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Genetics

A transposable element that splits the promoter region inactivates a *Drosophila* cuticle protein gene*

(insertion mutation/promoter mutation/T-A-T-A box/repeated sequence)

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ABSTRACT **Two mutations that affect larval cuticle protein gene expression in the 2/3 variant *Drosophila melanogaster* strain were investigated. We demonstrate that this strain synthesizes an electrophoretic variant, fast 2 (CPf2), of wild-type cuticle protein 2 (CP2). It also lacks detectable amounts of cuticle protein 3 (CP3). The other major cuticle proteins are still present. Protein and DNA sequence analyses indicate that point mutations cause two amino acid substitutions that change the electrophoretic mobility of CPf2 relative to that of CP2. The mutation abolishing the expression of CP3 was found to be a 7.3-kilobase DNA insertion located within the T-A-T-A box region of this gene, at −31 base pairs from the mRNA start site. This DNA insertion, called H.M.S. Beagle, belongs to a conserved family of repeated DNA elements that have characteristics similar to those of previously characterized *Drosophila* transposable elements. H.M.S. Beagle elements are repeated approximately 50 times in the haploid genome and exhibit restriction fragment-length polymorphisms around points of insertion between Canton S, Oregon R, and 2/3 *Drosophila* strains. Sequence analysis indicates that H.M.S. Beagle contains 266-base-pair direct repeats at its termini and is flanked by a duplication of 4 base pairs of target DNA sequence, T-A-T-A, in the *CP3* gene insertion. Thus, insertion of a transposable element into the putative promoter region of the *CP3* gene is evidently responsible for inactivating *CP3* gene expression.**

The analysis of mutants that exhibit changes in the expression of a particular gene holds promise for increasing our understanding of the molecular events involved in eukaryotic gene expression. This paper describes such an analysis of a *Drosophila* strain that has variant cuticle proteins. In wild-type *Drosophila melanogaster*, five major cuticle proteins are synthesized and secreted by the epidermal cells of third instar larvae (1). Genes for four of the five major proteins are clustered within a small [7.9-kilobase (kb)] segment of the *Drosophila* genome located at region 44D on chromosome 2 (2, 3). Naturally occurring variants have been found for three of the four cuticle protein genes (1). The four genes in the cluster are related in sequence; although there is a homologous pseudogene in this cluster, no other closely related genes exist in the *Drosophila* genome (2, 3). The region encoding these genes has been cloned; its sequence has been almost entirely determined (2, 3) and matched with the sequence of amino terminal residues of all four proteins (3). We can now apply this information to comparative studies of genetic variants to investigate factors that regulate expression of the third instar cuticle protein genes.

The *D. melanogaster* cuticle protein variant, called 2/3 (1), studied here synthesizes only three of the five major wild-type proteins and one electrophoretic variant protein (fast 2, CPf2). A molecular characterization of cuticle genes in this variant is reported below, including the finding of an insertion of a transposable element in the T-A-T-A box region of an unexpressed cuticle gene.

MATERIALS AND METHODS

Clones and Fly Stocks. λ clones and pBR322 subclones containing 44D Canton S *Drosophila* cuticle genes have been described (2, 3). The 2/3 variant (1) was recovered from a natural population of *D. melanogaster* in Australia in 1977. For some experiments, chromosomes 1 and 3 of the 2/3 stock were replaced with chromosomes from an Oregon R strain.

Construction and Screening of 2/3 Libraries. One recombinant library was constructed by ligating *Eco*RI-restricted genomic 2/3 DNA into the *Eco*RI site of pBR322 (4). From 50,000 colonies screened (5) with ^{32}P-labeled probes to pCPII-7 and pCPIII-9 (2), a positive one, pDm2/3-1, was obtained (see Fig. 2). Another library of 2/3 DNA was constructed by using the λ vector λL47 (6); 30,000 plaques were screened (2) with ^{32}P-labeled probes to pCPII-7, pCPIII-9, and pCPIV-8, and five positive phage were isolated (see Fig. 2).

Other Techniques. General nucleic acid procedures were as described (2–4, 7). Total cuticle proteins from late third instar larvae were analyzed on two-dimensional O'Farrell gels as described (2). CPf2 was purified by the procedure described for CP3 (3), and its sequence was determined as described (8).

