant deleted to -162 was not significantly above the background level of detection determined from an *Adh*^{null} control, indicating that the effect of deleting the -194 to -162 segment has to be at least a fivefold reduction. However, in this line, the histochemical assay revealed a significant staining in the fat body, indicating that *Fbp1* sequences downstream of -162 are still able to promote expression of a

FIGURE 8.—Expression of the *Adh* reporter gene in the $\Delta(\text{ry-}Fbp1)$ mutant lines. ADH activity of each deleted line (ordinates) is plotted as the ratio to the ADH activity of the original transgenic line (open circles), against the coordinate of the deletion breakpoint within the *Fbp1* sequences (abscissae) (coordinate +1 being the transcription start). The solid bars represent the confidence intervals at $P = 0.05$ (see Appendix 2). The structural features of interest in the -1386 to $+1$ *Fbp1* region drawn at the same scale are indicated under this diagram. The enhancer element (coordinates -138 to -68) containing a functional EcRE (position of the central base -97) is boxed. The AEF-1 binding sites are drawn (position of the central base -1109). Three putative EcREs (position of the central base -194 , -310 , -946 , respectively) are indicated.

reporter gene, albeit at a considerably reduced level.

Binding of the Adh-Enhancer-Factor-1 (AEF-1) to the negative *Fbp1* element: With the aim of obtaining some insight into the mechanism leading to the increase of ADH activity observed when deleting the most upstream *Fbp1* sequences, a closer examination of the breakpoints and the sequences deleted in the first seven mutants was undertaken. This revealed the presence between positions -1121 and -1097 of a direct perfect repeat of a CAACAAAAA motif (see Figure 1B) with nine intervening bp. This motif is very similar to the binding site of the zinc finger transcription factor AEF-1 found in three fat body enhancers (the *Adh* genes of *D. melanogaster* and *Drosophila mulleri*, and the *Yp* genes of *D. melanogaster*) (FALB and MANIATIS 1992). The most upstream deletion breakpoint (H12, coordinate -1105) eliminates the first motif and breaks a single base pair upstream of the second one. All other breakpoints are located downstream of both of them. Therefore, all seven mutants presenting an increase in the expression of the reporter gene bear either an alteration of these motives or a deletion of them. Since AEF-1 has been shown to behave like a repressor (FALB and MANIATIS 1992), we tested the negative *cis*-acting *Fbp1* upstream region for AEF-1 fixation. Thanks to a generous gift of a cDNA clone from T. ABEL and T. MANIATIS, the AEF-1 protein was produced *in vitro* in a coupled transcription and translation experiment. As revealed by gel retardation assays (see MATERIALS AND METHODS and Figure 9), the translation product from the

sense RNA bound to a 189-bp *Fbp1* fragment encompassing the two repeats (Figure 1B), while the translation products from an anti-sense RNA did not present any retardation signal (Figure 9, lanes 1 and 4). A very similar pattern was obtained with a *D. melanogaster* *Adh* probe of a similar size, covering the previously identified AEF-1 binding site (Figure 9, lanes 6 and 9). A 37-bp *Fbp1* fragment (coordinates -1126 to -1090) comprising both CAACAAAAA repeats (see Figure 1B) was synthesized. Addition of a 10-fold excess of this cold 37-mer led to a 86% competition with the homologous *Fbp1* probe and a 74% with the *Adh* one, while the same excess of cold nonspecific DNA did not compete more than 7–8% (Figure 9, lanes 2 and 3, 7 and 8, respectively).

DNA fragments were prepared by specific PCR amplification of the transposon sequences from three of the mutants, deleted up to position -1105 , within the segment comprising the couple of AEF-1 binding sites (H12), positions -1096 (A1) and -995 (R5), downstream of both sites, respectively. When these fragments were used as competitor DNA in gel shift assay, competition was drastically reduced as compared with that achieved with wild-type *Fbp1* sequences (Figure 10). The effect observed in the mutants is thus correlated with a loss of the affinity of binding to the AEF-1 protein of the DNA sequence surrounding the breakpoints.

