Molecular Screening for P-Element Insertions in a Large Genomic Region of Drosophila melanogaster Using Polymerase Chain Reaction Mediated by the Vectorette

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ABSTRACT

As an alternative to existing methods for the detection of new insertions during a transposon mutagenesis, we adapted the method of vectorette ligation to genomic restriction fragments followed by PCR to obtain genomic sequences flanking the transposon. By combining flies containing a defined genomic transposon with an excess of flies containing unrelated insertion sites, we demonstrate the specificity and sensitivity of the procedure in the detection of integration events. This method was applied in a transposon-tagging screen for BJ1, the Drosophila homolog of the vertebrate gene Regulator of Chromosome Condensation (RCCI). Genetic mobilization of a single genomic P element was used to generate preferentially new local insertions from which integrations into a genomic region surrounding the BJ1 gene were screened. Flies harboring new insertions were phenotypically selected on the basis of the zeste-dependent transvection of white. We detected a single transposition to a 13-kb region close to the BJ1 gene among 6650 progeny that were analyzed. Southern analysis of the homozygous line confirmed the integration 3 kb downstream of BJ1.

With the advent of sequencing projects for different model organisms, the number of genes with unknown or only presumed functions will inevitably increase, and, therefore, methods to investigate gene functions will be invaluable tools in deciphering the genetic content. A prime choice is the creation of null mutations for the genes of interest. The creation of directed gene knockouts is easily achieved by homologous recombination in organisms such as yeast and mice, whereas this is not a routine technique for Drosophila. In this organism, different screening strategies for mutations in genes without previous knowledge of a phenotype have been developed. These include the generation of random insertions of genetically marked mobile elements within the whole genome and subsequent screening for stable insertions in the regions under investigation (Cooley et al. 1988). Either the insertion itself leads to inactivation of the gene function or a deletion can be induced by imprecise excision of the primary insertion (Voelker et al. 1984; Daniels et al. 1985).

For the detection of the primary insertion, several thousand chromosomes have to be screened at the molecular level (Segal et al. 1992; Dalby et al. 1995), which can only be accomplished with reasonable effort in pools of flies. Such a screening procedure must therefore be sensitive enough to detect a single positive event among ~100 flies. One method is based on the rescue of plasmid DNA together with flanking genomic DNA, which is used as a hybridization probe for the genes of interest (Hamilton et al. 1991, 1995). Other investigators used the powerful PCR technology either to screen with gene and P-element-specific primers for new insertions, or generated P-element flanking genomic DNA by inverse PCR, which again could be used as a hybridization probe (Ballinger and Benzer 1989; Kaiser and Goodwin 1990; Segal et al. 1992; Littleton et al. 1993; Dalby et al. 1995).

Besides applying an efficient molecular screening procedure for P-element insertions in a given genomic region, it is equally desirable to generate a maximum number of P-element insertions within that region from the starting element. One strategy of raising insertion frequencies for a given locus in comparison with the rest of the genome is to use a previous P-element insertion close to that locus as a starter element to perform a “local hop” (Tower et al. 1993). The intention to perform a local hop unfortunately restricts the application of plasmid rescue for insertion screening to those regions where P elements with integrated plasmid sequences have been mapped. Another restriction for the screening by plasmid rescue and by inverse PCR is the necessity to determine DNA concentrations correctly to achieve the circularization of linear genomic restriction fragments (Sambrook et al. 1989), which is a bit uncertain from crude DNA preparations. A further critical step for plasmid rescue is the need for very high transformation rates to recover the desired piece of DNA from the complex genomes derived from a pool of ~50–100 adult flies.

The PCR screening methods also suffer from some
inherent limitations that might influence the efficiency of recovering desired insertions. Restriction fragments flanking P elements shorter than 200–300 bp might be difficult to detect by inverse PCR as they are rather refractile to circularization (Shore et al. 1981). Screening by PCR with a gene-specific primer limits the region to be scanned close to the selected primer or demands the setup of a battery of different PCRs for the same DNA with primers covering a larger region.