RESULTS

The 2/3 *Drosophila* Strain Has Two Genetic Differences Affecting Cuticle Protein Synthesis. In most *D. melanogaster* strains, five major cuticle proteins are extracted from cuticles of late third instar larvae (1). The two-dimensional electrophoretic pattern of these five proteins is shown in Fig. 1 *Upper*. A naturally occurring *D. melanogaster* strain called 2/3 has third instar cuticle proteins that show the two-dimensional gel pattern shown in Fig. 1 *Lower*. Only three of the major cuticle proteins—CP1, CP4, and CP5—are found; both CP2 and CP3 are undetectable. Instead there is one new protein of altered electrophoretic mobility; its pI (5.5) and apparent molecular weight are less than that of CP2. The protein called 2/3 in Fristrom *et al.* (1) and 3V in Snyder *et al.* (2) is now renamed fast 2 (CPf2) because it is actually a variant of CP2. We have determined the sequence of the 55 amino terminal residues of CPf2. Comparison with that previously determined for CP2

Abbreviations: CP2, CPf2, and CP3, cuticle proteins 2, fast 2, and 3; kb, kilobase(s); bp, base pair(s).

* We wish to note that the first analyses of the genome blots and clone maps showing a major DNA change that was quite possibly an insertion in the gene *III* region were made by D.K. in Berkeley. Further genomic DNA blot experiments demonstrating an insert were carried out essentially concurrently by both groups. 2/3 λ clones were isolated and sequences were determined in Pasadena.

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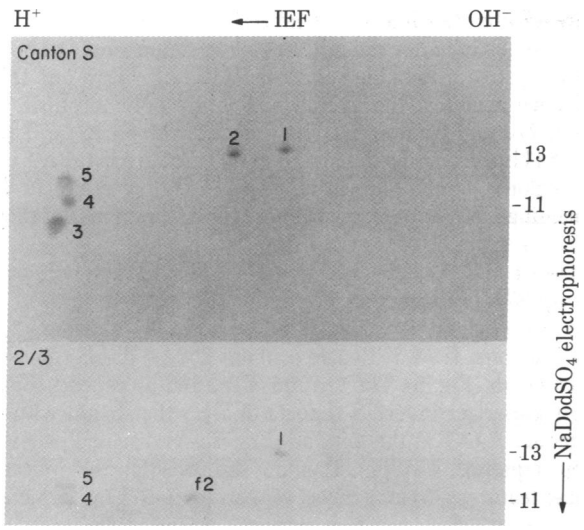

FIG. 1. Comparison of third instar cuticle proteins of Canton S and 2/3 *Drosophila* strains: Coomassie blue-stained two-dimensional O'Farrell gels (2). Most wild-type *D. melanogaster* strains contain cuticle proteins CP1–CP5 (1). The 2/3 *D. melanogaster* strain lacks detectable amounts of CP2 and CP3 in purified cuticles but makes a different protein now called CPf2. Molecular masses are given in kilodaltons; those of CP1 and CP2 have been revised slightly based on DNA sequence analyses (3). IEF, isoelectric focusing.

(3) shows that 54/55 residues match perfectly; one amino acid substitution occurs at position 18. The CPf2 sequence does not match any other cuticle protein sequence. Hence, for the 2/3 *Drosophila* strain, the following questions may be asked: (*i*) What is the nature of the mutation affecting the electrophoretic mobility of CP2 and, more interestingly, (*ii*) what is the nature of mutation such that no CP3 is found?

A 7.3-Kilobase (kb) DNA Insertion Is Located at the 5′ End of the Unexpressed Gene *III*. The genes encoding CP1–CP4, denoted genes *I–IV*, respectively, have been cloned (2, 3). All four genes are clustered within 7.9 kb of DNA (Fig. 2). A pseudogene, *ψI*, also lies within this cluster (3). Each cuticle gene contains a short [56- to 64-base-pair (bp)] intron interrupting the signal peptide coding sequence.

