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Isolation and analysis of the breakpoint sequences of chromosome inversion *In(3L)Payne* in *Drosophila melanogaster*

(polymorphism/nonspecific PCR/chromosome rearrangements/genome evolution)

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ABSTRACT **Chromosomal rearrangements constitute a significant feature of genome evolution, and inversion polymorphisms in *Drosophila* have been studied intensely for decades. Population geneticists have long recognized that the sequence features associated with inversion breakpoints would reveal much about the mutational origin, uniqueness, and genealogical history of individual inversion polymorphisms, but the cloning of breakpoint sequences is not trivial. With the aid of a method for rapid recovery of DNA clones spanning rearrangement breakpoints, we recover and examine the DNA sequences spanning the breakpoints of the cosmopolitan inversion *In(3L)Payne* in *Drosophila melanogaster*. By examining the sequence diversity associated with six standard and seven inverted chromosomes from natural populations, we find that the inversion is monophyletic in origin, the sequences are genetically isolated from recombination at the breakpoints, and there is no association with features such as transposable elements. The inverted sequences show 17-fold less nucleotide polymorphism, but there are eight fixed differences in the region spanning both breakpoints. This suggests that this inversion is not recently derived. Finally, Northern analysis and transcript mapping find that the distal breakpoint has disrupted three transcripts that are normally expressed in the standard arrangement. Incidentally, the method introduced here can be used to isolate breakpoint sequences of arrangements associated with many human diseases.**

Chromosomal inversions were first discovered by Sturtevant in 1917 as recombination modifiers in *Drosophila melanogaster* (1), and the subsequent study of chromosomal rearrangements in *Drosophila* became important in evolutionary biology and population genetics (2, 3). Nevertheless, after decades of study, a number of questions and assumptions about inversions have persisted. For instance, inversions are assumed to be mutationally unique (i.e., monophyletic in origin); however, it is now known that transposable elements exhibit genomic site preferences, can generate rearrangements, and therefore are potential causal agents of chromosomal inversions in natural populations (4–6). It has also been proposed that inversions persist in natural populations as recombination-protected coadapted gene complexes (3), but the possibility that mutations unique to the inversion event are involved in early establishment has not been addressed. These and other questions can be addressed by molecular analysis of chromosomal breakpoint sequences.

The historical cosegregation of large segments of associated chromosomal arms with inversions (by suppression of products of recombination in heterokaryotypes) is not absolute at the population level but is expected to decrease with distance from the inversion breakpoints, where recombination is most suppressed. Taken to the limit, the nucleotide sequences immediately flanking rearrangement breakpoints should contain information about the origin, genealogical history, and mutational effects of associated arrangements. Unfortunately, the isolation of rearrangement breakpoint sequences is a challenging technical problem. In this report, we introduce a method that facilitates the rapid isolation of clones spanning the breakpoints of any chromosomal rearrangement. We use this method to isolate and analyze sequences† at the breakpoints of a cosmopolitan inversion and one of the first recombination modifiers described by Sturtevant (1), *In(3L)Payne* [hereafter referred to as *In(3L)P*] in *D. melanogaster*.

EXPERIMENTAL PROCEDURES

Chromosome Microdissection. The procedure described in ref. 7 was adopted with the following modifications. Third-instar larvae of 709-6, a line homozygous for *In(3L)P* (collected in Maryland, in 1986), were reared at low density on glucose medium at 15°C. Salivary glands were squashed (8) taking care to keep the exposure time to acetic acid (from dissection to 70% ethanol dip) to about 2 min to minimize depurination of DNA. Dissections were transferred into 5 μl of digestion solution [proteinase K at 0.2 μg/μl, 1.7 mM SDS, 10 mM Tris·HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin] with a needle wetted in a 1% bovine serum albumin/0.1 mM EDTA solution, the tubes were incubated at 65°C for 1 hr, and the enzyme was inactivated by placing the tubes in boiling water for 5 min. Three to five dissections were pooled for each amplification.