To circumvent these limitations, we used a special adapter called vectorette (Riley et al. 1990), which we ligated to genomic restriction fragments. PCR with a P-element-specific primer and a vectorette primer generated fragments of DNA flanking any genomic P element. Screening for the presence of defined P-element insertions was done by hybridization of these fragments to genomic probes. In a model situation, the specificity and sensitivity of the method was tested, with the result that detection of a single P-element insertion within a large region can be accomplished in a background with a large excess of a complex mixture of different P-element insertions. Finally, the application of this powerful screening method with the aim of mutagenizing the B[1] gene, the Drosophila homolog of the vertebrate gene RCC1 (Regulator of Chromosome Condensation; Ohlsubo et al. 1987; Frasch 1991), is demonstrated.

**MATERIALS AND METHODS**

**Oligonucleotides**: Primers used for PCR were of following sequence: PF2 CGACGGGACACCTGCTTTGAT, PF3 CCACCTTATGTATTTTCTCATG, VRe2 GACTGACAACGAAACGAACGGT, V1GATCTCCTCGGTACATAGCTGAGGAGCTGAC AACGAACGACAAGGGTGAGAAGGGAGAG, V2 CGCTTCTCC TCTCTCTCGGTAAAACGACGGCCAGTCCTCGATCATGT ACCGGGA. The vectorette was produced by hybridizing 18 μg V1 with 17 μg V2 in 100 μl containing 100 mm TrisHCl, pH 7.5, 10 mm MgCl2. Annealing occurred by placing the oligonucleotides in an Eppendorf tube into a beaker with 1 liter of H2O at 95° and cooling the water continuously in a cold room to 4°. The partially double-stranded structure of the vectorette and the position of the vectorette primer VRe2 are shown in Figure 1. Primers PF2 and PF3 recognize sequences within the 31-bp P-element-terminal repeat (O'Hare and Rubin 1983).

**Detection of genomic sequences flanking P elements**: For the isolation of genomic DNA, 50–60 flies were frozen in 200 μl lysis buffer (100 mm TrisHCl, pH 9, 100 mm EDTA, 1% SDS), thawed on ice, and homogenized. After the addition of 600 μl lysis buffer, the homogenate was incubated at 70° for 30 min. A total of 150 μl 8 m K-acetate was added, and the homogenate was left on ice for 20 min and centrifuged in a microcentrifuge at 10,000 rpm for 20 min at 4°. DNA from the supernatant was precipitated with 0.9 vol of isopropanol, washed with 75% ethanol, and resuspended in 100 μl TE (10 mm TrisHCl, pH 8.1, 1 mm EDTA) containing 2 μg/μl RNase A. Genomic DNA (20 μl) was digested with 10 units of restriction enzyme (TagI, HpaII, or HinfI from Biolabs) in a final volume of 50 μl for 3–4 h at the recommended temperature, and the DNA was purified by extraction with an equal volume of phenol and chloroform and was precipitated with ethanol. The DNA was finally resuspended in 50 μl TE. About 100 ng of digested DNA was ligated to 200 ng of annealed vectorette with 6 units ligase (Biolabs) in 20 μl for 16 hr at 16°. After incubation at 70° for 10 min, the DNA was precipitated with ethanol and resuspended in 30 μl H2O. Two microliters of ligated DNA was used for amplification (10 pmol of the primers PF2 and VRe2, 250 μm dNTP, 2 mm MgCl2, 0.01% gelatine, 0.5 units Taq polymerase (Boehringer Mannheim) in 25 μl, 20 cycles of 1 min 95°, 1 min 63°, and 1.5 min 72° in a thermal cycler). Two microliters of this products was diluted 25-fold, and 2 μl was used for nested PCR under identical conditions, except that primer PF3 was used instead of PF2, and the PCR conditions were changed (27 cycles of 1 min 95°, 1 min 65°, and 1.5 min 72°). For the analysis of amplification products, 12 μl of the final reaction was loaded on a 1% agarose gel. All other techniques used were standard methods and were performed as described (Sambrook et al. 1989).

