Reconstitutional Mutagenesis of the Maize *P* Gene by Short-Range *Ac* Transpositions

Maria A. Moreno, Jychian Chen,[1] Irwin Greenblatt[2] and Stephen L. Dellaporta[3]

Yale University, Department of Biology, New Haven, Connecticut 06511

Manuscript received August 16, 1991
Accepted for publication April 8, 1992

ABSTRACT

The tendency for *Ac* to transpose over short intervals has been utilized to develop insertional mutagenesis and fine structure genetic mapping strategies in maize. We recovered excisions of *Ac* from the *P* gene and insertions into nearby chromosomal sites. These closely linked *Ac* elements reinserted into the *P* gene, reconstituting over 250 unstable variegated alleles. Reconstituted alleles condition a variety of variegation patterns that reflect the position and orientation of *Ac* within the *P* gene. Molecular mapping and DNA sequence analyses have shown that reinsertion sites are dispersed throughout a 12.3-kb chromosomal region in the promoter, exons and introns of the *P* gene, but in some regions insertions sites were clustered in a nonrandom fashion. Transposition profiles and target site sequence data obtained from these studies have revealed several features of *Ac* transposition including its preference for certain target sites. These results clearly demonstrate the tendency of *Ac* to transpose to nearby sites in both proximal and distal directions from the donor site. With minor modifications, reconstitutional mutagenesis should be applicable to many *Ac*-induced mutations in maize and in other plant species and can possibly be extended to other eukaryotic transposon systems as well.

SINCE its discovery (McCLINTOCK 1945, 1946, 1947), the maize transposable element *Activator* (*Ac*) has been the subject of numerous investigations. The genetic behavior of *Ac* (McCLINTOCK 1948, 1949, 1950), molecular cloning (FEDOROFF, WESSLER and SHURE 1983; BEHRENS *et al.* 1984), sequencing (BEHRENS *et al.* 1984; POHLMAN, FEDOROFF and MESSING 1984), transcriptional patterns (KUNZE *et al.* 1987), and its utility for gene tagging (FEDOROFF, FURTEK and NELSON 1984; DELLAPORTA *et al.* 1988) and cell lineage analysis (FINNEGAN *et al.* 1989; DAWE and FREELING 1990; DELLAPORTA, MORENO and DELONG 1991) have been well documented. Studies of transposition patterns have demonstrated the tendency of *Ac* to transpose to linked sites (VAN SCHAIK and BRINK 1959; GREENBLATT and BRINK 1962; GREENBLATT 1968; DOONER and BELACHEW 1989; DOONER *et al.* 1991). These sites can be over short physical distances, as demonstrated by intragenic transposition of *Ac* at the *Waxy* and *P* genes (CHOMET 1988; PETERSON 1990)

The most detailed genetic studies of *Ac* transposition have been at the *P* locus of maize. The *P* locus of maize conditions red pigmentation of pericarp and cob tissues of the female inflorescence of maize. These conspicuous red pigments are flavonoids synthesized through several enzymatic conversions of the precursor chalcone (STYLES and CESKA 1977). The *P* locus appears to regulate the production and tissue-specific deposition of these pigments. An alternatively spliced transcript encodes a *myb*-like protein that may act as a transcriptional regulator of the structural genes in the biosynthetic pathway (GROTEWOLD, ATHMA and PETERSON 1991). A variegated *P* allele, termed *P-VV* for variegated pericarp and variegated cob, originally described by EMERSON (1914, 1917) has been the subject of numerous transposition studies. EMERSON's *P-VV* mutation (*P-VV:E*) is caused by insertion of *Ac* into the *P* gene (BARCLAY and BRINK 1954). Because most excisions of *Ac* from the *P-VV:E* allele restore *P* function, each red pericarp stripe represents a somatic excision of *Ac* in a cell lineage which forms pericarp.

Full red pericarp ears are recovered by *Ac* excision from *P-VV:E* when transposition precedes gamete formation. These red ears sometimes exhibit an unstable phenotype consisting of somatic sectors of colorless or variegated pericarp on a background of uniformly red pericarp (ORTON and BRINK 1966). When these somatic sectors occur in lineages that form sexual gametes, new variegated *P* alleles are recovered in offspring caused by reinsertion of *Ac*. These new "reconstituted" alleles often condition new patterns of pericarp variegation (ORTON and BRINK 1966). Using reconstitution strategies, *Ac*-induced and *Ds*-induced reconstituted alleles of the *R* locus have been obtained (BRINK and WILLIAMS 1973; KERMICLE, ALLEMAN and

[1] Present address: Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan.
[2] While on sabbatical leave from the University of Connecticut, Storrs, Connecticut 06269.
[3] To whom correspondence should be addressed.

DELLAPORTA 1989). *Ds* elements are members of the same *Ac* transposon family but are nonautonomous. In the absence of *Ac*, the reconstituted *Ds* alleles of *R* are stable, permitting the genetic positions of *Ds* to be mapped by intragenic recombination tests (KERMICLE, ALLEMAN and DELLAPORTA 1989). The *Ds* elements mapped at different genetic positions, suggesting the reinsertions were dispersed throughout the locus.

The experiments described here were designed to examine the molecular basis of reconstituted alleles and their utility for insertional mutagenesis and fine structure genetic mapping. We undertook this study in order to obtain a detailed characterization of transposition profiles and target site selection of *Ac* in maize. Over 250 reconstituted *P-VV* alleles were recovered by reinsertion of *Ac* from nearby chromosomal sites. Our results clearly demonstrate the tendency of *Ac* to select nearby chromosomal sites for transposition. Most importantly, these nearby sites can be over short physical distances in both the proximal and distal directions from the donor site. Reinsertions occur in the promoter, exons and introns in a dispersed but nonrandom fashion throughout a 12.3-kb chromosomal region that defines the *P* gene. Analysis of target sequences indicates the existence of preferred insertion sites for *Ac*. The variegation patterns conditioned by reconstituted alleles may reflect structural features about the *P* gene, such as exon and intron locations, and information concerning its product, such as critical sites for protein function. Finally, we discuss the utility of reconstitutional mutagenesis and fine structure genetic analysis in higher eukaryotic systems.

MATERIALS AND METHODS

Genetic analysis: The alleles and genetic stocks used in this study are listed in Table 1. *P* alleles are designated with a two-letter suffix that denotes pericarp and cob color (V = variegated, R = red, W = white). In order to reconstitute *P-VV* alleles, a modified strategy of ORTON and BRINK (1966) was employed. The starting allele was *P-VV:E*. Stock S55 (*P-VV:E/P-VV:E r-g/r-g*) was crossed as females by tester T21 (*P-WR/P-WR r-g/r-g*) to generate *P-VV:E/P-WR* seed. This F_1 seed was grown and testcrossed as females to tester T43 (*P-WR/P-WR r-sc:m3/r-sc:m3*) to generate approximately 3,500 medium variegated ears with approximately 50% of the kernel progeny showing aleurone variegation. The *r-sc:m3* allele, introduced in this cross to monitor *Ac* activity, conditions aleurone variegation only in the presence of an active *Ac* element. A total of 500 red pericarp, variegated aleurone kernels from these ears were grown and testcrossed as females by T43. Unstable red pericarp (UR) classification was assigned to ears when two or more colorless or variegated sectors appeared in the pericarp. From each initial UR ear, all kernels showing *Ac* activity (spotted aleurone) were grown and crossed as females by stock T59 (*P-WW/P-WW r-r/r-r*). These crosses increased the number of UR ears to approximately 3,200. Kernels underlying colorless or variegated pericarp sectors on all UR ears were grown and testcrossed as females by the T43 stock; 257 variegated ears were recovered from these crosses. The pericarp and cob phenotypes of the reconstituted *P-VV* alleles were determined visually.

The *Ac* dosage was scored for each reconstituted allele by examining the spotting pattern in the aleurone conditioned by the *r-sc:m3* allele. Half of the progeny kernels were expected to receive *P-VV* and at least one *Ac* element if the *P-VV* mutation was *Ac* induced. The aleurone is part of the triploid endosperm which receives two identical maternally derived chromosomes and one paternally derived chromosome. Hence, if a single *Ac* was present at *P* in the female parent, two copies of *Ac* would be transmitted to the progeny endosperm. This results in a coarsely spotted aleurone phenotype. If two *Ac* elements are transmitted from the female parent, the endosperm would receive four copies of *Ac* which results in a very finely spotted aleurone phenotype. The difference between coarse spotting and fine spotting is due to a dosage effect exhibited by *Ac* elements–as the number of *Ac* elements increases, transposition is developmentally delayed which results in a change in aleurone spotting patterns. Both one-*Ac* lines and two-*Ac* lines were recovered in reconstitution experiments (see RESULTS). In two-*Ac* lines, a reduction in *Ac* copy number from four copies to two copies would change aleurone spotting patterns from finely spotted to coarsely spotted. Recombination between two *Ac* elements or transposition and loss of one copy of *Ac* would result in a reduction from four to two copies of *Ac* in the endosperm. Therefore, on two-*Ac* ears the coarsely spotted aleurone phenotype could also result from dosage reduction by a transposition event followed by loss, through mitotic or meiotic segregation of one *Ac*.