DISCUSSION

The *P* element mutagenesis: The loss of expression of a marker gene has been widely used as a criterion

FIGURE 9.—Binding of the AEF-1 factor to the *Fbp1* and the *Adh* sequences. Gel retardation assays were performed in the presence of the *Fbp1* (coordinates -1140 to -952) radioactive probe (lanes 1 to 5) and the 190-bp *Adh* (see MATERIALS AND METHODS) radioactive probe (lanes 6 to 10); 0.5 μ l of the *in vitro* translation lysate supplemented with either AEF-1 sense mRNA or anti-sense mRNA were used for each assay. Competition experiments were carried out with the indicated amounts of cold competitor. Nonspecific competitor (NS) was denatured salmon sperm DNA (Sigma). *Fbp1* specific cold competitor was a 37-bp fragment from coordinates -1126 to -1090 (see Figure 1B) containing both AEF-1 binding sites.

for excision events in *P* element mutagenesis studies (DANIELS *et al.* 1985; ROBERTSON *et al.* 1988; ENGELS 1989; BERG and SPRADLING 1991; DANIELS and CHOVNICK 1993). Excision frequencies have been shown to be extremely variable (ENGELS 1989). A very high frequency of excision was obtained in our experiments. On average, as many as 70% of gametes originating from each “pseudo-dysgenic” male, bearing both the target transposon and the source of transposase, produced (*ry*⁻) progeny. This figure is a minimal estimate of the frequency of excision since our molecular analysis showed that excision events also occurred among the (*ry*⁺) progeny. It is in fact the highest figure reported until now, although high rates have also been reported by others (DANIELS and CHOVNICK 1993). This high rate creates favorable

FIGURE 10.—Competition assays with the Δ (*ry-Fbp1*) DNA fragments. Gel retardation assays were performed using the *Fbp1* radioactive probe (coordinates -1140 to -952); 0.5 μ l of the *in vitro* translation lysate supplemented with AEF-1 sense mRNA were used for each assay. Competition experiments were carried out with the indicated amounts of cold competitors. Nonspecific competition was performed with denatured salmon sperm DNA and specific competition with the cold *Fbp1* probe. DNA fragments of the Δ (*ry-Fbp1*) transposons were generated by PCR (see MATERIALS AND METHODS and Table 1). Coordinates and length of the fragments were the following: A1: coordinates *ry* +185 (primer 8) *Fbp1* -952 (primer 11), length 1523 bp; H12: coordinates *ry* +1158 (primer 7) *Fbp1* -952 (primer 11), length 1309 bp; R5: coordinates *ry* +185 (primer 8) *Fbp1* -952 (primer 11), length 1697 bp.

circumstances with regard to our mutagenesis project. The lengths of the generated deletions varied greatly, ranging from 450 bp to 8 kb. They covered almost the entire range of what could be expected, taking into account our screening constraints, *i.e.*, preservation of the 5' and 3' *P* ends and the *Adh* reporter gene. In 17 Δ (*ry-Fbp1*) mutants, the deletion breakpoints were determined at the nucleotide level by sequencing. They were distributed throughout the 1.5-kb *Fbp1* sequence scanned. Our Southern blot screening procedure probably led to an underrepresentation of the most extreme class of deficiencies in which a few nucleotides of the *Fbp1* sequences were preserved but

did not give a detectable hybridization signal. No deletion breakpoint was recovered within the *rosy* sequences upstream from coordinate -1468, (i.e., +177 from the *rosy* transcription start) (CURTIS *et al.* 1989). The random distribution of breakpoints throughout the *rosy* transcribed sequence makes this lack of deletion breakpoints within upstream sequences significant. As indicated by the screen used—the loss of phenotypic (*ry*⁺) expression—only a few nucleotides upstream from the transcription start are actually needed to drive expression of the *rosy* gene sufficiently to yield a wild-type eye phenotype. This is consistent with our finding that a mutant lacking 450 bp within the *rosy* upstream sequences is still phenotypically (*ry*⁺), and also with the location of enhancer-like regulatory sequences within the large first intron of the gene (DUTTON and CHOVIK 1988; CURTIS *et al.* 1989; RILEY 1989).