To understand the nature of the mutations affecting CP2 and CP3, we have investigated the organization of the 44D cuticle genes in the 2/3 *Drosophila* strain by gel blot analysis of genomic DNA and of cloned DNA derived from the 2/3 strain and finally by DNA sequence analysis of the latter. The cloned DNAs constructed and used in this study are shown in Fig. 2.

The initial gel blot analysis of genomic DNA strongly suggested that there was an insertion of foreign DNA or a DNA rearrangement in the region of gene *III* (Fig. 3). (The salivary gland chromosomes appear cytologically normal in the 44D region.) Analysis of the DNA from clone pDm2/3-1 showed that the restriction endonuclease cleavage sites (see Fig. 2) in and near genes *I* and *II* are unchanged in the variant as compared with those of the wild type (2, 3). However, between the *Sal* I site 119 bp upstream from the mRNA start position of gene *III* and the *Eco*RI site at the 3′ end of gene *III*, a new segment of DNA with a different restriction site pattern is present.

A definitive demonstration of an insertion of foreign DNA between genes *II* and *III* was then obtained by gel blot analysis of genomic 2/3 DNA. Gel blots of 2/3 genomic DNA and, for comparison, of Canton S DNA, were probed with the subclones for Canton S genes *I*, *II*, *III*, and *IV* (examples in Fig. 4). The restriction map so derived is shown in Fig. 2. The results indicate that the region within and around genes *I*, *II*, and *IV* is identical in Canton S and 2/3 DNAs at a resolution of 100 bp. However, a 7.3-kb DNA insertion is located within 50 bp of the mRNA start site of gene *III*. (For example, compare lanes 1 vs. 1′, 3 vs. 3′, and 7 vs. 7′ in Fig. 4.) We name this insertion element H.M.S. Beagle, in honor of the voyage of Charles Darwin.

Gene *II* Mutations Are Single Nucleotide Substitutions. The precise nature of the changes in the 2/3 DNA was determined by sequence analysis of cloned DNAs. The strategy used and the results are presented in Fig. 5. The DNA sequence of gene *II* of the variant differs by only three single base changes from that of Canton S DNA. One lies within the 3′-untranslated re-