To analyze the pericarp variegation phenotypes of reconstituted *P-VV* alleles *Ac* copy number in reconstituted lines were standardized to one. On two-dose ears, progeny kernels showing a coarsely spotted pattern in the aleurone were grown and testcrossed by T59 (inbred W22) to reduce *Ac* copy number to one. To introduce alleles into a common genetic background all reconstituted lines were crossed at least four times by the inbred W22. The phenotype of the one-*Ac* lines carrying a reconstituted *P-VV* allele was compared to that of the *P-VV:E* allele heterozygous with *P-WR*. At least 10 siblings from each *P-VV* family were used for comparisons. *P-VV:E/P-WR* conditions a medium variegated phenotype (see Figure 1A). In reconstituted lines, several classes of pericarp striping (medium variegated = MV, medium-light variegated = MLV, light variegated = LV, very light variegated = VLV) as well as levels of basal *P* expression were visually determined (colorless, very light orange = VLO, light orange = LO, orange = O, dark orange = DO).

DNA isolation and Southern hybridization analysis: Maize genomic DNA was isolated from seedlings and leaf tissue using the procedure of SHURE, WESSLER and FEDOROFF (1983) or DELLAPORTA, WOOD and HICKS (1983). DNA was subjected to Southern hybridization (SOUTHERN 1975) modified according to CHOMET, WESSLER and DELLAPORTA (1987). The genomic DNAs were sequentially hybridized to the three *P-RR* specific probes pM (0.3-kb *Xba*I-*Sst*I fragment) pR (1.2-kb *Sal*I-*Hin*dIII fragment) and pG (0.5-kb *Bam* HI-*Xho*I fragment). The *Ac* probe was an internal 1.6-kb *Hin*d III fragment of *Ac*. The *P* probes used in the genomic mapping and Southern hybridization experiments are indicated in Figure 2A. Restriction fragments from plasmids were excised from low melting agarose gels after gel electrophoresis and labelled by the random primer method of FEINBERG and VOGELSTEIN (1984).

Polymerase chain reaction (PCR) amplification and DNA sequencing *Ac* junction fragments: Maize genomic DNA was amplified by PCR using a modified procedure

TABLE 1

Genetic stocks and alleles used in this study

Allele	Phenotype	Source
P-VV:E	Variegated pericarp, variegated cob	EMERSON (1914)
P-WR	Colorless pericarp, red cob	Supplied by J. L. KERMICLE
P-WW	Colorless pericarp, white cob	Supplied by J. L. KERMICLE
r-sc:m3	Colorless aleurone (−*Ac*), variegated aleurone (+*Ac*)	KERMICLE (1984)
r-r	Colorless aleurone, red plant	Supplied by J. L. KERMICLE
Stock	**Genotype**	**Genetic background**
T43	*P-WR/P-WR r-sc:m3/r-sc:m3*	W22
T21	*P-WR/P-WR r-g/r-g*	W22
T59	*P-WW/P-WW r-r/r-r c1/c1 y1/y1*	W22
S55	*P-VV:E/P-VV:E r-g/r-g*	4Co63

TABLE 2

Oligonucleotide primers used in the PCR amplification of *Ac::P-RR* junction fragments

Primer	Sequence (5′ to 3′)[a]	Position[b]
OP-1	AGCATCGTCTCACGGTCTCACCAC	6–25
OP-2	TTGGCCATCTGAACCAAGACCCAC	2139–2115
OP-3	GCCTGCCTAGTACATACTCCCTCC	1150–1125
OP-4	ATGAGTGAGCTAGTTCGTTGCACC	965–988
OP-5	CAGCGCTAGTAGCTTGAATGAAGAGC	528–503
OP-6	GGAGCCAAGTCTCGAACGCCTCCA	2024–2001
OP-7	TGAGAGGACCACGCGAAAGAAACG	431–454
OP-8	CATTAATCCCACTGTTTGCCACGGAC	757–732
OP-9	CGATAGAGCTCGGGGACCTAGTCT	1708–1731
OP-10	ATGTGACTCATGCGAGTGTGCACAG	2711–2686
OA-1	CCTCGGGTTCGAAATCGATCGGG	4470–4492
OA-2	GGATCGTATCGGTTTTCGATTACC	147–124
OA-3	ccggtaCCCGTTTTCGTTTCCGTCCCGC	89–68
OA-4	GGTATATCCCGTTTTCGTTTCCGTCCCGC	96–68

[a] Mismatches with *Ac* sequences (indicated in lower case letters) were introducted to create a restriction endonuclease cleavage site for cloning purposes.

[b] The positions of the 5′ and 3′ bases of *P* oligonucleotides (OP) in the sequence of the 3.2-kb *SalI* fragment (T. PETERSON, personal communication) or positions of the 5′ and 3′ nucleotides of *Ac* oligonucleotides (OA) in the *Ac* sequence (GenBank entry K01964).

from that of MULLIS and FALOONA (1987). The oligonucleotide primers used for amplification and their position's are listed in Table 2. Standard PCR reaction conditions on genomic DNA included 10 mM Tris, pH 8.3 (at 25°), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 100 μM of each dNTP, 0.5 unit of Taq polymerase (Perkin-Elmer Cetus) for 35 cycles (95° for 45 sec, 55° for 1 min, 72° for 2 min) (SAIKI *et al.* 1988). The double-stranded amplification product was fractionated by agarose gel electrophoresis and subjected to a second round of asymmetric amplification (GYLLENSTEN and ERLICH 1988). The single-stranded amplification products were sequenced using the procedure of SANGER, NICKLEN and COULSON (1977).

RESULTS

Recovery of unstable red ears: We recovered 500 transpositions of *Ac* from *P-VV:E* from approximately 3500 variegated pericarp ears pollinated with homozygous *r-sc:m3* tester (Figure 1A). *Ac* transpositions were selected as individual kernels with red pericarp and spotted aleurone (Figure 1B). The red pericarp phenotype indicated *Ac* had excised from *P-VV:E* giving rise to the *P-RR* phenotype. The *r-sc:m3* tester carries a *Ds* insertion in the *R-sc* gene and conditions a spotted aleurone phenotype only when *Ac* is present (KERMICLE 1984). The spotted aleurone indicated that the red pericarp kernel that lost *Ac* from *P-VV:E* must carry a transposed *Ac* element (tr-*Ac*). The intervening meiosis that gives rise to the megaspore will cause independent assortment of the *P* homologs. The expected maternal chromosome *1* constitution of these kernels is either *P-RR* + tr-*Ac* or *P-WR* + tr-*Ac*. Due to the tendency of *Ac* to transpose to linked sites, however, *P-RR* chromosomes carrying a tr-*Ac* should be overrepresented (VAN SCHAIK and BRINK 1959; GREENBLATT and BRINK 1962; GREENBLATT 1974). Red pericarp kernels were grown, testcrossed by *r-sc:m3* males, the ears harvested and pericarp phenotypes were scored. Results from these experiments are summarized in Table 3. Approximately 63% of the resultant ears displayed red pericarp (*P-RR*), 33% were colorless (*P-WR*), 4% were variegated pericarp