The deletion breakpoints within the *Fbp1* sequences were within one or two nucleotides of each other in some lines but the corresponding *rosy* breakpoints were not correlated, indicating that recurrent deletions did not occur. We can conclude that the deletions within the *Fbp1* and the *ry* sequences relevant for (*ry*⁻) selection are randomly distributed and that hot spots of excision within the transgene were not observed. This is consistent with data previously obtained by various authors in similar analyses (DANIELS *et al.* 1985; DELIDAKIS and KAFATOS 1989; O'HARE *et al.* 1992).

In conclusion, when analyzed at the nucleotide level, each deletion turned out to be unique with regard to its length, location and breakpoint structure. In this respect, they fulfilled the required conditions for a systematic deletion scanning of a sequence of interest.

The effect of genomic and of *rosy* sequences: Our selection procedure ensured that there was no variation in the genomic environment of the mutant transposons. However, it could be argued that the deletion itself shortened the distance between the external genomic DNA and the *Fbp1* promoter. This in turn might have been responsible for variations in the expression of the *Adh* reporter gene. Our results showed that within both the $\Delta(\textit{ry})$ and the $\Delta(\textit{ry-Fbp1})$ mutant series, ADH activity did not correlate with the overall extent of the deficiency. This indicates that the influence of the flanking genomic sequences, if any, remains constant in the mutant lines.

Another important question is the potential effect of variations in the internal *rosy* sequences on the activity of the *Adh* reporter gene. The $\Delta(\textit{ry})$ mutants, deleted in *rosy* sequences only and not in *Fbp1* ones, were studied in order to examine this point. No significant effect was found in four of seven mutants

studied, and a minimal effect was detected in the other three.

We also carefully examined the potential effect of *rosy* sequences within the $\Delta(\textit{ry-Fbp1})$ class of mutants. Particular attention was paid to the presence or absence of sequences belonging to the large first *rosy* intron, in which a fat body-specific enhancer-like sequence has been identified (DUTTON and CHOVIK 1988). It turned out that the mere presence of complete or partial intronic sequences cannot be responsible for any effect.

In summary, we believe that the method whereby *in vivo* deficiencies are generated by means of a secondary exposure to the *P* transposase efficiently ensures that the expression of the gene under study is prevented from variations in genomic flanking sequences and is not greatly influenced by any variation in unrelated internal sequences.

Deletion analysis reveals new important *cis*-acting sequences of the *Fbp1* gene: The general view emerging from our results is that the level of expression of the *Fbp1* gene is controlled by multiple *cis*-acting elements dispersed along the upstream sequences of the gene. In other words, the regulation of a gene presenting the most simple pattern, being expressed in a single tissue within a single short developmental period, requires a complex interaction of multiple *cis*-acting elements. We shall now focus on some of them.

A deletion of a small sequence between positions -194 and -162 drastically reduced the level of the ADH activity. This corresponds to an AT rich sequence (87%) that does not contain the binding site of any previously known transcription factor. This "amplifier" element lies close to a 70-bp more proximal element (from -138 to -68) that has been shown to contain a biologically active EcRE and to function in an enhancer-like manner (LAVAL *et al.* 1993). The molecular characterization of a factor that specifically binds to this 32-bp positively acting sequence, present in larval fat body extracts, has been undertaken.

A negative control sequence has been located near or upstream of the -1105 coordinate. A similar modulating negative regulation was observed for the *Adh* gene. The AEF-1 transcription factor, expressed in the fat body, appears to be responsible for this regulation and has been shown to bind to a specific sequence within the control element of the *Adh* distal promoter (FALB and MANIATIS 1992). We propose that the *Fbp1* gene could be negatively modulated through the same mechanism. The following data support this hypothesis: two repeats similar to the AEF-1 site are located close to each other in the negative upstream region of the *Fbp1* gene. Gel retardation assays clearly demonstrated that these repeats are *bona fide* AEF-1 binding sites. In the mutant lines that show an increase of the reporter gene activity

these two AEF-1 sites are partially or totally deleted. AEF-1 binding of DNA fragments from these mutant lines is impaired. Current work is aimed at a better characterization of the *Fbp1* AEF-1 binding sites.