**Fly strains and crosses**: Flies were kept on standard medium at 20°. Mobilization of a single genomic P element in map position 65A in a derivative of the transformant 47.1 N8 (Gehring et al. 1984) was performed as shown in Figure 2A. w, z2; P[w]47.1 N8 homozygous virgins were crossed with w+/Y; P[ry-12Δ2-3]99B, Ki males (Robertson et al. 1988). Ki males with a mosaic eye color were collected from the next generation (F1) and backcrossed to w, z2; P[w]47.1 N8 virgins. Descendant males and females (F2) with a red eye color indicative of an unpaired copy of P[w+] resulting from excision of the P element or integration into a new site were separately backcrossed to w, z2; P[w+]47.1 N8. After 4–5 days of mating, red-eyed flies were removed and analyzed for new P-element insertions by vectorette-mediated PCR. Flies were mated in groups of 20 red-eyed males with 40 females per bottle or 20 red-eyed females with 30 males. Red-eyed flies from three bottles comprising 50–60 individuals were combined for the PCR analysis, schematically shown in Figure 2B. 180 male descendants (F2) with a positive integration event from a group of three bottles were again crossed to virgins of the original transformant 47.1 N8 in groups of 10 males with 25 females per bottle. Males were removed after 4–5 days of mating, and their DNA, in groups of 10 individuals, was analyzed by PCR. From a single positive bottle in F3, 30 red-eyed
The amplification of a sequence by PCR, where one end of that sequence for primer binding is known, can be accomplished by ligation of an adapter to restriction fragments and using the adapter for the binding of the second primer. The specificity of amplification can be improved by designing a special adapter called a vectorette. An essential feature of this adapter is a centrally unpaired region (see Figure 1). The vectorette primer chosen for PCR has the same sequence as one of the mismatched strands of the vectorette, with the effect that in a PCR reaction, this primer, on its own, cannot amplify genomic fragments containing ligated adapter sequences at both ends. Only the synthesis of a complementary vectorette sequence from a specific second primer generates a binding site for the vectorette primer and results in the amplification of a genomic fragment flanking the second primer. The design of the vectorette is intended to reduce background amplification of any genomic fragments and has been used to amplify and isolate terminal sequences from YAC clones (Riley et al. 1990).

To test reliable amplification of defined sequences from the background of a more complex genome by this hemi-specific PCR, we intended to isolate genomic sequences flanking the P insertion in the transformant 47.1 N8 (Gehring et al. 1984). Genomic DNA was cut with TaqI, ligated to the vectorette, and subjected to 45 cycles of nested PCR with the P-element-specific primer and the vectorette primer (see materials and methods). A 700-bp fragment visible on the ethidium bromide-stained agarose gel was cloned. To verify its origin from the P-element integration site in the transformant 47.1 N8, DNA from this line and from wild type and males (F₁) were collected and individually mated to three to five virgins of the strain 47.1 N8. 50% of the F₂ generation had a zeste eye color and 50% had red eyes. Flies with red eyes were collected and analyzed by Southern blot for new P insertions.

For the establishment of a homozygous line from a positive heterozygous line in F₁, red-eyed males were crossed with w·; e, st, spo/TM3, Sb virgins. Several red-eyed Sb males (either containing the chromosome with the new insertion or containing the chromosome with the original insertion) from the next generation were singly crossed to w·; st, spo/TM3, Sb virgins. For the determination of the respective chromosome, the same males were mated to zeste-eyed homozygous virgins from line 47.1 N8. Only males containing a chromosome with a new insertion gave rise to red-eyed Sb+ males. Red-eyed Sb descendants from these males were crossed inter se to establish a homozygous stock.

Strains P1749 (P[ry = PZ]1(3)10567 ryRK TM3, ryRK Sb1) and 72 were generous gifts from the Bloomington Stock Center (Indiana University, Bloomington). Genes, chromosomes, and symbols are described in Lindsley and Zimm (1992).

RESULTS

Amplification of genomic sequences flanking P elements: The amplification of a sequence by PCR, where only one end of that sequence for primer binding is known, can be accomplished by ligation of an adapter to restriction fragments and using the adapter for the binding of the second primer. The specificity of amplification can be improved by designing a special adapter called a vectorette. An essential feature of this adapter is a centrally unpaired region (see Figure 1). The vectorette primer chosen for PCR has the same sequence as one of the mismatched strands of the vectorette, with the effect that in a PCR reaction, this primer, on its own, cannot amplify genomic fragments containing ligated adapter sequences at both ends. Only the synthesis of a complementary vectorette sequence from a specific second primer generates a binding site for the vectorette primer and results in the amplification of a genomic fragment flanking the second primer. The design of the vectorette is intended to reduce background amplification of any genomic fragments and has been used to amplify and isolate terminal sequences from YAC clones (Riley et al. 1990).