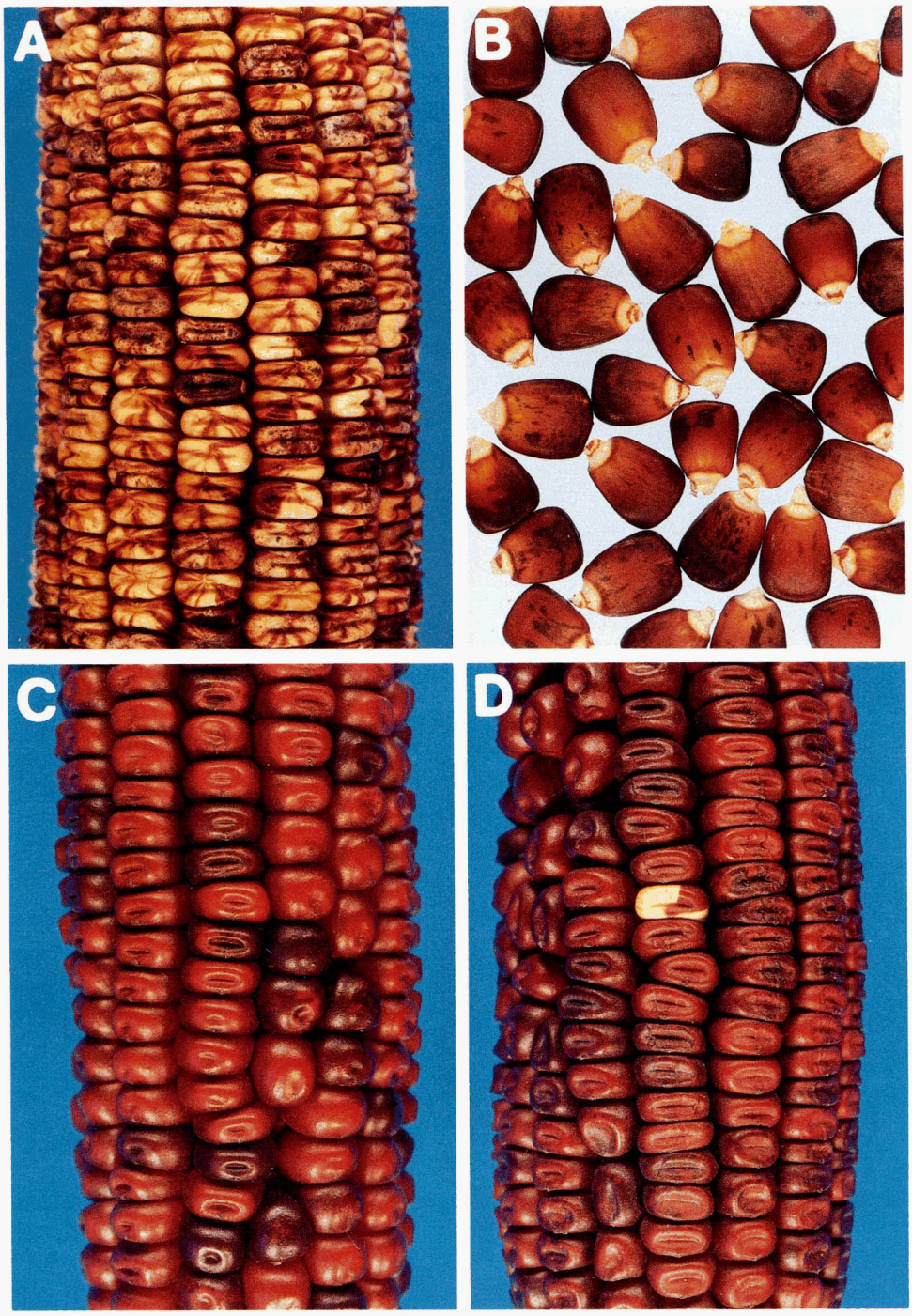

FIGURE 1.—Pericarp and kernel phenotypes used for reconstitution. (**A**) Medium variegated pericarp ear (*P-VV:E/P-WR*) pollinated with *r-sc:m3* tester. Kernels show a spotted aleurone phenotype when *Ac* is present and a colorless aleurone phenotype when *Ac* is absent. (**B**) Kernel selections from medium variegated ears from red pericarp sectors with spotted aleurones were grown and testcrossed to *r-sc:m3* males. There were 500 such kernels selected and crossed. (**C**) A stable red pericarp ear derived from kernels selections shown in (**B**). The spotted aleurone phenotype indicates that the female parent carried a transposed *Ac* element. (**D**) An unstable red pericarp ear showing *P* instability. The kernels show the spotted aleurone phenotype when carrying the *P-RR* + tr-*Ac* element. These kernels were selected and grown to generate a total of over an additional 2500 red pericarp ears. Kernels underlying colorless or variegated pericarp sectors on UR ears were tested for reconstitution. Eight hundred twenty kernels of this type were recovered which yielded a total of 250 reconstituted alleles.

(*P-VV*). All ears segregated for kernels with spotted aleurones indicating the effectiveness of the selection for *Ac* activity by the *r-sc:m3* response. The presence of variegated ears may be due to excision of *Ac* after separation of pericarp and sporogenous tissue lineages or to reinsertion of *Ac* back into the *P* gene.

The red pericarp ears were examined for instability. Both stable and unstable red ears were recovered (examples shown in Figure 1, C and D). The size and frequency of the unstable sectors (colorless or variegated pericarp) varied considerably, ranging from 1 to 10 sectors per ear reflecting different levels of

TABLE 3

Pericarp phenotypes derived from red pericarp kernels showing spotted aleurone

Pericarp phenotype	No. of ears	Percent
Stable red	266	54
Unstable red	46	9
Colorless	161	33
Variegated	18	4
Total	491	

instability. Red ears were classified as unstable red (UR) if two or more colorless or variegated sectors were present on the ear. From a population of 312 red pericarp ears, a total of 46 ears were classified as UR (14.7%) (Table 3). Each ear was assigned an UR family number. From each UR ear, kernels underlying unstable sectors were removed and tested for reconstitution of the *P-VV* phenotype (see below). Pericarp is maternal tissue derived from ovary wall and shares a common lineage with the megaspore. Sectors that cover the germinal face or larger are often transmitted to the megaspore (ANDERSON and BRINK 1952). Therefore, we selected only those kernels where the unstable sector covered minimally the germinal face of the kernel. Of the remaining red pericarp kernels on each UR ear, kernels with *Ac* activity (spotted aleurones) were grown to generate additional UR ears. These crosses generated an additional 3144 red pericarp ears, most of which (approximately 80%) displayed an unstable red phenotype. All kernels underlying unstable sectors from these secondary ears were also tested for reconstitution (see below).

Molecular analysis of unstable red chromosomes: Because *Ac* transposition is often short range, we investigated whether UR phenotypes might result from transposition of *Ac* from *P-VV:E* to nearby sites and its subsequent reinsertion into the *P* gene. We reasoned that if very closely linked *Ac* elements were responsible for the UR phenotype these elements may be within detectable limits of the donor *P* locus. DNA from plants containing UR chromosomes was examined by Southern blot analysis with *P* probes. These *P* probes, shown in Figure 2A, were derived from genomic clones of the *P* locus; in *Sal*I digests of genomic DNA they can monitor 24.7 kb of *P* and flanking DNA (CHEN, GREENBLATT and DELLAPORTA 1987; LECHELT *et al.* 1989). The pM probe detects four *Sal*I fragments in *P-RR* DNA because it contains a 250-bp motif repeated four times in this region: a 1.2-kb doublet, 3.0, and 3.2-kb *Sal*I fragments. The pR probe is a 1.2-kb *Sal*I-*Hin*dIII fragment, part of a 5.2-kb direct repeat found twice in this region (LECHELT *et al.* 1989; ATHMA and PETERSON 1991). This probe hybridizes to two *Sal*I fragments of 9.1 and 7.0 kb. The pG probe is a 500-bp *Bam*HI-*Xho*I fragment that spans the *Sal*I site joining the 9.1- and 3.2-kb *Sal*I fragments. The organization of these five *Sal*I fragments is shown in Figure 2A. By sequentially hybridizing Southern filters with these three probes, the *Sal*I position of *Ac* in 7 out of 11 independently derived UR chromosomes was determined (data summarized in Figure 2B). UR280 was derived as an unstable red derivative of UR22 and not directly from *P-VV:E*. *Sal*I/*Eco*RI and *Sal*I/*Bam*HI double digests of genomic DNA were examined by Southern hybridization to localize further and to orient the *Ac* elements on these 7 chromosomes. We could not detect the *Ac* in 4 UR chromosomes. However, genetic data (data not shown) indicated all UR chromosomes contained *Ac* elements closely linked (within two map units) to *P*.

The distribution of the *Ac* elements on these UR chromosomes was concentrated at the 5′ and 3′ ends of the *P* gene. Both orientations of *Ac* were detected in these flanking segments. There is an equal number of transpositions in each direction (3:3) from the starting position of *Ac* in *P-VV:E*. The location and orientation of the transposed elements with reference to the starting position demonstrates that short-range transpositions occur in both proximal and distal directions and that the original orientation of *Ac* is not a major factor in determining the direction of transposition nor its orientation upon reinsertion. In conclusion, Southern blot analysis indicated that many UR chromosomes contain a flanking *Ac* element just outside the *P* gene. It is the subsequent reinsertion of *Ac* from these sites into the *P* gene (see below) that gave rise to the unstable red pericarp phenotype of these ears.

Recovery of reconstituted *P-VV* alleles: From a total of 3192 UR ears, 820 kernels underlying somatic colorless or variegated pericarp sectors were tested for reconstitution. These crosses yielded a total of 257 variegated pericarp ears, each ear representing an independent reconstitution event. This recovery rate represents approximately 63% of the potential reconstitution events (only ½ of the kernels are expected to receive the appropriate homolog due to meiotic assortment during megaspore formation). A total of 120 reconstituted alleles were further studied and are listed in Table 5 (in the APPENDIX). The pericarp phenotype observed for these ears showed considerable variation in striping, background pigmentation, and *r-sc:m3* response (*Ds* transactivation). We investigated the basis of this variation by genetic and molecular means as discussed below.

Ac shows a unique dosage effect–as the number of elements increase, transposition is developmentally delayed (MCCLINTOCK 1949, 1950, 1951). Based on the *r-sc:m3* response, we recovered two types of *P-VV* reconstitution events with respect to *Ac* copy number. The first class, termed one-*Ac* lines, displayed an *r-*