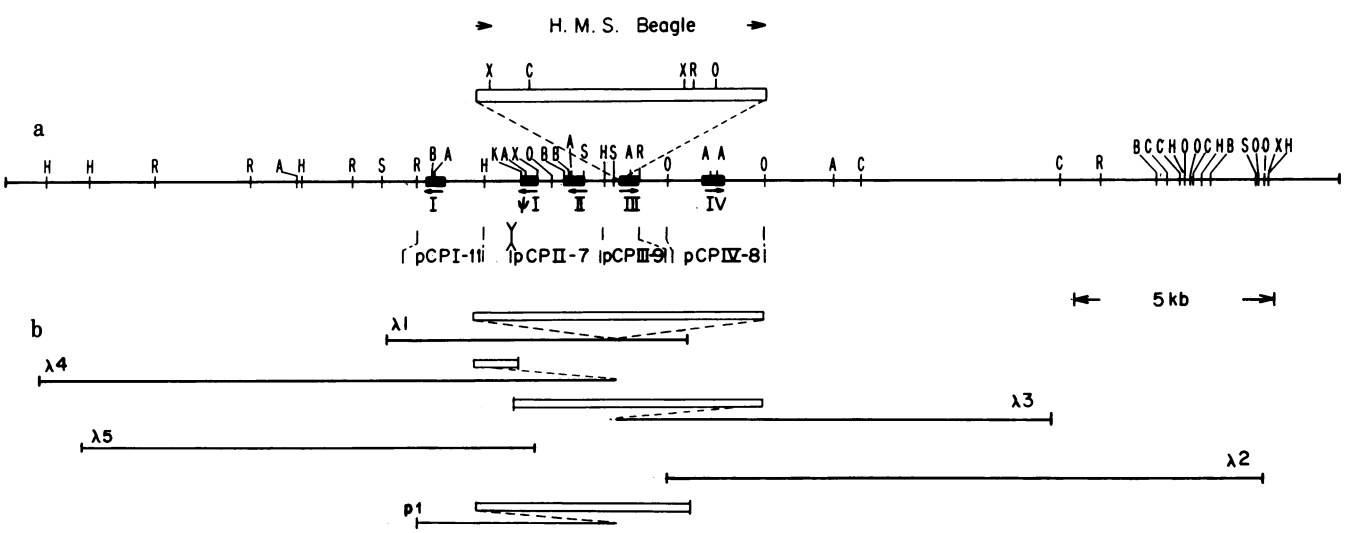

FIG. 2. Organization of the 44D cuticle genes. (*a*) Restriction endonuclease cleavage map of the cloned region of the Canton S genes. Genes *I–IV* encode CP1–CP4, respectively (2, 3). *ψI* is a putative *Drosophila* cuticle pseudogene. Arrows indicate the 5′ → 3′ direction of transcription (3). pCPI-11, pCPII-7, pCPIII-9, and pCPIV-8 are subclones of Canton S DNA encoding genes *I–IV*, respectively, and contain the regions indicated. The restriction map of 2/3 DNA is identical except for the presence of the 7.3-kb DNA insertion, H.M.S. Beagle. (*b*) The overlapping cloned inserts of 2/3 DNA. λ1–λ5, λDm2/3 LCP1–5, respectively; p1, pDm2/3-1. All sites from the *Bam*HI site 1.3 kb to the right of gene *IV* to the *Hind*III site 1.0 kb to the left of gene *I* have been mapped by genomic Southern blotting experiments using 2/3 DNA (see text and Fig. 3). Sites outside this region were mapped only on cloned DNA inserts. The *Ava* I map is incomplete; only sites in the *I–IV* region are shown and closely spaced *Ava* I sites are not indicated. *Ava* I also cleaves at *Xho* I sites. Sites: R, *Eco*RI; B, *Bam*HI; O, *Bgl* II; S, *Sal* I; C, *Sac* I; H, *Hin*dIII; A, *Ava* I; K, *Kpn* I; X, *Xho* I.

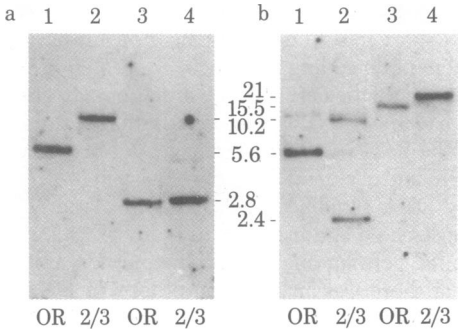

FIG. 3. Genomic DNA gel blots showing differences in 2/3 and wild-type DNAs. 2/3 and Oregon R (OR) DNAs were digested with *Hin*dIII (lanes 3 and 4) or *Eco*RI (lanes 1 and 2), and gel blots were prepared and probed with ^{32}P-labeled pCPII-7 (*a*) and pCPIII-9 (*b*). The sizes in kb of hybridizing bands are indicated. Note that the *Eco*RI-digested material probed with pCPIII-9 shows faint hybridization to a 12.4-kb band; this is due to cross-hybridization with gene *IV*.

gion; the two other changes lead to a substitution of leucine for serine (position 18) and of serine for arginine (position 108) in the amino acid sequence of CP2 (Fig. 5*c*).

The Insertion Is in the T-A-T-A-Box Region of Gene *III*. We have determined the DNA sequence at the junctions of the insertion element with the wild-type DNA. Novel DNA sequences begin (Fig. 5*b*) at position −32 bp from the mRNA site, immediately adjacent to the −31 to −24 T-A-T-A-T-A-A-A sequence (T-A-T-A box) of gene *III*. [The T-A-T-A box is placed to start at −31 rather than −29 based on its homology to the other cuticle genes and also by its position relative to the mRNA start site (3).] At the left junction of the insertion, we find another copy of part of the T-A-T-A box, T-A-T-A, together with the expected upstream flanking sequence. We conclude that the insertion occurred within or immediately adjacent to the T-A-T-A box and duplicated 4 bp of T-A-T-A-box sequence (see below). In the remainder of gene *III*, we find no other nucleotide changes in either the mRNA coding sequences (489 bp) or in the upstream flanking sequence (119 bp). From our DNA sequence analyses, the overall nucleotide polymorphism between 2/3 and Canton S DNA was determined to be 0.16% (3/1,844 bp) excluding the insertion (3/≈985 are in mRNA coding regions; 0/≈859 are in non-mRNA coding regions). The remaining DNA sequences of genes *II* and *III* regions not shown in Fig. 4 are in Snyder *et al.* (3).