***In vivo* deletion scanning using *P* transposase mutagenesis:** A characteristic of the present project was to scan potential *cis*-acting sequences on the basis of a quantitative assay for the expression of a reporter gene suitable for this purpose, such as the *Adh* gene. Statistical analysis of the data obtained with the various mutant lines allows us to conclude that the accuracy of the method is between 15 and 20% (see Figures 7 and 8 and Appendix 2), a satisfactory limit for any quantitative biological assay. The deletion scanning procedure has thus been proved to be powerful in detecting *mild* effects in the level of expression of a gene. Such effects are notoriously difficult to substantiate, and for that reason quantitative effects on gene expression are not frequently studied. It must be pointed out that the method can detect such mild effects *in vivo*, in the context of the whole organism. It is reasonable to think that in this context, where the chromatin structure and the whole transcriptional machinery are preserved, with all the transcription factors present at their normal titer, even mild effects could be important. However moderate they may be *in vivo* at the level of the whole organism, these effects could be very drastic when studied *in vitro*, with analytical procedures dealing with naked DNA and considering transcription factors one by one.

The predictive value of the method is supported by the molecular analysis of one of the regions of interest detected. The rather mild, but significant 50% increase in the expression of the reporter gene noted in mutants deleted in the most upstream *Fbp1* sequence under study was found to correlate with a loss of sites binding to the previously known negative regulator AEF-1.

An alternative classical approach to this deletion scanning procedure would be to engineer *in vitro* deletions, either at random or at sequence-defined locations, and to study their *in vivo* effects after germline *P* transformation. The advantage of this strategy is that the molecular structure of the deletions is more easily determined. The drawback is that a quantitative analysis of the expression of the constructs is severely impaired by genomic position effects. From the computation made by LAURIE-AHLBERG and STAM (1987) after their quantitative study on *Adh^F* and *Adh^S* alleles, it can be inferred that, in order to take into account position effects, 8–59 transgenic lines *per deficiency* would have to be tested to achieve an accuracy similar to the one reached in our study.

During the course of this work, new tools were provided for *Drosophila* molecular geneticists. R. KELLUM and P. SCHEDL (KELLUM and SCHEDL 1991;

KELLUM and SCHEDL 1992) took advantage of the “*scs*” sequences isolated from the *hsp70* locus, and proved that they were able to insulate a promoter from the effect of an enhancer located either within flanking genomic sequences or internal to the transposon. A similar insulating effect has been recently demonstrated when sequences known to be targets for the product of the *Su(Hw)* gene were introduced between a gene and enhancers (ROSEMAN, PIRROTTA and GEYER 1993). Although they have not yet been studied at the quantitative level, such vectors will clearly permit in the future the reconciliation of quantitative studies with *in vitro* engineered constructs.

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LITERATURE CITED

- ANTONIEWSKI, A., M. LAVAL and J.-A. LEPESANT, 1993 Structural features critical to the activity of an ecdysone receptor binding site. *Insect Biochem. Mol. Biol.* **33**: 105–114.
- BAINBRIDGE, S. P., and M. BOWNES, 1981 Staging the metamorphosis of *Drosophila melanogaster*. *J. Embryol. Exp. Morph.* **66**: 57–80.
- BENDER, W., P. SPIERER and D. S. HOGNESS, 1983 Chromosomal walking and jumping to isolate DNA from the *Ace* and *rosy* loci and the *Bithorax Complex* in *Drosophila melanogaster*. *J. Mol. Biol.* **168**: 17–33.
- BERG, C. A., and A. C. SPRADLING, 1991 Studies on the rate and site-specificity of *P* element transposition. *Genetics* **127**: 515–524.
- BONNER, J. J., C. PARKS, J. PARKER-THORNBURG, M. A. MARTIN and H. R. B. PELHAM, 1984 The use of promoter fusions in *Drosophila* genetics: isolation of mutations affecting the heat shock response. *Cell* **37**: 979–991.
- CHERBAS, L., K. LEE and P. CHERBAS, 1991 Identification of ecdysone response elements by analysis of the *Drosophila Eip28/29* gene. *Genes Dev.* **5**: 120–131.
- COTÉ, B., W. BENDER, D. CURTIS and A. CHOVNICK, 1986 Molecular mapping of the *rosy* locus in *Drosophila melanogaster*. *Genetics* **112**: 769–783.
- CURTIS, D., S. H. CLARK, A. CHOVNICK and W. BENDER, 1989 Molecular analysis of recombination events in *Drosophila*. *Genetics* **122**: 653–661.
- DANIELS, S. B., and A. CHOVNICK, 1993 *P*-element transposition in *Drosophila melanogaster*: an analysis of sister-chromatid pairs