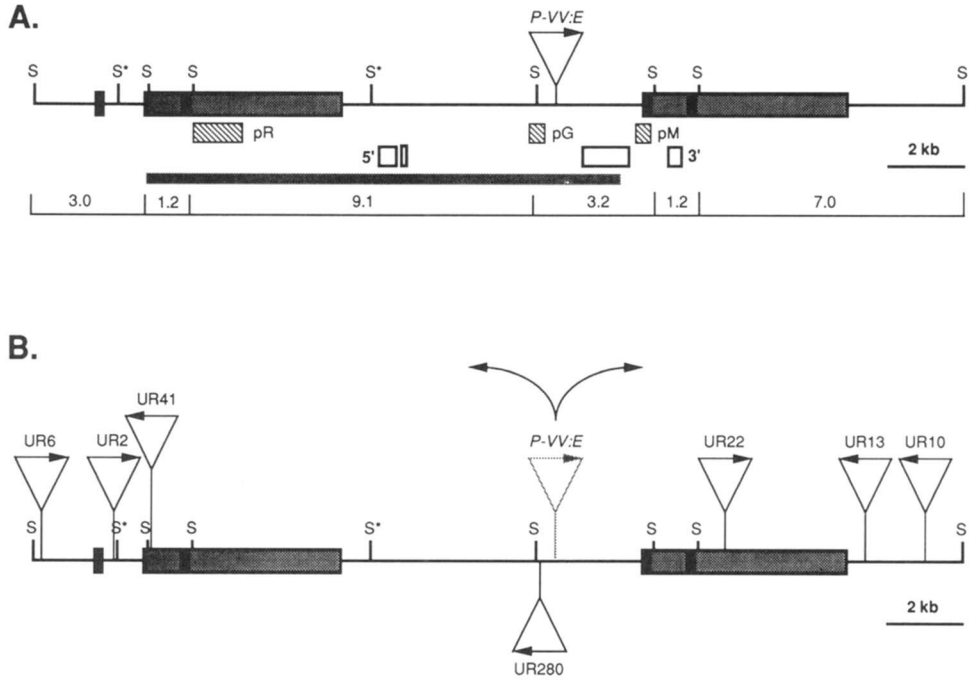

FIGURE 2.—Structure of the *P* region and UR insertion sites. (**A**) A 24.7-kb region including the *P-RR* allele on chromosome *1* of maize is shown. The position of *Sal*I sites (S) is shown and the size (in kb) of genomic fragments detected by Southern hybridization is diagramed below the map. S* indicates *Sal*I sites that are methylated in genomic DNA and therefore uncut. The positions of the three hybridization probes derived from genomic *P* clones (CHEN, GREENBLATT and DELLAPORTA 1987; LECHELT *et al.* 1989) are shown as cross-hatched boxes. The black boxes show the four locations of a 250-bp repeated motif detected by the pM probe. Two shaded boxes represent the position of a 5.2-kb direct repeat detected by probe pR. The *P* gene, is transcribed from left (5′) to right (3′) (LECHELT *et al.* 1989) and the positions of four known exons (white boxes) are shown (GROTEWOLD, ATHMA and PETERSON 1991). The minimum region of *P* (12.3 kb) defined by reconstitutional mutagenesis (see DISCUSSION) is indicated by the gray bar. The insertion site of *Ac* (triangle) within the *P-VV:E* allele is shown with the arrow pointing in the direction of the major *Ac* transcript (KUNZE *et al.* 1987). The size of *Ac* elements in all figures is not shown to scale with the *P* map. This information was compiled from genomic mapping experiments (J. CHEN, unpublished results) and published data (CHEN, GREENBLATT and DELLAPORTA 1987; LECHELT *et al.* 1989; ATHMA and PETERSON 1991; GROTEWOLD, ATHMA and PETERSON 1991). (**B**) The position and orientation of 7 *Ac* insertion sites which condition a UR phenotype (the resolution of Southern mapping data is within 100 bp). Each UR chromosome, with the exception of UR280, was derived from the *P-VV:E* allele by transposition of *Ac* to nearby sites. UR280 was derived from UR22. Both orientations of *Ac* are represented at the 5′ and 3′ regions.

sc:m3 response (coarsely spotted aleurone) indicating that the female parent carried a single *Ac* (an example is shown in Figure 3A). The remaining ears showed a *r-sc:m3* response (finely spotted aleurone) that indicated the female parent contained two tightly linked *Ac* elements (an example shown in Figure 3B). These two classes were recovered in nearly equal frequencies (data summarized in the APPENDIX, Table 5, under the column headed "Original *Ac* dosage"). Moreover, a single UR family could give rise to both classes. For instance, 9 one-*Ac* lines and 10 two-*Ac* lines were recovered from UR2 reconstitution events.

We tested whether *Ac* copy number differences were due to the retention of the donor *Ac* during the reconstitution process. Based on the current models for *Ac* transposition, two factors might influence the *Ac* dosage outcome–which of two daughter chromatids was recovered and whether the target site was replicated at the time of reinsertion (see DISCUSSION). We analyzed one- and two-*Ac* reconstituted lines derived from UR2; this chromosome carries the donor *Ac* in the 3.0-kb *Sal*I fragment resulting in a novel 7.5-kb fragment (Figure 4, lane 4) and several reconstituted alleles were recovered carrying one *Ac* and two *Ac* elements from this UR family (examples shown in lanes 2, 3 and 5). In DNA from two-*Ac* lines, the donor *Ac* in the 7.5-kb *Sal*I fragment was retained in addition to a second *Ac* element inserted into the 3.2-kb *Sal*I fragment of *P* (the 3.2-kb fragment shifts to 7.7 kb in lane 3) or the 1.2-kb *Sal*I fragment (the 1.2-kb fragment shifts to 5.7 kb in lane 5). In one-*Ac* lines, only the reinserted *Ac* was present, the donor *Ac* was lost from the 7.5-kb band as demonstrated by the reappearance of the 3.0-kb *Sal*I fragment. A one-*Ac* line is shown in lane 2; the reconstituted allele has lost *Ac* from the 7.5-kb *Sal*I fragment resulting in a 3.0-kb fragment and contains an insertion of *Ac* into the 3.2-kb *Sal*I fragment of *P* resulting in a shift to 7.7 kb. These results demonstrate that the distinction in *Ac* dosage results from whether the donor *Ac* was retained during the reconstitution process.

To eliminate the effects of *Ac* dosage on reconstitution phenotypes in the two-*Ac* lines we reduced the *Ac* copy number to one by removing the original UR

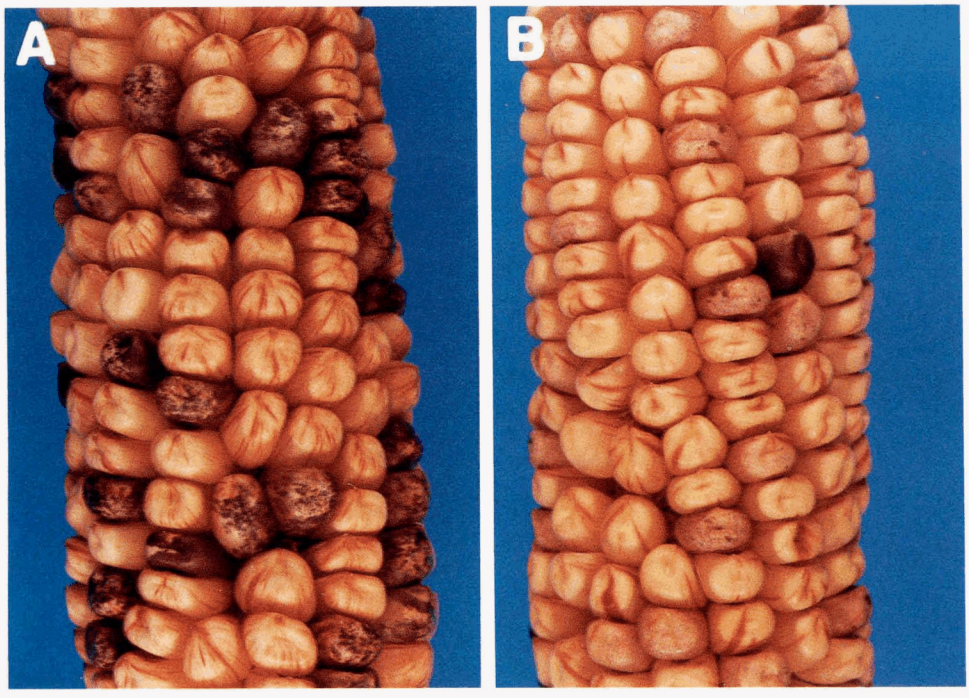

FIGURE 3.—Two classes of reconstituted lines. (**A**) Representative ear from a one-*Ac* line The pericarp is maternally derived from the ovary wall, therefore female diploid tissue, and contains a single *P-VV:151* chromosome. The *P* genotype of the pericarp is *P-VV:151/P-WR*. The triploid endosperm is derived from double fertilization and contains two maternal and one paternal genomes, therefore one-half of the progeny kernels contain two copies of the maternally derived *P-VV:151* chromosome. The coarsely spotted aleurone phenotype is a typical *r-sc:m3* response (two maternal *Ac* copies) in one-*Ac* lines. The *R* genotype of the kernels is *r-sc:m3/r-g* or *r-g/r-g*. Only 25% show spotting because one-half of the kernels containing *r-sc:m3* are homozygous for *c1*, a colorless aleurone mutation epistatic to *R*. (**B**) Representative ear from a two-*Ac* line. The *P* genotype of the ear is *P-VV:169/P-WR*. The somatic pericarp tissue contains one copy of the maternal *P-VV:169* chromosome carrying two *Ac* elements (both donor UR and reconstituted element); the triploid endosperm receives two maternally derived *P-VV:169* chromosomes, each chromosome carries two *Ac* elements. This results in four copies of *Ac* in the triploid endosperm which shows a very finely spotted aleurone phenotype. Only 25% of the aleurones are spotted due to the segregation of the epistatic *c1* mutation. The exceptional coarsely spotted kernels (not shown) on these ears represent instances where *Ac* copy number is reduced by recombination between the two *Ac* elements or by transposition and loss of one element.

Ac element. In two-*Ac* lines, transmission of the two tightly linked *Ac* elements to kernel offspring resulted in a finely spotted aleurone phenotype (Figure 3B). The infrequent occurrence of coarsely spotted kernels on these ears, which comprised about 1–2% of the kernel offspring, should represent a reduction in *Ac* dosage from two copies to one copy by separation of the linked elements by recombination or transposition. Kernels with coarsely spotted aleurone were selected on reconstituted ears, grown and testcrossed by *P-WW* males. The resultant ears were either variegated or UR depending on which of two *Ac* elements was left present in the chromosome. When the *Ac* element found on the UR chromosome was retained, the resultant ear was unstable red. However, when the *Ac* element inserted into the *P* gene was retained, the resultant ear was variegated. Often, when the *Ac* copy number was reduced, there was an increase in *P* striping on variegated ears (data not shown), a result consistent with reported *Ac* dosage effects on pericarp striping (BRINK and NILAN 1952). By copy number reduction, together with the original one-*Ac* lines, we recovered a total of 120 reconstituted alleles containing only a single active *Ac* element inserted into the *P* gene. This allowed us to map unambiguously the *Ac* element causing the reconstitution phenotype.