PCRs. *Nonspecific PCR.* Amplifications were done as described (7) with the following modifications. Native *Taq* polymerase (Perkin–Elmer/Cetus) was treated in a solution of DNase I at 0.025 μg/μl, micrococcal nuclease at 0.15 unit/μl, 10 mM Tris·HCl (pH 7.5), 1 mM $CaCl_2$, and 2 mM $MgCl_2$ for 45 min at 37°C, and the DNases were inactivated at 85°C for 15 min (with addition of sufficient EGTA for a final concentration of 0.5 mM) before being taken into the mixture for PCR. Thirty nanograms of a 17-mer nonspecific primer (5′-CTTAGGTAGANNNNTTC-3′) and 2 mM $MgCl_2$ were used in 50-μl reaction mixtures. The first three cycles used were denaturation at 94°C (30 sec), annealing at 25°C (5 min), and extension at 75°C (2 min) with a 4-min ramp to 75°C; these cycles were followed by 35 relatively stringent cycles with annealing changed to 48°C and without a ramp. Ten microliters of the first amplification and 1 μl of the second amplification were used as templates in second and third amplifications, which were done according to stringent cycles with 250 ng of the primer. Amplified DNA was precipitated in 1 mM ammonium acetate solution to remove excess nucleotides and resuspended in 10 mM Tris·HCl, pH 7.5, with

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L19785–L19836).

0.01 mM EDTA. *Inverse PCR.* The procedure described in ref. 9 was followed. The temperature cycling conditions were 35 cycles of 95°C (30 sec), 50°C (50 sec), and 75°C (1 min). *Specific PCR.* One microliter (for 50-μl reaction mixtures) of genomic DNA template, prepared according to the single fly procedure (10), plus the required amount of water were boiled for 5 min and amplified with 100 ng of each primer. Temperature cycling conditions were four cycles of 97°C (30 sec), 54°C (30 sec), and 75°C (70 sec) followed by 36 cycles with denaturation at 95°C. *Nested PCR.* One microliter of heat-denatured lysate (titer of 10^9/ml) was first amplified with 100 ng of the outer set of vector-specific and insert-specific primers, followed by reamplification of 1 μl of the first amplification product with 100 ng of the inner set of vector-specific and insert-specific primers. Temperature cycling conditions were 35 cycles of 95°C (30 sec), 46°C (30 sec), and 75°C (70 sec). All PCR products were checked in 2% NuSieve GTG agarose (FMC)/1% regular agarose gel electrophoresis with 0.04 M Tris–acetate/0.001 M EDTA and stained with ethidium bromide.

***In Situ* Hybridizations.** The procedure in ref. 8 was followed with modifications suggested by others (11, 12). Random hexamer-labeled biotin-14-dATP (BRL) probes produced from 20 ng of template were used on each slide.

Cloning Procedures. Oregon R genomic library was purchased from Promega. The library was screened on GeneScreen*Plus* (DuPont) membranes following described procedures (13) and the manufacturer's protocols. Phage DNAs were prepared according to the miniprep procedure (13), plasmid DNA preparations and subclonings used the boiling method and rapid procedure (14), and genomic DNAs were prepared according to ref. 10. Radioisotope-labeled probes were prepared by the random hexamer method.

DNA Sequencing. Plasmid DNAs were sequenced as described (15). For sequencing PCR products, the amplified fragments were excised from 1% low-melting-point agarose after electrophoresis with 0.5× Tris/borate/EDTA buffer and reamplified for production of single-stranded templates by the λ exonuclease procedure (16). Single-stranded templates were sequenced using Sequenase (United States Biochemical). The products of nested PCR were excised out after electrophoresis in 1% low-melting-point agarose in 0.5× Tris/borate/EDTA buffer, and 1 μl of this gel solution was directly sequenced using the CircumVent thermal cycle dideoxy DNA sequencing kit (New England Biolabs).

Northern Hybridization. RNAs were extracted by the hot phenol method, and poly(A)$^+$ RNA was purified using mAP Hybond messenger affinity paper (Amersham) (10). Poly(A)$^+$ RNA purified from 300 μg of total RNA was loaded in each lane. Electrophoresis, capillary transfer, and hybridizations were done according to methods described in ref. 17.