- and the formation of intragenic secondary insertions during meiosis. *Genetics* **133**: 623-636.
- DANIELS, S. B., M. MCGARRON, C. LOVE and A. CHOVIK, 1985 Dysgenesis-induced instability of *rosy* locus transformation in *Drosophila melanogaster*: analysis of excision events and the selective recovery of control element deletions. *Genetics* **109**: 95-117.
- DELIDAKIS, C., and F. C. KAFATOS, 1989 Amplification enhancers and replication origins in the autosomal chorion gene cluster of *Drosophila*. *EMBO J.* **8**: 891-901.
- DEUTSCH, J., M. LAVAL, J.-A. LEPESANT, F. MASCHAT, F. POURRAIN and L. RAT, 1989 Larval fat body-specific gene expression in *D. melanogaster*. *Dev. Genet.* **10**: 220-231.
- DUTTON, F. L., and A. CHOVIK, 1988 Developmental regulation of the *rosy* locus in *Drosophila melanogaster*, pp. 267-316 in *Developmental Biology. A Comprehensive Synthesis*, Vol. 5, edited by L. W. BROWDER. Plenum Press, New York.
- DUTTON, F. L., and A. CHOVIK, 1991 The *l(3)S12* locus of *Drosophila melanogaster*: heterochromatic position effects and stage-specific misexpression of the gene in *P* element transposons. *Genetics* **228**: 103-118.
- ENGELS, W. R., 1979 The estimation of mutation rates when premeiotic events are involved. *Environ. Mutagen.* **1**: 37-43.
- ENGELS, W. R., 1989 *P* elements in *Drosophila*, pp. 437-484 in *Mobile DNA*, edited by D. E. BERG and M. M. HOWE. American Society for Microbiology, Washington, D.C.
- FALB, D., and T. MANIATIS, 1992 *Drosophila* transcriptional repressor protein that binds specifically to negative control elements in fat body enhancers. *Mol. Cell. Biol.* **12**: 4093-4103.
- JUNAKOVIC, N., R. CANEVA and P. BALLARIO, 1984 Genomic distribution of copia-like elements in laboratory stocks of *Drosophila melanogaster*. *Chromosoma* **90**: 378-382.
- KEITH, T. P., M. A. RILEY, M. KREITMAN, R. C. LEWONTIN, D. CURTIS and G. CHAMBERS, 1987 Sequence of the structural gene for xanthine dehydrogenase (*rosy* locus) in *Drosophila melanogaster*. *Genetics* **116**: 67-73.
- KELLUM, R., and P. SCHEDL, 1991 A position-effect assay for boundaries of higher order chromosomal domains. *Cell* **64**: 941-950.
- KELLUM, R., and P. SCHEDL, 1992 A group of *scs* elements function as domain boundaries in an enhancer-blocking assay. *Mol. Cell. Biol.* **12**: 2424-2431.
- KENDALL, M., and A. STUART, 1977 *The Advanced Theory of Statistics*, Vol. 1. C. Griffin and Company, U.K.
- KREITMAN, M. 1983 Nucleotide polymorphism at the *Alcohol dehydrogenase* locus of the *Drosophila melanogaster*. *Nature* **304**: 412-416.
- LAURIE-AHLBERG, C. C., and L. F. STAM, 1987 Use of *P*-element-mediated transformation to identify the molecular basis of naturally occurring variants affecting *Adh* expression in *Drosophila melanogaster*. *Genetics* **115**: 129-140.
- LAVAL, M., F. POURRAIN, J. DEUTSCH and J.-A. LEPESANT 1993 *In vivo* functional characterization of an ecdysone-response enhancer in the proximal upstream region of the *Fbp1* gene of *D. melanogaster*. *Mech. Dev.* (in press).
- LEE, C. S., D. CURTIS, M. MCCARRON, C. LOVE, M. GRAY, W. BENDER and A. CHOVIK, 1987 Mutations affecting expression of the *rosy* locus in *Drosophila melanogaster*. *Genetics* **116**: 55-66.
- LEPESANT, J.-A., J. KEJZLAROVÀ-LEPESANT and A. GAREN, 1978 Ecdysone-inducible functions of larval fat bodies in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **75**: 5570-5574.
- LEPESANT, J.-A., M. LEVINE, A. GAREN, J. LEPESANT-KEJZLAROVÀ, L. RAT and G. SOMME-MARTIN, 1982 Developmentally regulated gene expression in *Drosophila* larval fat bodies. *J. Mol. Appl. Genet.* **1**: 371-383.