***Ac* position and orientation in reconstituted *P-VV* alleles:** To investigate further the basis of reconstitution, we performed a molecular analysis of 94 reconstituted *P-VV* alleles. The position and orientation of the inserted *Ac* element in each of the reconstituted alleles was determined by Southern analysis using *P* probes. DNA was isolated from plants heterozygous for a *P-VV* allele and the *P-WW* tester allele, digested with *Sal*I, Southern blotted, and hybridized with the pM, pR, and pG probes (examples of pM and pR blots are shown in Figure 5, A and B). After positioning *Ac* within a *Sal*I fragment, the *Ac* was further localized and orientated by digesting genomic DNA with *Sal*I together with a second enzyme which cuts *Ac* at a single defined site such as *Bam*HI, *Xho*I, and *Eco*RI to position and orient the *Ac* element within the *Sal*I fragment (data not shown). All blots were rehybridized with an internal *Ac* probe to confirm that the altered fragments contained *Ac* (an example is shown in Figure 5C). Southern hybridization results are sum-

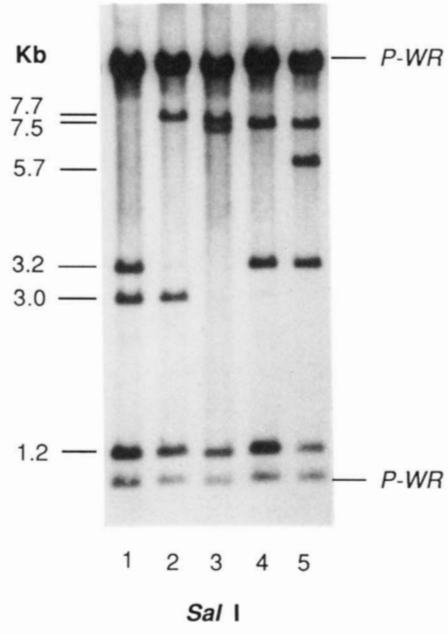

FIGURE 4.—Southern blot analysis of UR and reconstituted lines. Genomic DNA digested with *Sal*I, Southern blotted, and hybridized with the pM probe which detects *Sal*I fragments of 1.2, 3.0 and 3.2 kb of the *P-RR* allele. *P-RR/P-WR* (lane 1); *P-VV:168/P-WR* (lane 2) a one-*Ac* line showing the reinserted 4.56-kb *Ac* element in the 3.2-kb *Sal*I fragment which shifts to 7.7 kb and loss of the donor *Ac* in the 7.5-kb fragment which shifts to 3.0 kb; *P-VV:173/P-WR* (lane 3) a two-*Ac* line showing both donor *Ac* in the 7.5-kb *Sal*I fragment and reinserted *Ac* in the 3.2-kb fragment which shifts to 7.7 kb; UR2/*P-WR* (lane 4) showing the donor UR *Ac* inserted into the 3.0-kb *Sal*I fragment which shifts to 7.5 kb; and *P-VV:165/P-WR* (lane 5) a second two-*Ac* line with the donor *Ac* in the 7.5-kb *Sal*I fragment and reinserted *Ac* in the 1.2-kb *Sal*I fragment which shifts to 5.7 kb. (See legend to Figure 2 for positions of the *Sal*I fragments and pM probe.)

marized in Figure 6 which indicates that reconstituted *P-VV* alleles were caused by insertions of *Ac* dispersed throughout a 12.3-kb region. The position of *Ac* in *P-VV:424* defined the most 5′ insertion and in *P-VV:190* defined the most 3′ insertion site. Insertions of *Ac* in either orientation disrupted *P* function. The target sites were dispersed nonrandomly and many insertions were clustered in a few regions with other regions devoid of insertions. One reconstituted allele, *P-VV:134*, showed a complex hybridization pattern, indicating that a structural rearrangement within the *P* gene had occurred (data not shown). The mechanism and molecular basis of this rearrangement is currently under study. In summary, 93 out of 94 reconstituted *P-VV* alleles were caused by simple reinsertions of *Ac* in a dispersed but nonrandom pattern into *P*. We conclude that the minimum physical limits of the *P* gene are defined by the insertion sites of *Ac* in *P-VV:424* and *P-VV:190* alleles. By this estimate, at least 12.3 kb of genomic DNA are required for full *P-RR* expression.

The transposition profile of *Ac* from its flanking position to reconstituted sites in the *P* gene was analyzed in greater detail for two UR chromosomes, UR2 and UR10. Figure 7, A and B, shows the reinsertion patterns of *Ac* from the two different UR start points. In each UR chromosome, both orientations of *Ac* insertion relative to the donor element occurred at about equal frequency. In both examples, the reinsertion sites were dispersed.

Sequence analysis of *Ac* target sites: PCR amplification and DNA sequencing of the junction sequences between *Ac* and *P* was performed on 28 *Ac* insertions in the 3.2-kb *Sal*I fragment. Results of these experiments are shown in Table 4. The accuracy of the position of the *Ac* element when mapped by Southern blots compared to the PCR sequencing analysis was found to be within 100 bp. From these data we found five instances, representing 10 reconstituted alleles, in which the same target site was used more than once by *Ac*. The first instance, reconstituted alleles *P-VV:252* and *P-VV:303*, have *Ac* insertions at precisely the same location and in the same orientation within the *P* gene (Figure 8). Two additional target sites received multiple insertions with *Ac* elements in the same orientation. These cases are *P-VV:132*, *P-VV:262* and *P-VV:310* insertions at position 702 in the 3.2-kb *Sal*I fragment, and *P-VV:135* and *P-VV:302* insertions at position 726. Another target site at position 401 contained *Ac* elements in opposite orientation (*P-VV:168* and *P-VV:240*). *P-VV:360* represents another example; its target site at position 312 is identical to a previously characterized insertion of *Ac* (PETERSON 1990) but contains *Ac* in the opposite orientation. To confirm that these insertions were caused by independent transposition events, we determined the sequence of the *P-VV:E* excision footprint for several alleles. Excision footprints would provide molecular evidence on whether reconstituted alleles were borne on a unique chromosome and therefore derived from independent transposition events. Figure 8 shows these footprints indicating the independent origin for many of these insertions. The phenotypes of these alleles are discussed below.

Variegation phenotypes of reconstituted alleles: Pericarp phenotypes were compared in all one-*Ac* lines (data summarized in the APPENDIX). There were still major differences in striping phenotypes and background pigmentation levels apparent in lines containing a single *Ac* inserted at *P*. We classified the striping phenotypes into four categories: medium variegated (MV) the highest rate of striping, medium light variegated (MLV), light variegated (LV), and very light variegated (VLV), the least amount of striping per ear. Examples of these striping phenotypes are provided in Figure 9A–D. From a total of 120 one-*Ac* lines, we recovered 45 MV, 35 MLV, 22 LV and 18 VLV phenotypes (see APPENDIX, Tabele 5, column 5). We also classified the level of basal *P* expression or

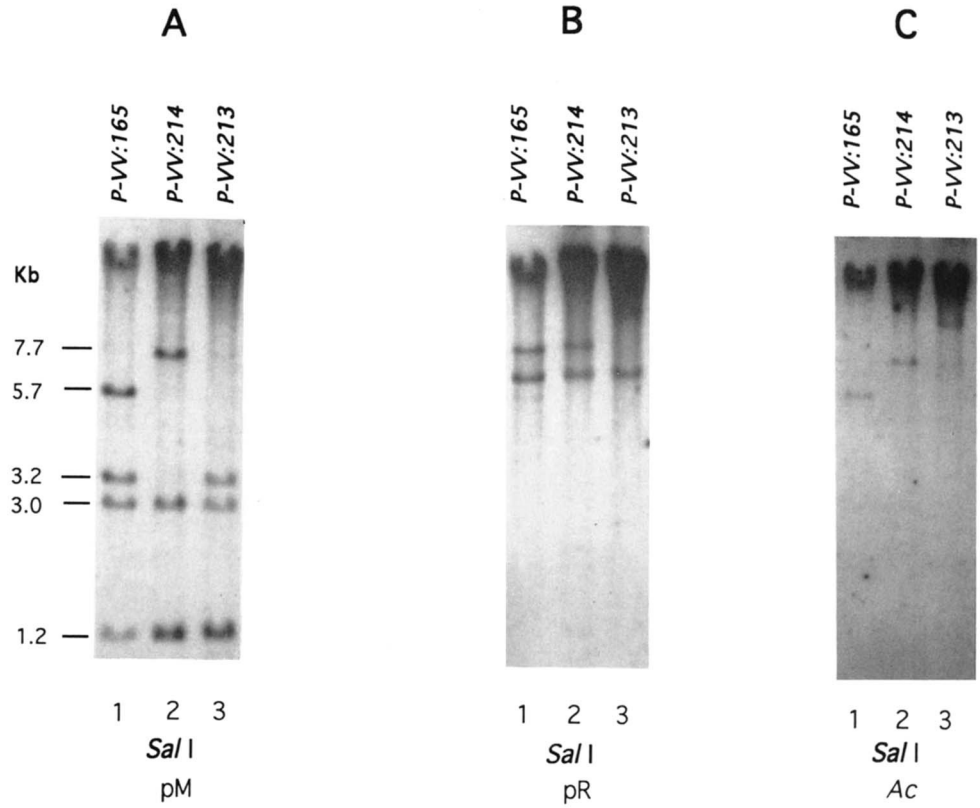

FIGURE 5.—Southern blot analysis of reconstituted alleles. (**A**) Genomic DNA of reconstituted alleles digested with *Sal*I, Southern blotted and probed with the pM probe which detects 1.2-, 3.0- and 3.2-kb *Sal*I fragments in *P-RR* DNA (see text and Figure 2 for details on probes and *Sal*I fragments). Reconstituted alleles containing an insertion of *Ac* (4.56 kb) into the 1.2-kb *Sal*I fragment which shifts to 5.7 kb (lane 1) and insertion into the 3.2-kb *Sal*I fragment which shifts to 7.7 kb (lane 2). (**B**) Same DNAs as in (**A**) probed with pR which detects a 9.0- and 7.0-kb *Sal*I fragment in *P-RR* DNA. Lane 3 shows a reconstituted allele containing an insertion of *Ac* in the 9.1-kb *Sal*I fragment which shifts to 13.5 kb. (**C**) Same DNAs probed with the *Ac* probe which confirms the shifted bands seen in (**A**) and (**B**) contain an *Ac* element.