The H.M.S. Beagle Insertion Has the Characteristics of a *Drosophila* Transposable Element. The exact termini of the insertion element were identified from the DNA sequences at the junctions. H.M.S. Beagle contains 266-bp direct repeats at its two ends (Fig. 5). The termini of the 266-bp repeats contain short imperfect inverted repeats of 7 bp that begin with the sequence A-G-T at the left end (Fig. 5). As discussed above, a 4-bp sequence, T-A-T-A, flanking the insertion is duplicated. These features—(*i*) long direct repeats (276–571 bp) (which begin with the sequence $\frac{T}{A}$GT or $\frac{A}{T}$CA in other instances studied so far), (*ii*) short inverted repeats (14 bp or less) at the direct repeat termini, (*iii*) the 4- or 5-bp duplication of target DNA sequence, and (*iv*) the repetitive nature of the element (see below)—are all properties common to many *Drosophila* transposable elements (for review, see ref. 9). By restriction mapping and the limited sequence data, the element differs from previously described elements.

Gel blots of appropriately digested genomic DNAs probed with internal restriction fragments of the H.M.S. Beagle insert and with fragments that include both insert and flanking DNA show the following: (*i*) the H.M.S. Beagle element is repeated approximately 50 times in the *D. melanogaster* haploid genome, (*ii*) there is a high degree of sequence conservation within the element, and (*iii*) there are differences in H.M.S. Beagle band positions among several *D. melanogaster* strains (Oregon R, Canton S, and 2/3) because of either restriction site polymorphisms or of different chromosomal locations of the insert. In