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Detection of specific vector integrations in the background of many other integrations: Successful screening of mutated flies requires the detection of a positive event in the background of many other integrations distributed within the genomes. Therefore, we checked the sensitivity of the vectorette PCR by mixing one fly of the transformant 47.1 N8 with an increasing number of flies containing P-element insertions unrelated to the genomic insertion of strain 47.1 N8. The DNA isolated from these pools of flies was digested, ligated with the vectorette, subjected to PCR, and the blotted products were probed with the genomic clone isolated from 47.1 N8. The result shown in Figure 4A demonstrates that a single insertion can still be detected in a 100-fold dilu-
numbers of flies from 47.1 N8 with the P strain π2, resulting in an increased amount of different fragments from the P-element ends competing in the amplification reaction, the specific end fragment from the insertion line 47.1 N8 could be detected (Figure 4B). In principle, genomic sequences from both ends of the P elements are subject to amplification by the vectorette method. This means that we detected the desired P-element insertion in a background of 60–100 amplified unrelated fragments. Even a 10-fold dilution leading to a relation of one targeted P-element end to 600–1000 unrelated P-element ends allowed detection of the target (Figure 4B, lane 2), and this band is still visible in a 100-fold dilution of 47.1 N8 with π2. We conclude that amplification of P-element ends by the vectorette is a reliable and sensitive method to detect any P-element insertion in a desired region from the background of at least 100 independent insertions, which is suitable for a large insertion mutagenesis screen.

Generating P-element insertions in the region of BJ1: To maximize the efficiency of recovering P-element transpositions to the cytologic region 64F, the location of the BJ1 gene, we used a combination of two strategies: strain 47.1 N8 containing a single P element located in 65A (Gehring et al. 1984) was used as a source for a genetic cross with the Δ2-3 strain to generate elevated insertion rates within this local region (Tower et al. 1993; Zhang and Spradling 1993). The presence of a white gene with its 5′-regulatory sequences containing the binding site for the Zeste protein allowed the selection for transposition events among the F2 generation; as in a z1 background, flies with paired copies of white have yellow eyes, whereas single unpaired copies produce red eyes (Wu and Goldberg 1989).

Flies were crossed as shown in Figure 2A, and the DNA from pools of 60 red-eyed flies, each of the F2 generation (see Figure 2B), was prepared and subjected to PCR amplification (see materials and methods). The blotted products were hybridized with 13 kb of genomic DNA containing the BJ1 gene. From 111 pools containing a total of 6650 flies, a single pool with a positive event was detected (Figure 5). Subdivision of the positive pool into 18 bottles containing 10 red-eyed flies each in the F3 generation, and 30 single-pair crosses in F4 from a positive pool in F3 allowed selection and establishment of a transformed line containing an insertion within the BJ1 region. The length of the hybridizing PCR product generated during rescreening F3 flies was identical in length to the product generated from F2 flies when genomic DNA was cut with TaqI, indicating that the same insertion had been studied throughout. The hybridizing products generated from HpaII- or HinPI-restricted genomic DNA were of different length (data not shown), suggesting a specific P-element end fragment amplification from the positive pool.

Identification of the integration site: Progeny from single crosses in F4 were subjected to Southern analysis
transposon insertion within the BJ1 products from a pool of 60 red-eyed mutagenized flies were homozygous (see materials and methods). A single positive signal in the W19 pool of flies is visible, indicative of the presence of at least one fly with a transposon insertion within the BJ1 region tested.

to detect an RFLP within the 13-kb BJ1 region. The positive line heterozygous for the insertion was made homozygous (see materials and methods), and the integration event was localized within this homozygous line by Southern analysis using probes from the 13-kb genomic region. Figure 6A shows restriction fragments obtained from wild type and the homozygous line BJ1-19.4 hybridizing with a probe derived from the 3’-side of BJ1 (Figure 6B). The 5.6-kb EcoRI fragment and the 4-kb HindIII fragments from wild type are split into two fragments, each of nearly identical length in the homozygous transformant, which confirms an integration event in the middle of these fragments and 3 kb downstream of the BJ1 gene (Figure 6B). Flies homozygous for this insertion are viable and fertile and have no obvious morphological or behavioral phenotype.