RESULTS AND DISCUSSION

Isolation of Breakpoint Sequences of *In(3L)P*. The basic steps and logic of our approach are as follows: Chromosome regions spanning the proximal and distal breakpoints are microdissected separately from a line homozygous for the inversion and nonspecifically amplified via PCR as described by Wesley *et al.* (7). Amplified DNAs are labeled and *in situ* hybridized to chromosomes from both standard and inverted lines to confirm amplification of regions spanning the breakpoints. Appropriate amplifications are then used to separately screen duplicate lifts from a genomic library of the standard chromosome. Each probe yields numerous positive clones of the regions, but positives common to *both* distal and proximal breakpoint probes actually span the breakpoints. This method permitted the isolation of the first *In(3L)P* breakpoint clone in 4 weeks.

Specifically for *In(3L)P*, replicate regions spanning bands 63B8-11 (distal breakpoint) and 72E1-2 (proximal breakpoint) from 709-6 were microdissected and nonspecifically amplified. Amplifications that adequately covered the breakpoint regions, as determined by *in situ* hybridization signals on the polytene chromosomes of Oregon R (standard arrangement) and 709-6, were combined into separate distal (designated 63B-MD) and proximal (72E-MD) DNA pools. 72E-MD included a transposable element. It was determined to be the *I* element by a dot-blot hybridization of the labeled 72E-MD probe to 31 of the known *D. melanogaster* transposable elements that were available in our laboratory. However, unless the same transposable element is included in both dissections (an unlikely event if the size of dissected region is limited) or the element is directly involved in both breakpoints, the elements will not interfere with the procedure. Duplicate transfers of the Oregon R genomic library were screened in parallel with 63B-MD and 72E-MD probes. Out of ≈200 positive clones in about 72,000 plaques, 8 unambiguous copositives were identified. DNA of these copositive clones were *in situ* hybridized to Oregon R and 709-6 polytene chromosomes. All the clones proved to be distal or AB breakpoint clones. In retrospect, the failure to pick up the proximal breakpoint is not surprising, since we used only one degenerate primer, with a specific 3-bp anchor at the 3′ end. We subsequently isolated the proximal breakpoint sequence by inverse PCR as discussed below.

One copositive phage clone, λPGL15, was arbitrarily chosen, and its DNA was subjected to restriction enzyme and Southern analyses. A 4-kb *Bam* HI fragment that hybridized to both 63B-MD and 72E-MD probes was identified (Fig. 1*A* and *B*) and subcloned, and a terminal 1.0-kb *Pst* I–*Bam*HI fragment was determined to contain the AB breakpoint (Fig. 1*C*). This fragment was subcloned (pGB-P1.0/7) and sequenced with SP6 and T7 promoter primers, and the sequence was utilized in inverse PCR to recover the unknown sequences associated with the proximal 72E (CD) breakpoint. To increase the limits of CD sequence, we further localized the breakpoint in a 342-bp *Ava* I–*Hin*dIII fragment (Fig. 2*A*). Sequence D was then obtained from 709-6 genomic DNA using primers located in B, and sequence C was obtained from Oregon R genomic DNA using primers located in region D (Fig. 2*B*). In total, 600 bp of putative C and 645 bp of putative D sequence were obtained. Finally, primers were synthesized for the outer limits of the known sequences of A, B, C, and D and used in PCR with Oregon R and 709-6 genomic DNA. PCR products of appropriate sizes were observed only in appropriate combinations of primers and templates (Fig. 2*C*). The final confirmation that the sequences solely acquired by inverse PCR were that of the 72E breakpoint region was obtained by *in situ* hybridization of the 13^+ to 9^- PCR-generated CD fragment to the polytene chromosomes of Oregon R and 709-6.

Sequence Feature and Population Genetics of the *In(3L)P* Breakpoint Regions. Including Oregon R and 709-6, we sequenced six *Standard* and seven *In(3L)P* lines collected in the United States, Mexico, and Africa for the AB (14^+ to 8^-) and CD (13^+ to 9^-) breakpoint regions (deposited in GenBank). The polymorphic sites are shown in Fig. 3. Overall there are 50 variable positions, represented by 39 polymorphisms within the *Standard* chromosome, 3 within *In(3L)P* chromosomes, and 8 apparently fixed differences between the two arrangements. All the *In(3L)P* chromosomes in this geographically diverse set have identical breakpoints (including probable repair-related deletions of 4 bp in D at the breakpoint and 2 bp in B, 6 bases away from the breakpoint), indicating that *In(3L)P* has a mutationally unique (monophyletic) origin and ruling out the possibility that the 63B and 72E breakpoint regions are general "hot spots" for repeated breakage. Two observations also rule out the involvement of