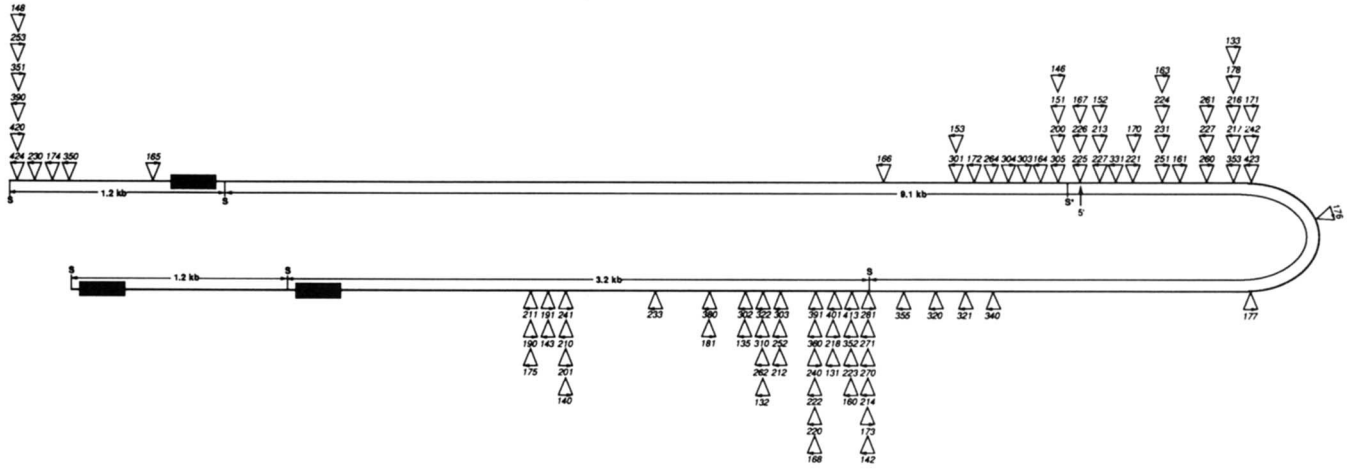

FIGURE 6.—Position and orientation of *Ac* insertions in reconstituted alleles. Data from Southern blot analysis of reconstituted alleles are summarized here. The position (within 100 bp) and orientation of *Ac* are shown within a region of *P* containing the 5′ 1.2-, 9.1-, 3.2- and 1.2-kb *Sal*I genomic fragments and three repeated motifs (black boxes) (see Figure 1A for map details). The transcription initiation site and direction of *P* transcription are indicated. The most 5′ insertion (*P-VV:424*) and most 3′ insertion (*P-VV:190*) define the minimum physical segment of DNA (12.3 kb) required for full *P* function.

background pigmentation conditioned by reconstituted alleles into five categories: colorless (C), very light orange (VLO), light orange (LO), orange (O), to dark orange (DO) (Table 5, column 4). The colorless

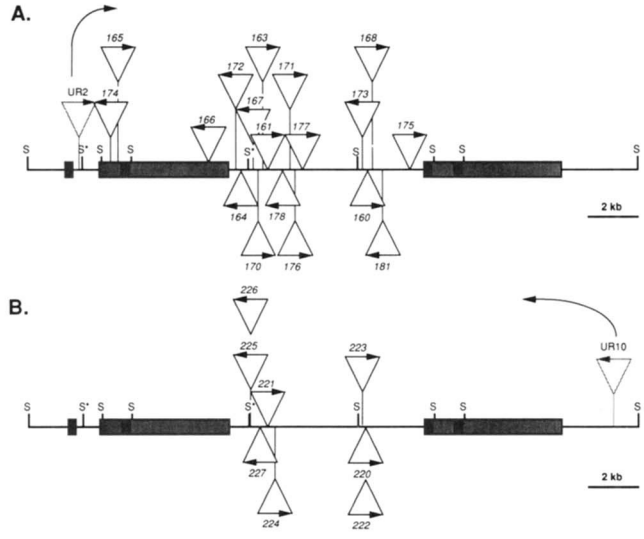

FIGURE 7.—Short range transposition profiles of *Ac*. (**A**) Location of the donor (UR2) *Ac* element and transpositions which resulted in reconstitution are shown (see Figure 1A for details of map). The distance between donor and target sites (transposition interval) ranged from 1.3 to 13.3 kp. (**B**) Location of the donor (UR10) *Ac* element and transposition which resulted in reconstitution. Transposition intervals ranged from 10.6 to 15.8 kb.

variegated phenotypes (90 alleles) represented about 75% of all reconstituted ears; VLO, LO, O and DO (30 alleles) constituted the remaining 25%. From a single reconstituted line, however, nearly all offspring ears displayed a constant variegation pattern demonstrating the reconstituted phenotype was heritable (data not shown). In conclusion, while *Ac* copy number did affect variegated pericarp phenotypes conditioned by reconstituted *P-VV* alleles, major differences in variegation patterns were caused by other factors.

We then compared the location of *Ac* in the 3.2-*Sal*I fragment with the reconstitution phenotypes (Figure 10). The presence of a large 1220-nt exon in this region allowed us to assign an intron or exon position to these insertions. All insertions in the exon showed reduced striping (LV or VLV) and most were colorless or VLO in background pigmentation. While most intron insertions show heavier striping, both LV and VLV insertions in introns were found to occur. Nine out of 10 insertions in introns that showed background pigmentation were found in the same *Ac* orientation.

To investigate the basis of reconstituted phenotypes further, we compared the phenotypes of several insertions with same target sites (see Figure 8). Insertions in the same target site and with *Ac* in the same orientation showed variegation patterns that were indistinguishable (an example is shown in Figure 11, A and B). However, insertions in the same target site but in opposite orientations show distinct differences in background pigmentation. The phenotypes of *P-VV:168* and *P-VV:240* demonstrate this difference (Figure 11, C and D). Both insertions are located in the same target site but in opposite orientation. In one orientation, the insertion causes no background expression while in the opposite orientation the insertion causes a distinct orange pigmentation to the pericarp. A similar result emerges when comparing the phenotypes of the *P-OVOV* allele, an orange variegated allele (PETERSON 1990) and *P-VV:360*, a colorless variegated allele with an insertion in the same target site but in opposite orientation (data not shown). There is a remarkable similarity in the striping frequency of insertions at the same target sites regardless of *Ac*'s orientation (*e.g.*, compare Figure 11, A and B, or C and D). Therefore, while the background phenotype of reconstituted alleles is affected by the orientation of *Ac* insertion, the striping frequency, a reflection of *Ac* transposition rates, appears to be dependent on the position and not the orientation of *Ac*.

TABLE 4

Target site sequences for *Ac* insertions into the 3.2-kb *Sal*I fragment of *P*

Allele	UR origin	Target site[a]	*P* position[b]	*Ac* orientation[c]
P-VV:131	49	taat GATAGATA tgcg	286	≫
P-VV:132	49	tagc TTCCCTGT tgcc	702	≫
P-VV:135	49	atca TAGAGGGG tccc	726	≫
P-VV:140	50	ctga TGCCTATC gggc	1772	≪
P-VV:143	50	gtcg GCTGCGAG gccc	1828	≫
P-VV:168	2	gcta GCTGTGAG agag	401	≫
P-VV:173	2	aacc CAAACTGA tatg	98	≪
P-VV:175	2	tgct GCTGCTGC gacg	1967	≫
P-VV:181	2	gttc GTTGCACC aaat	988	≪
P-VV:190	3	tgct GCGACGGC ggcc	1973	≪
P-VV:191	3	ggct GCGAGGCC cagg	1831	≪
P-VV:201	4	cgcc CTGATGCC tatc	1768	≪
P-VV:212	6	cact CGCTCTTC attc	510	≫
P-VV:214	6	cggt CTCACCAC acca	30	≫
P-VV:218	6	cgtg CATGCATG ccac	255	≪
P-VV:220	10	tagc GGTGCCAC acag	343	≫
P-VV:223	10	atct AACAAAAC tggc	189	≫
P-VV:240	13	gcta GCTGTGAG agag	401	≪
P-VV:252	16	tagc GCTGGGAG cgaa	532	≫
P-VV:262	17	tagc TTCCCTGT tgcc	702	≫
P-VV:271	18	tgga GCTTCTTC ggtg	58	≫
P-VV:302	23	atca TAGAGGGG tccc	726	≫
P-VV:303	23	tagc GCTGGGAG cgaa	532	≫
P-VV:310	30	tagc TTCCCTGT tgcc	702	≫
P-VV:322	32	cacc GCCGGCCG gtac	666	≫
P-VV:360	36	taca ACTACAAC ccac	312	≫
P-VV:391	39	tagc TGTGAGAG agtc	403	≫
P-VV:401	41	aata TCCCTCGT gcat	246	≫

[a] Sixteen base-pairs of the sequence of the *P* gene are shown with the 8 bp that are duplicated flanking the *Ac* insertion in upper case.