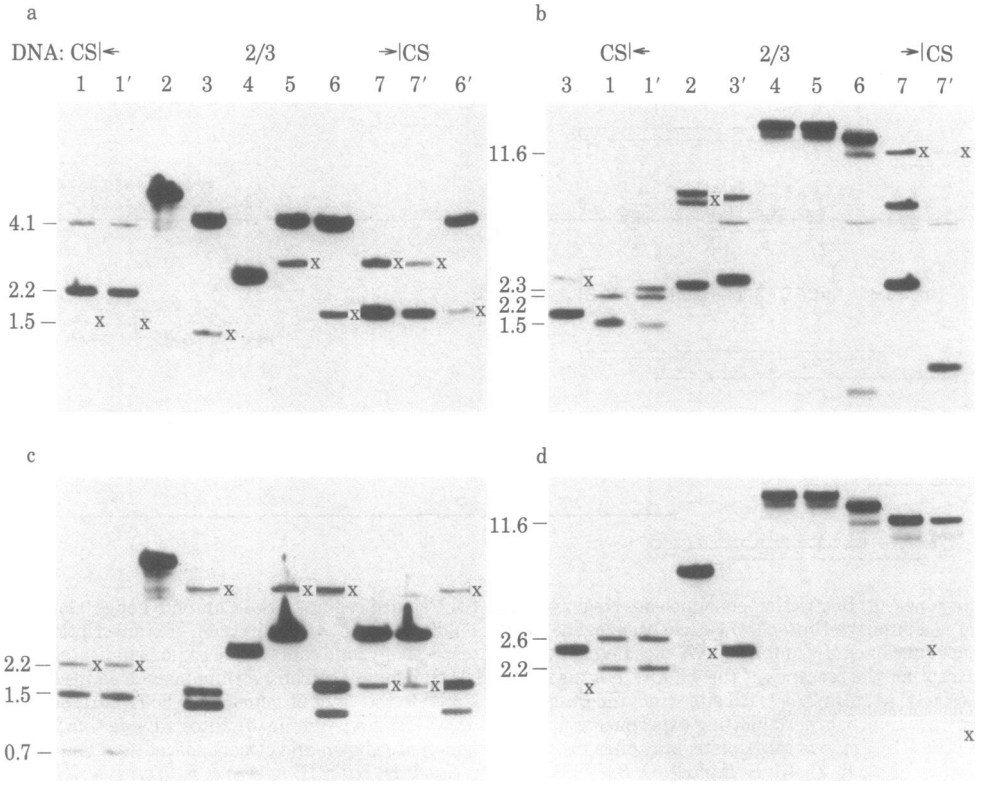

FIG. 4. Identification of an insertion near the 5′ end of gene *III*. Genomic DNA gel blots of 2/3 DNA were probed with pCPI-11 (*a*), pCPII-7 (*b*), pCIII-9 (*c*), and pCPIV-8 (*d*). Lanes: 1 and 1′, *Ava* I; 2, Sac I; 3 and 3′ Bgl I/*Hin*dIII; 4, *Sal* I/*Hin*dIII; 5, *Hin*dIII; 6 and 6′, *Xho* I/*Hin*dIII; 7 and 7′, *Eco*RI/*Hin*dIII. Note the gene *I* and *II* fragments cross-hybridize in the mRNA coding regions as do the *III* and *IV* fragments. This cross-hybridization is indicated by an X when the homologous genes are separated on different-sized restriction fragments. For comparison Canton S (CS) DNAs are also shown. Additional gel blots of 2/3 DNA digested with other combinations of restriction endonucleases and probed with pCPIII-9 and pCPIV-8 have been carried out but are not shown. The sizes in kb of *Ava* I-hybridizing bands are indicated.