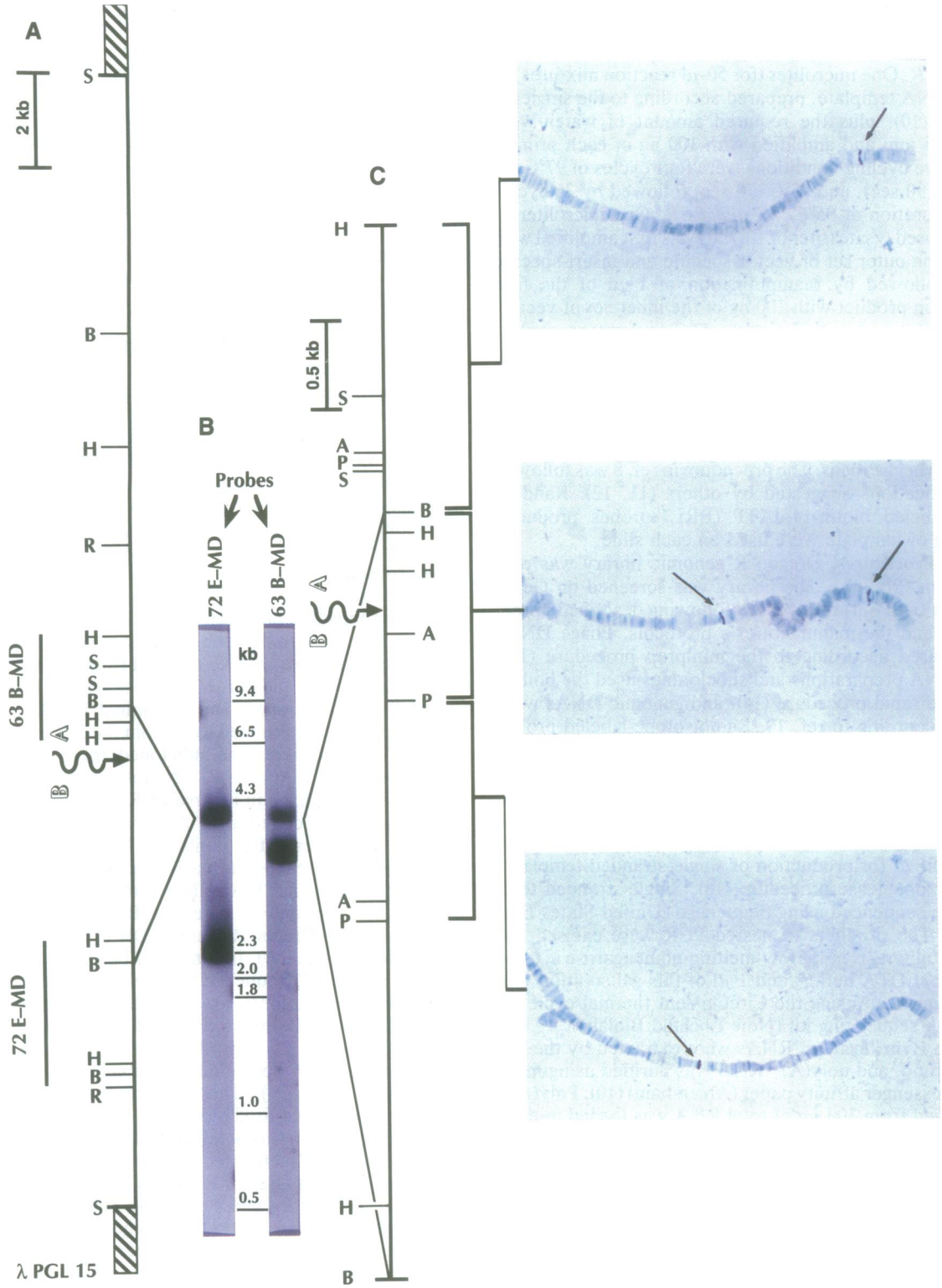

FIG. 1. Localization of the 63B (AB) breakpoint of 709-6. (*A*) Restriction enzyme map of the copositive clone λPGL15. The segments recognized by 63B-MD and 72E-MD are indicated. Squiggly arrows point to the breakpoints. (*B*) Identification of the smallest copositive fragment. One microgram of *Eco*RI–*Bam*HI-digested λPGL15 DNA was loaded in each lane. The autoradiographs show the 4-kb *Bam*HI fragment that hybridizes to both probes. (*C*) Identification of a sequenceable fragment that includes the breakpoint. Subclone pGBam4.0/2 of the 4-kb *Bam*HI fragment was digested with *Pst* I and *Hin*dIII, and the resulting three fragments, along with the two *Bam*HI–*Hin*dIII fragments that flank the 4-kb fragment (see *A*), were *in situ* hybridized to polytene chromosomes of 709-6. The photographs show the signals shifting from the distal to proximal regions of *In(3L)P* via a two-signal step due to displacement of sequence in the 1.0-kb *Bam*HI–*Pst* I fragment. A, *Ava* I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I; S, Sst I.