- MARONI, G., and S. STAMEY, 1983 Use of blue food to select synchronous late third instar larvae. *Drosophila Inf. Serv.* **59**: 142-143.
- MASCHAT, F., M. L. DUBERTRET and J.-A. LEPESANT, 1991 Transformation mapping of the regulatory elements of the ecdysone-inducible *P1* gene of *Drosophila melanogaster*. *Mol. Cell. Biol.* **11**: 2913-2917.
- MASCHAT, F., M. L. DUBERTRET, P. THÉRON, J. M. CLAVERIE and J.-A. LEPESANT, 1990 Structure of the ecdysone-inducible *P1* gene of *Drosophila melanogaster*. *J. Mol. Biol.* **214**: 359-372.
- MASCHAT, F., J. ROUX, H. BENES, R. PICTET, J. JAMI and J.-A. LEPESANT, 1986 Hormonal and developmental specificities of transcription lie within a 1.5 kb region 5' to the inducible *Drosophila P1* gene. *EMBO J.* **5**: 583-588.
- MERÇOT, H., and D. HIGUET, 1987 *ADH* activity in two *Drosophila* strains from selection on body weight. *Drosophila Inf. Ser.* **66**: 98-99.
- O'HARE, K., A. DRIVER, S. MCGRATH and D. M. JOHNSON-SCHILTZ, 1992 Distribution and structure of cloned *P*-elements from the *Drosophila melanogaster P*-strain pi. *Genet. Res.* **60**: 33-41.
- O'HARE, K., and G. M. RUBIN, 1983 Structures of *P* transposable elements and their sites of insertion and excision in the *Drosophila melanogaster* genome. *Cell* **34**: 25-35.
- RABINOW, L., and J. A. BIRCHLER, 1989 A dosage-sensitive modifier of retrotransposon-induced alleles of the *Drosophila white* locus. *EMBO J.* **8**: 879-889.
- RIGBY, P. W. J., M. DIEKMANN, C. RHODES and P. BERG, 1977 Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**: 237-251.
- RILEY, M. A., 1989 Nucleotide sequence of the *Xdh* region in *Drosophila pseudoobscura* and an analysis of the evolution of synonymous codons. *Mol. Biol. Evol.* **6**: 33-52.
- ROARK, M., V. RAGHAVAN, T. TODO, C. A. MAYEDA and E. M. MEYEROWITZ, 1990 Cooperative enhancement at the *Drosophila sgs-3* locus. *Dev. Biol.* **139**: 121-133.
- ROBERTSON, H. M., C. R. PRESTON, R. W. PHILLIS, D. M. JOHNSON-SCHILTZ, W. K. BENZ and W. R. ENGELS, 1988 A stable genomic source of *P* element transposase in *Drosophila melanogaster*. *Genetics* **118**: 461-470.
- ROSEMAN, R. R., V. PIRROTTA and P. K. GEYER, 1993 The Su(Hw) protein insulates expression of the *Drosophila melanogaster white* gene from chromosomal position-effects. *EMBO J.* **12**: 435-442.
- RUBIN, G. M., and A. C. SPRADLING, 1983 Vectors for *P* element-mediated gene transfer in *Drosophila*. *Nucleic Acids Res.* **11**: 6341-6351.
- SACRAMENTO, D., H. BOURHY and N. TORDO, 1991 PCR technique as an alternative method for diagnosis and molecular epidemiology of rabies virus. *Mol. Cell. Probes* **5**: 229-240.
- SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York.
- SANGER, F., S. NICKLEN and A. R. COULSON, 1977 DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**: 5463-5467.
- SEARLES, L. L., A. L. GREENLEAF, W. E. KEMP and R. A. VOELKER, 1986 Sites of *P* element insertion and structures of *P* element deletions in the 5' region of *Drosophila melanogaster Rp11215*. *Mol. Cell. Biol.* **6**: 3312-3319.
- SPRADLING, A. C. and G. M. RUBIN, 1983 The effect of chromosomal position on the expression of the *Drosophila xanthine dehydrogenase* gene. *Cell* **34**: 47-57.
- TAKASU-ISHIKAWA, E., M. YOSHIHARA and Y. HOTTA, 1992 Extra sequences found at *P*-Element excision sites in *Drosophila melanogaster*. *Mol. Gen. Genet.* **232**: 17-23.
- WILSON, C., H. J. BELLEN and W. J. GEHRING, 1990 Position effects on eucaryotic gene expression. *Ann. Rev. Cell Biol.* **6**: 679-714.