[b] The nucleotide position of the last base of the first (5′) eight bp duplication in the 3.2-kb *Sal*I fragment of *P*.

[c] The arrow points in the direction of the major *Ac* transcript (*Bam*HI to *Xho*I orientation).

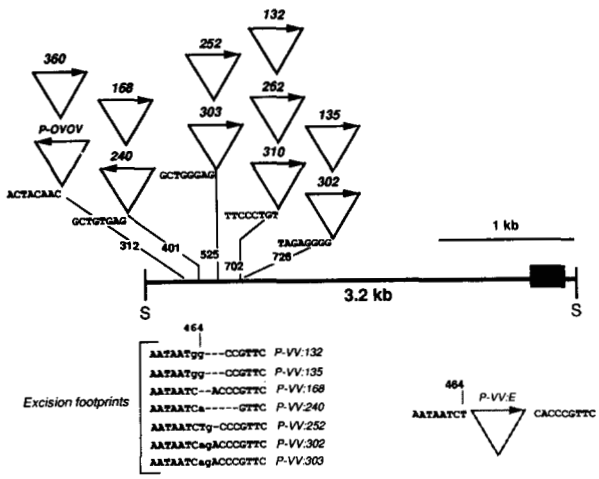

FIGURE 8.—Hot spots for *Ac* insertion. The 3.2-kb *SalI* fragment contains several examples of independent insertions at identical target sites. The nucleotide position (the first base in the 3.2-kb *SalI* fragment is designated position 1) of the last base of the 8-bp target site duplication is given. The excision footprints for *P-VV:E* at position 464 on reconstituted chromosomes were used to confirm the independent origin of these insertions. The sequence of the 8-bp target site duplication flanking each insertion is shown. The repeated motif (black box) in this region is shown for orientation. Note that at positions 401 and 312 insertions are recovered in opposite orientations at the same target site.

DISCUSSION

Transpositions of *Ac* often occur over short DNA intervals: Red pericarp ears that result from excision of *Ac* from the *P* gene, can show somatic instability in *P* expression. This instability can be explained by several mechanisms including: (1) breakage and loss of the chromosomal segment carrying the *P* gene, or (2) *P* inactivation by reinsertion of nearby *Ac* elements. The heritability of the unstable red (UR) phenotype and the tight genetic linkage of the *Ac* element to *P* suggests that the proximity of *Ac* to *P* confers the UR phenotype. Our results confirmed that 7 out of 11 UR chromosomes contained an *Ac* element within detectable limits of the *P* gene. Although the remaining UR chromosomes contained an *Ac* element tightly linked to *P*, we were unable to examine DNA regions outside this interval to determine their location. Therefore, the unstable red phenotype can be explained by the tendency of *Ac* to transpose to nearby sites. Short-range transposition would explain both the common occurrence of the unstable red phenotype among red pericarp ears (15%), and the frequent somatic and germinal reinsertion of nearby *Ac* elements back into the *P* gene. Our molecular analysis of unstable red chromosomes and reconstituted *P-VV* alleles clearly demonstrates this feature of *Ac* transposition. The high frequency of short-range transpositions would be inconsistent with a transposition mechanism involving a free DNA intermediate and may reflect a process that requires a physical interaction between donor and target sites.

Transposition profiles: Unstable red chromosomes contain reinsertions that map both 5′ and 3′ to the donor *Ac* element at *P-VV:E*. There is an approximately equal number of transpositions in both directions from the starting position and reinsertions are found in both orientations. Previous genetic studies suggested that a 4-cM region just proximal to *P* was refractory to *Ac* transposition perhaps because of chromosomal replication patterns (GREENBLATT 1984). Our results are inconsistent with *Ac* transposing preferentially to closely linked distal sites. Rather our results indicate the direction of transposition is random over short physical intervals.

The *Ac* copy number differences in reconstituted lines may reflect the replication status of donor and target sites during transposition. Approximately 50% of the reconstituted lines contain two *Ac* elements, and the remaining 50% contain a single *Ac* (*e.g.*, see UR2- and UR10-derived alleles in APPENDIX 1). This pattern was consistent with the overall recovery rates of one- and two-*Ac* lines. Both types can be explained according to the current models of the mechanism of *Ac* transposition (GREENBLATT 1984; CHEN, GREENBLATT and DELLAPORTA 1987, 1992). An interpretation consistent with molecular data presented in Figure 4 is that reconstitution takes place by transposition from one chromatid after replication of the donor site and reinsertion into a target site within the *P* gene that may or may not be replicated. In one *Ac* lines, the donor element has excised from the chromatid that receives the transposed element inserted at *P*. In two-*Ac* lines, the sister chromatid is recovered–that which receives the transposed *Ac* and retains the donor *Ac*. Alternatively, both donor and transposed *Ac* would be recovered by an interchromatid transposition to a replicated target site within *P*. These events might take place entirely during the period of DNA synthesis, perhaps as a replication fork passes through the donor site. The distribution of reinserted *Ac* elements in one- and two-*Ac* lines may therefore reflect a localized pattern of DNA replication.

***Ac* insertions are nonrandomly dispersed:** Using a modified reconstitution strategy of ORTON and BRINK (1966), we were able to reconstitute 257 variegated *P* alleles. The distribution of the *Ac* elements in these reconstituted alleles clearly demonstrates that *Ac* reinsertions are dispersed (Figure 6). Insertions occurred into introns, exons and promoter sequences. However, this dispersion pattern is not random–there appear to be distinct clusters of insertions in some regions and other regions apparently devoid of insertions. This dispersion pattern could be explained by several factors. First, since the recovery of reconstituted alleles was based on a phenotypic change from red to colorless or variegated pericarp, regions devoid of reconstituted *Ac* elements may represent target

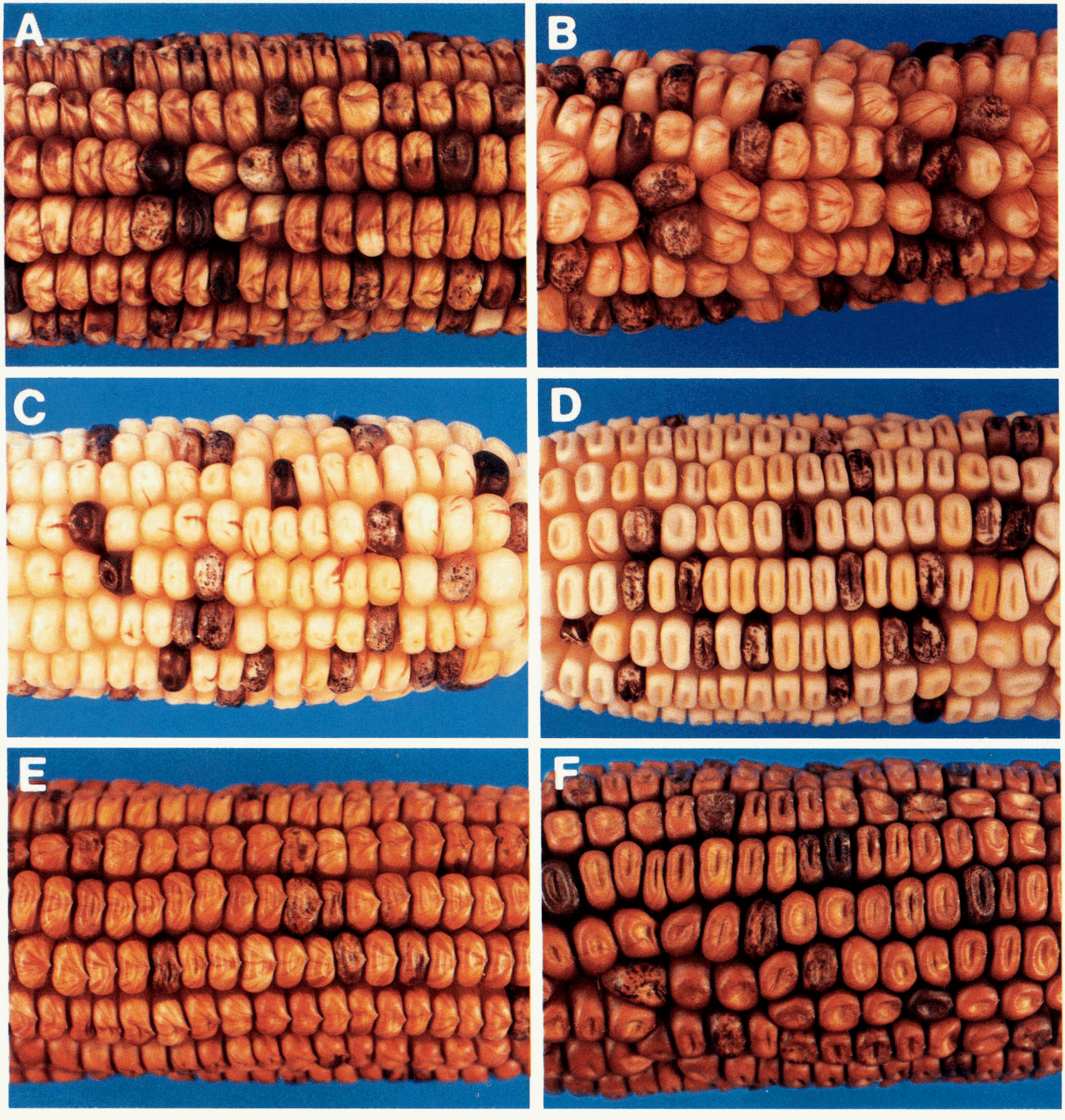

FIGURE 9.—Phenotypes of reconstituted alleles. The copy number of *Ac* was maintained as one in all reconstituted lines for comparison of variegation phenotypes. All lines are heterozygous for *P-WR* and are inbred W22. Variation in striping rate ranged from medium variegated (**A**), the maximum striping rate observed for variegated pericarp alleles, medium light variegated (**B**), light variegated (**C**) to very light variegated (**D**) the minimum striping rate with only one or two red stripes per ear. Background pigmentation levels ranged from colorless (**A–D**), very light and light orange (not shown), medium orange (**E**), to dark orange (**F**). Darker level of pigmentation would be classified as unstable red. Superimposed on the background level of pigmentation are stripes (red or colorless) caused by the excision of *Ac* from the *P* gene. Alleles shown are *P-VV:170* (**A**), *P-VV:151* (**B**), *P-VV:175* (**C**), *P-VV:201* (**D**), *P-VV:351* (**E**) and *P-VV:352* (**F**).