DISCUSSION

A transposon mutagenesis seems to be the only reasonable way in Drosophila for obtaining mutations in genes for which a prediction of a phenotype cannot be made because these alterations are unambiguously recognized at the molecular level (Spradling et al. 1995). The detection of transposon insertions in a mutagenesis screen demands a high degree of specificity and sensitivity for the method applied when a large region is to be scanned for insertions. To allow screening within large genomic regions, we used the semi-specific PCR on vectorette-ligated genomic fragments, which was supposed to be useful and had been applied only for DNA of low complexity, e.g., cDNA and yeast artificial chromosomes (Riley et al. 1990; Silver 1991; Valdes et al. 1994). The specificity of the method is demonstrated by the cloning of a genomic fragment flanking a single P-element insertion (see Figure 3), and this was also achieved for two other P-element insertion strains tested (data not shown). At the same time, this method is very sensitive, as a single genomic end fragment is detectable in the background of genomic DNA containing 60–100 different amplifiable fragments. Flies with this large number of different P elements could even be used in 100-fold excess, resulting in a ratio of 1 P-element end to 6000–10,000 different P-element ends in the PCR reaction. This high degree of sensitivity could not be obtained in the generation and detection of genomic fragments from P-element ends with the inverse PCR. By this method, a specific integration was detected in a 100-fold dilution with flies containing 4 unrelated P elements, but not in a 500-fold dilution (Dalby et al. 1995). The lower sensitivity with the inverse PCR method may be caused by the need to circularize linear fragments, which is only efficient for and depends on certain fragment lengths and DNA concentrations (Sambrook et al. 1989). Additionally, covalently closed circular, double-stranded DNA is a rather poor template for PCR amplification (Silver 1991). These disadvantages are circumvented by the ligation of the vectorette, which could be used in excess with genomic restriction fragments, and this ensures a high yield of template DNA for PCR. As a result of its high sensitivity, this strategy gives the confidence that a transposon integration within a desired region will not go undetected during a comprehensive transposon mutagenesis. At the same time, the sensitivity allows pooling of flies in groups of 100 individuals or more, which reduces the number of single PCR reactions for screening and enables the investigation of several thousand mutagenized flies within a few months.

A further advantage of the vectorette-mediated PCR compared to alternative methods, such as plasmid rescue or inverse PCR, lies in the restriction on P-element-terminal repeat sequences for screening. Therefore, vectorette-mediated PCR with a set of two primers detects all kinds of nonautonomous P elements in a single reaction, facilitating a mutagenesis using the 17 incomplete P elements from the Birmingham 2 strain (Robertson et al. 1988).

As transposition frequencies of single marked P elements are highly variable and can be as low as 1% per chromosome and generation (Engels 1989), it is preferable to preselect flies with new insertions to keep the number of flies that have to be screened at a minimum. This can be achieved genetically by selecting for the presence of the marker of the transposon and simultaneously counterselecting the chromosome containing the starting element. Transpositions to other chromosomes occur with a significantly lower frequency than transpositions to the vicinity of the starting element on the same chromosome (Tower et al. 1993; Zhang and Spradling 1993). Thus, a local source of a P element for insertion mutagenesis is often desirable. A problem associated with local transposition concerns the phenotypic recognition and selection of descendants harboring new transpositions. A preselection could be achieved by “reversion jumping,” which includes the counterselection...
of a starting transposon causing recessive lethality (Tower et al. 1993). The success of the strong positive selection for transpositions by this method is counteracted by the tendency of the starting element to remain at the original site (Engels 1996), which leads to low frequencies of obtaining new insertions.