transposable elements in the origin of *In(3L)P*. First, there is no significant insertion/deletion difference at the breakpoints (other than the two 4-bp and 2-bp ones mentioned above) between the *In(3L)P* and *Standard* chromosomes or among *Standard* chromosomes. Second, probes of 63B-MD, 72E-MD, λPGL15, and the CD PCR fragment do not hybridize to

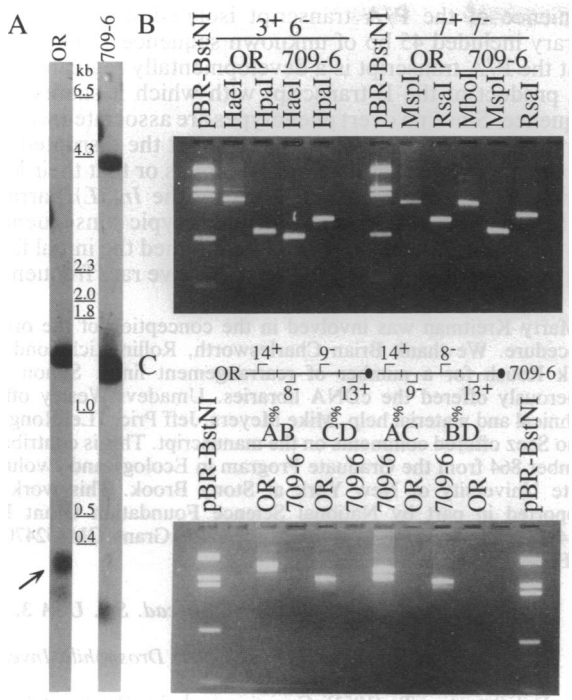

FIG. 2. Isolation of the 72E (CD) breakpoint of 709-6. (*A*) Fine-scale localization of the AB breakpoint. Five micrograms of Oregon R (OR) and 709-6 genomic DNA, digested with *Bam*HI, *Ava* I, and *Hin*dIII, were loaded in each lane. pGB1.0/7 (see Fig. 1*C*) was used as the probe. The arrow points to the 342-bp *Ava* I–*Hin*dIII fragment in Oregon R that is altered in 709-6. (*B*) Inverse PCR products containing the D sequence (709-6 DNA with 3^+ and 6^- primers) and the C sequence (Oregon R DNA with 7^+ and 7^- primers). Consult Fig. 3 for primer locations. (*C*) PCR products of AB, CD, AC, and BD fragments from Oregon R and 709-6 genomic DNA. Secondary bands in some lanes are single-stranded DNA. pBR-*Bst*NI is included as a size marker.

any of the 31 known transposable elements in *D. melanogaster* or to other sites on the chromosomes, even under relaxed stringency known to detect small segments of DNA with about 50% homology to the probe (unpublished results).