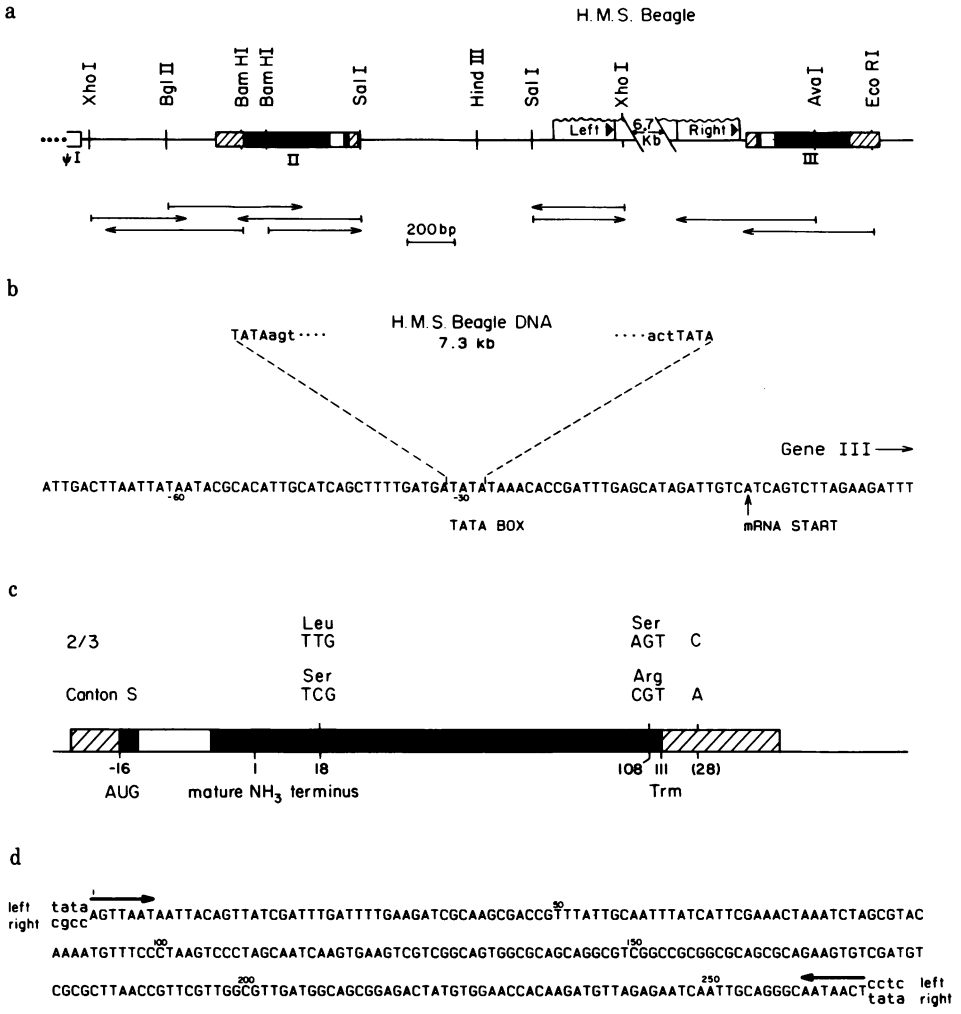

FIG. 5. Sequence analysis of H.M.S. Beagle insertion junctions and genes *II* and *III*. (*a*) Strategy used. Arrows indicate direction and extent of DNA sequence analysis of fragments that were ^{32}P labeled at their 3′ termini and analyzed as described (3). To the left of H.M.S. Beagle, the sequence was determined from λDm2/3LCP1 and, to the right, it was determined from a 2.5-kb *Eco*RI fragment of λ1 subcloned into pBR322. Only restriction sites pertinent to the analysis are indicated. ■, protein coding region; ▨, mRNA untranslated region; □, intron. (*b*) Sequence of gene *III* and H.M.S. Beagle insertion regions. The wild-type sequence is shown with the insertion in the 2/3 DNA as indicated above it. The sequence, T-A-T-A, of the T-A-T-A box is present on both sides of the insertion. No other nucleotide changes in the gene *III* region have been found; the complete sequence from the *Sal* I site (−119) to the *Eco*RI site at the 3′ end of gene *III* is identical to the Canton S sequence (3). (*c*) Sequence of the gene *II* region. Only the three differences between wild-type and 2/3 DNA are indicated. The remainder of the DNA sequence, which extends to the *Xho* I site (Fig. 5*a*), is identical in 2/3 and Canton S DNAs (3). From amino acid sequence analyses, the first 55 amino acid residues of CPf2 were identical to those predicted from the DNA sequence. Codons are numbered, with codon 1 the amino-terminal residue of the mature protein. (28) indicates a nucleotide substitution 28 bp from the termination codon. (*d*) Terminal repeats of H.M.S. Beagle. Upper-case letters indicate direct repeats, lowercase letters indicate flanking sequences and arrows indicate inverted repeats. Left and right indicate left and right repeats as shown in *a*.

this latter respect, the similarity between Oregon R and the 2/3 DNA used is partly due to the fact that chromosomes 1 and 3 of the 2/3 strain had been replaced with Oregon R chromosomes. Some relevant data are shown in Fig. 6.

DISCUSSION

The data show that two types of mutations affect cuticle protein synthesis in the *D. melanogaster* 2/3 strain. Two amino acid substitutions in gene *II* are evidently responsible for the altered electrophoretic mobility of CPf2 as compared with that of CP2. The substitution of a serine for an arginine is consistent with the observed pI shift of CPf2 from CP2. However, the increase of 10% in $NaDodSO_4$ gel electrophoretic mobility of CPf2 relative to that of CP2 is unexpected because our DNA sequence data predict that the two proteins have the same number of amino acids. Since no modification (other than signal peptide processing) is known for these proteins (3), the amino acid substitutions found in CPf2 may cause the mobility differences. The *in vitro* translation products of CPf2 also migrate faster than the *in vitro* translation products of CP2 on $NaDodSO_4$ gels (2, 3). Proteins of similar size that differ only in charge can display apparent molecular mass differences on $NaDodSO_4$ gels (10). A less likely cause of increased migration could be an undetected modification. Such a modification must lie in the carboxyl-terminal half of the protein, as the sequence of the amino-terminal half of the protein has been determined and no modified residues were found.