The visible detection of transpositions during a "local hop" is limited by the capability of the marker to create new eye color phenotypes. Markers suitable for this kind of selection are the mini-white gene able to create dosage dependent, distinguishable eye phenotypes and the rosy gene, which has been used on the basis of its sensitivity to position effects (Tower et al. 1993). The element we used for mobilization carried a white marker, which causes a normal wild-type eye color in its original position and in most cases of new insertions. Nevertheless, because of its large 5′-regulatory region containing the binding site for the Zeste protein, and because flies with this element are homozygously viable, preselection of flies with new transpositions was possible on the basis of the z1 repression of paired copies of white. Thus, flies containing the starting chromosome in a z1 background showed a zeste eye color, and new insertions have been selected for by their wild-type eye color. To estimate the enrichment of new P-element insertions in the F2 frequencies of obtaining new insertions. The number of flies representing the three phenotypic classes is shown in Table 1. Flies with the Ki1, yellow eyes phenotype had a starting P element, which was either unchanged or had minimal rearrangements not influencing the z1 repression, or contained a complete new P-element insertion elsewhere in the genome. Without any preselection method, new P-element insertions would have to be

![Figure 6.—Transposon insertion 3 kb downstream of B1. (A) Genomic DNA isolated from wild-type flies (a) and the established line homozygous for the P-element insertion in the B1 region (b) was cut with the restriction enzymes indicated, and the blotted products were hybridized with the probe shown in B. The length in kilobases of the expected fragments from wild type are denoted at the right. (B) The restriction map of the region including the B1 gene (open bar, coding region shaded) is shown as solid lines with relevant fragment lengths given in kilobases. The whole region shown was used as a hybridization probe during the mutagenesis screen, whereas the black bar denotes the probe used for the Southern in A. Above the map, the transposon used for mobilization with relevant restriction sites and the position of integration are shown. The transposon has a length of 13 kb and is not drawn to scale.](image)
screened from the \( w^+ \) population of flies from the \( F_2 \) generation, including the phenotypes \( K^{+}z^1 \), yellow eyes (29%), as well as a fraction of the phenotypic class \( K^{+}\), red eyes (0–19%), namely those without transpositions, but with minimal rearrangements not disturbing white expression, and those with new \( P \)-element insertions. With the preselection method relying on \( z^1 \) repression of white, new \( P \)-element insertions were included in 19% of the \( F_2 \) generation. The preselection by \( z^1 \) repression of white, therefore, resulted in about a twofold enrichment of flies containing new \( P \)-element insertions. The merely moderate enrichment is a consequence of the mechanism of \( P \)-element transpositions, which are very frequently associated with rearrangements within the region of the \( P \)-element insertion (Engels 1989; Delattre et al. 1995). True transpositions included in the phenotypic class of red-eyed flies in the \( F_2 \) generation were, therefore, to a large extent superimposed by rearrangements disturbing \( z^1 \) repression.

As two copies of white present on the same chromosome in tandem duplication are also repressible by \( z^1 \) in heterozygotes (Gubb et al. 1986), this kind of selection for new transposon insertions may fail for cases where the target is close to the starting element and this element remains at its original position, though the minimal distance between two copies of white disturbing the repressive effect of \( z^1 \) is unknown. The new insertion \( 3' \) of \( B_{J1} \) and the starting element used for the “local hop,” which remained in its original position, are close together at the cytogenetic level (64F and 65A, respectively), but presumably far enough at the molecular level, which is \( \geq 10 \) kb, to allow normal white expression in a \( z^1 \) background. The homozygous line \( B_{J1}-19.4 \) does not show \( z^1 \) repression possibly because of an alteration of the \( z\)-binding site of either of the two copies after \( J. \)

Flies containing the transposon insertion \( 3' \) of the \( B_{J1} \) gene are homozygous viable; the insertion does not influence the expression of this gene. In a secondary mutagenesis, this insertion 3 kb downstream of \( B_{J1} \) is currently being used to induce imprecise excisions of the \( P \) element to recover genomic deletions of \( B_{J1} \).

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**LITERATURE CITED**


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**TABLE 1**

<table>
<thead>
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<th>Phenotypes</th>
<th>Total number of flies</th>
<th>Percentage of total</th>
<th>Percentage of ( K^{+} )</th>
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<td>( K^{+}z^1 )</td>
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<td>( K^{+}, ) yellow eyes</td>
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<tr>
<td>( K^{+}, ) red eyes</td>
<td>569</td>
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**Representative sample of flies recovered in \( F_2 \)**