There are no shared polymorphisms, and therefore no evidence for recombination, between the *Standard* and *In(3L)P* regions covered by this sequence. Thus, the pattern of sequence diversity within and between these genetically isolated arrangements will reflect the historical demography of the *Standard* and *In(3L)P* arrangements. The global average frequency of *In(3L)P* is about 7%, but it exhibits latitudinal clines in frequency, varying from about 50% in some equatorial regions to only a few percent at higher latitudes in both the Northern and Southern hemispheres (18, 19). For both breakpoint regions, totaling 2433 nucleotides, the estimated per base heterozygosities are 0.00043 and 0.0074 for the inverted and standard regions, respectively, nearly a 17-fold difference. This low heterozygosity for the inversion could reflect a recent origin of *In(3L)P*. However, there are six fixed differences (excluding the two deletions at the breakpoints) between the *Standard* and *In(3L)P* lines, and the average pairwise difference (20) between the *Standard* and *In(3L)P* lines is 23.45 compared to 17.99 among the *Standard* lines alone. These features suggest that *In(3L)P* is not recently derived. The lower heterozygosity of *In(3L)P* is probably due to a historically smaller effective population size, a prediction under coalescence theory (21), or possibly periodic selection within the population of *In(3L)P* chromosomes. Periodic hitchhiking events have been proposed to explain the lower heterozygosity observed in other regions of low recombination in *D. melanogaster* (22, 23). The popu-

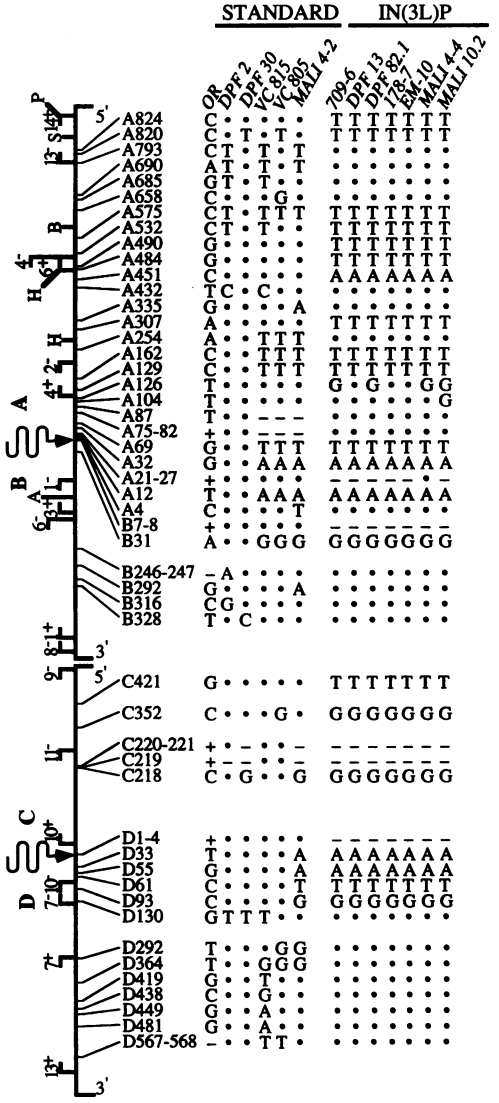

FIG. 3. Polymorphic sites in the breakpoint sequences in *Standard* and *In(3L)P* chromosomes. Locations of the primers and polymorphic sites are indicated on the line graph. The breakpoints are taken as the start point of A, B, C, and D sequences. The bases shown are those on the 5′ to 3′ strand when moving away from the breakpoints in Oregon R. The sign of the primers does not always correspond to the positive or negative strand. Oregon R (OR) is a laboratory strain; DPF 2, DPF 30, DPF 13, and DPF 82.1 were collected in New York, USA; 709-6 and 178-7 are from Maryland, USA; VC 815, VC 805, and EM-10 are from Vera Cruz, Mexico; Mali 4-2, Mali 4-4, and Mali 10.2 are from Mali, West Africa. A, *Ava* I; B, *Bam*HI; H, *Hin*dIII; S, *Sal* I. Dots indicate identity with OR sequence and dashes indicate deletions.

lation level recombination in an inversion with a frequency of 7% is 177-fold lower than the level in the majority arrangement (24), and this will result in an asymmetric vulnerability to the effects of periodic hitchhiking.

Gene Mutation and the *In(3L)P* Breakpoints. There is much evidence supporting the hypothesis that inversion polymorphisms in natural populations are maintained by natural selection (2) on coadapted gene complexes. Nevertheless, if the breakpoints disrupt the expression of one or more genes, then the immediate phenotypic consequences of these mutations may become the initial target of natural selection.