The H.M.S. Beagle insertion is immediately adjacent to the T-A-T-A-T-A-A sequence of gene *III*, and there is a duplication of 4 bp, T-A-T-A, of that sequence. We believe this mutation is responsible for inactivating gene *III* because (*i*) no other mutations within the gene *III* mRNA coding or upstream flanking sequences were found and (*ii*) data from other eukaryotic genes indicate that a T-A-T-A-box sequence together with sequences upstream of the T-A-T-A box are necessary for efficient tran-

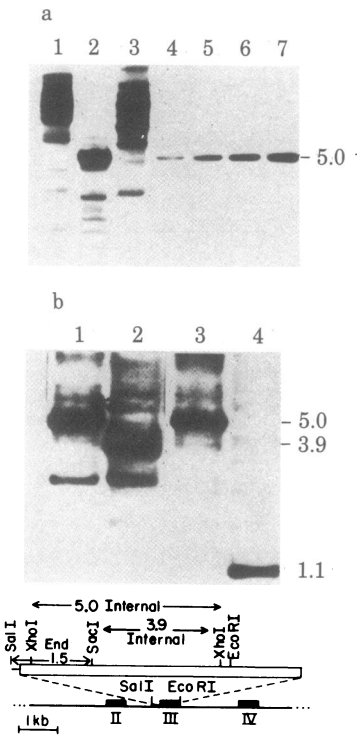

FIG. 6. Representation of H.M.S. Beagle sequences in 2/3 and Canton S DNAs. 2/3 and Canton S DNAs were digested with restriction endonucleases and analyzed on agarose gels. (*a*) Gel blots were probed with the internal 5.0-kb *Xho* I fragment of H.M.S. Beagle (isolated from λ1. Lanes: 1, *Eco*RI-digested Canton S DNA; 2 and 3, *Xho* I/EcoRI- and EcoRI-digested 2/3 DNA, respectively; 4–7, standards (mixtures of calf thymus DNA with 1, 3, 8, and 20 copies, respectively, of an internal 5.0-kb *Xho* I fragment isolated from λ1 were digested with *Xho* I/*Eco*RI). (*b*) 2/3 DNA gel blots were probed with the internal 3.9-kb *Xho* I/*Sac* I fragment (lanes 1 and 2) or with the 1.5-kb *Sal* I/*Sac* I end fragment from the left of the insert together with 119 bp of flanking DNA (lanes 3 and 4) of H.M.S. Beagle. (These probes were from pDm2/3-1.) Lanes: 1 and 3, *Xho* I-digested DNA; 2 and 4, *Sac* I/*Xho* I-digested DNA. The copy number of H.M.S. Beagle sequences was determined by comparing the results in lane 2 with standards made by using different exposure times.

scription *in vivo* (for example, see refs. 11 and 12).

The H.M.S. Beagle insert is a member of a copia-like middle repetitive *Drosophila* DNA family. Many such families, including copia, 297, 412, mdg 1, mdg 3, roo, and gypsy, have been well studied (for review, see ref. 9; for roo, see ref. 13). H.M.S. Beagle has a structure and properties similar to these transposable elements: (*i*) direct repeats at both ends, (*ii*) short inverted repeats at the direct repeat termini, (*iii*) restriction fragment length polymorphism around the insertions of family members in two *Drosophila* strains, (*iv*) a small duplication of target DNA sequence, and (*v*) a moderate degree of repetition in the genome with considerable conservation of internal sequences. Spontaneous mutations caused by transposable element insertions have been noted in the *Drosophila* white (14–16) and bithorax loci (17) as well as in yeast loci (18–21).

Recently, an additional example of a transposable element, 297, inserted into a T-A-T-A-T-A sequence has been reported, in this case for a *Drosophila* histone gene (22, 23). Since H.M.S. Beagle belongs to a different family of copia-like elements than 297, as judged by restriction mapping and sequence analysis, the occurrence of two such events suggests that insertion of such elements into the T-A-T-A-T-A sequence may be a general phenomenon and may have important biological effects in abolishing or altering the manner in which genes are regulated.

Thus far, more than 2,000 chromosomes have been examined from wild populations for variants of cuticle proteins (ref. 1; unpublished data). Other than rare variants with no or reduced levels of CP3, none has been found lacking other major third instar cuticle proteins. Structural variants of CP2 have been recovered only in association with elimination of CP3 (this study and another presumed independent one in a stock from Taiwan; C. Chihara, personal communication). The reasons for the toleration of the absence in CP3, but not in other major cuticle proteins, remain obscure. We speculate that the kinds of structural changes noted in fast 2 may have selective advantage in association with a CP3 deficiency.

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