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APPENDIX 1

Estimation of the excision rate. The rate of excision was estimated in two independent transgenic lines, namely PL1 and PL2. A total number of 34 F_1 crosses (6368 G1 progeny) for PL1 line and 36 crosses (6667 G1 progeny) for PL2 line were observed. The mean (m) of the frequency of excision rate was 0.7129 for PL1 and 0.7047 for PL2. The variances, calculated using the formula proposed by ENGELS (1979) in order to take into account the clustering due to premeiotic events are $s^2 = 4.65 \cdot 10^{-4}$ and $s^2 = 4.95 \cdot 10^{-4}$ for PL1 and PL2 lines, respectively. Comparison between the two variances did not show any significant difference ($F_{33}^{35} = 1.0645$; NS). The t -test did not show any significant difference between the two excision rates obtained in these two transgenic lines ($t = 1.566$; 68 d.f.; NS).

APPENDIX 2

Statistical analysis of the ADH activity. The mutant lines were dispatched in five groups comprising three to six different mutant lines and the nonmutated control line. ADH assays of each group constituted an independent experiment. In each experiment three successive extracts from a given line were assayed (three measures per extract which were pooled). A two-way ANOVA test was performed (experiment 1: $F_6^2 = 4.37$, NS; experiment 2: $F_{10}^2 = 3.83$, NS; experiment 3: $F_{12}^2 = 0.24$, NS; experiment 4: $F_6^2 = 2.46$,

NS; experiment 5: $F_6^2 = 0.19$, NS). Since a significant difference was not detected in any case, the values obtained from the three successive extracts were considered to be replicates. Moreover, comparison of the variances led to the consideration that the variance between replicates is constant along all experiments. This enabled us to calculate a weighted interreplicate variance common to all lines (including the control): $s^2 = 20.84$; d.f. = 56. To take into account any series effect, the ADH activity of each mutant line was divided by the activity of the nonmutated control line of the same experiment (see Figures 7 and 8). The variance of this ratio was calculated as proposed in KENDALL and STUART (1977). The confidence intervals ($\alpha = 0.05$) were calculated using the t -test of Student d.f. = 56; $t = 2.011$.

To test whether a given mutant line is different from the nonmutated control line, its ADH absolute activity was compared with that of the control of the same experiment using a t -test of Dünnett.

To compare the ADH activity of the seven $\Delta(ry)$ deleted lines and that of the first seven $\Delta(ry-Fbp1)$ mutant lines tested (deficiencies breakpoints from -1105 to -821) (group A), the nonparametric test of Mann and Whitney was applied ($e_U = 2.746$, $P < 0.01$). It can be concluded that the activity of the $\Delta(ry)$ deleted lines and that of the group A mutants are significantly different.

Analysis of $\Delta(ry-Fbp1)$ mutant lines (deficiencies from coordinates -543 to -162) (group B), following a nonparametric rank correlation of Spearman ($r' = 0.94$, $n = 6$, $\alpha < 0.01$) enables us to conclude that the ADH activity within this group significantly decreases.