For a preliminary examination of the hypothesis that *In(3L)P* breakpoints have disrupted a gene or genes, a Northern analysis was performed on the developmental stages of Oregon R and 709-6 lines. The CD (72E breakpoint)

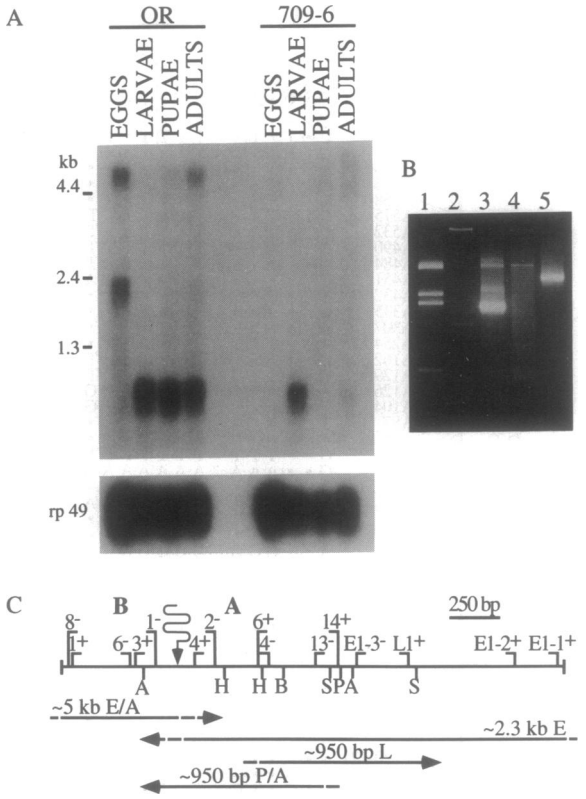

FIG. 4. Transcript analysis of the 63B (AB) breakpoint sequence. (*A*) Autoradiograph of a Northern transfer showing the transcripts that are altered or missing in 709-6. Corresponding levels of mRNA from the ribosomal protein 49 gene are shown as a control. (*B*) Nested PCR products amplified from an embryonic λZap II (Stratagene) cDNA library. Lane 3 shows the 3′ fragment of the E transcript amplified with the inner set of vector-specific (T3) and insert-specific (4^-) primers following an initial amplification with the outer set of vector-specific (M13 reverse) and insert-specific (14^+) primers (lane 2). Similarly, lane 5 shows the 5′ fragment of the E transcript amplified with the inner set T7 and 13^- primers following an initial amplification with the outer set of M13-forward and 6^+ primers (lane 4). Lane 1 is pBR322-*Bst*NI marker. (*C*) Transcript map of the 63B (AB) breakpoint sequence. The primers used in mapping and key restriction enzyme sites are shown. Arrowheads are at the 3′ ends of transcripts. Broken lines denote uncertainty of ends or unsequenced portions. A, *Ava* I; B, *Bam*HI; H, *Hin*dIII; S, *Sal* I; P, *Pst* I.

sequence failed to hybridize to any transcript. However, the AB (63B breakpoint) sequence was found to, in part, code for a set of four transcripts (Fig. 4*A*). In the 709-6 line, two of the transcripts (E and P/A) are missing, and one transcript (E/A) is altered in size. The exact nature of the association of the breakpoint with the disruption of transcripts was determined by mapping the termini of these transcripts within the 63B breakpoint. A crude mapping was first done by reprobing the Northern filter with the two complementary strands of the 14^+ to 1^- fragment and with the 14^+ to 4^+ double-stranded fragment (see Fig. 3 for primer locations). This was followed by sequencing of fragments amplified by nested PCR from available standard embryonic and pupal cDNA libraries. Both the 5′ and 3′ terminal fragments of E, P/A, and, unexpectedly, L transcripts were generated (Fig. 4*B*). As shown in Fig. 4*C*, the transcript differences are a consequence of physical disruption by the AB breakpoint. The 5′ sequence of the P/A transcript isolated from the cDNA library included 45 bp of unknown sequence. This suggests that the P/A transcript is a developmentally regulated splicing product of the E transcript with which it shares the 3′ sequence. Since no overt phenotypes are associated with flies homozygous for *In(3L)P*, it appears that the disrupted transcripts are not vital or have subtle effects or that their loss is compensated by modifiers carried by the *In(3L)P* arrangement. Nevertheless, it may be the phenotypic consequence of the loss of these transcripts that determined the initial fate of the inversion and permitted it to rise above rare frequencies.

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