areas where an insertion would not result in a phenotypic change on UR ears. The possibility of such "silent" insertions within *P* comes from the identification of an UR chromosome (UR280) with an insertion of *Ac* in the large 4.3-kb intron of *P*. A second factor is the possibility of insertional hot spots and other regions refractory to insertion. By analyzing 28 target sequences, we have identified several target sites that are used multiple times. Yet, our data also does not reveal any strong restrictions on *Ac* target

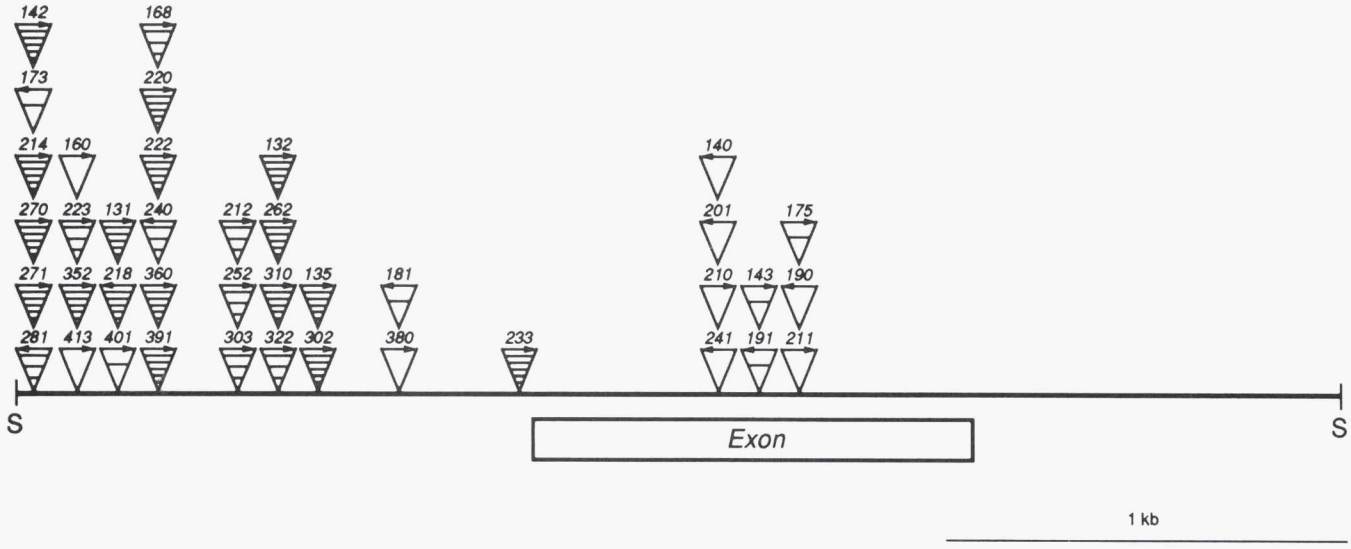

FIGURE 10.—Insertion sites and striping phenotypes. A comparison of *Ac* insertion sites in a region of the *P* gene containing the 1220-nt exon with striping phenotypes. The intervening segment is alternatively spliced to produce the two *P* transcripts [see Grotewold, Athma and PETERSON (1991) for details]. The degree of striping is indicated by the number of horizontal lines inside each triangle (5 = MV, 3 = MLV, 1 = LV and 0 = VLV). All *Ac* insertions within this exon condition a reduced striping phenotype (LV or VLV). Insertions within introns condition a variety of striping phenotypes from MV to VLV. All insertions in intron in the read-through orientation (see text) condition background levels of *P* expression from VLO to DO. The majority of insertions in the opposite orientation have a colorless background phenotype except one insertion *P-VV:352*) which show background pigmentation.

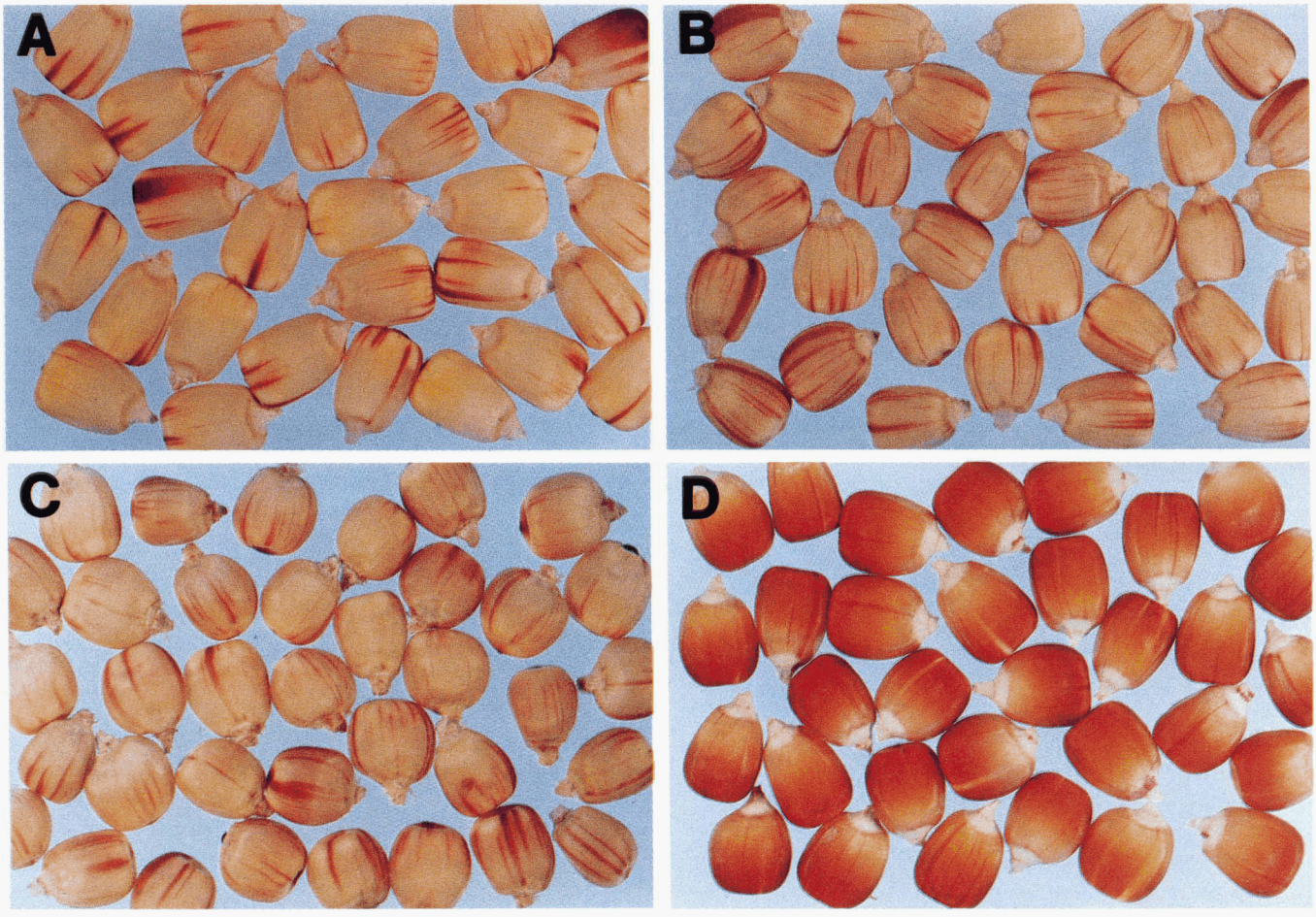

FIGURE 11.—*Ac* orientation and reconstitution phenotypes. Pericarp phenotypes of insertions at the same target site and in the same *Ac* orientation at position 525 in the 3.2-kb *Sal*I fragment shown in (**A**) *P-VV:252* and (**B**) *P-VV:303*. Insertions at the same target site (position 401 in the 3.2-kb *Sal*I fragment) but in opposite orientation are shown in (**C**) *P-VV:168* and (**D**) *P-VV:240*.

site selection. While a sequence preference may exist, we also find that any position within the 8-bp target sequence or in neighboring DNA sequences can be occupied by any nucleotide (Table 4). Finally, a combination of these and other unidentified factors may be involved in determining the distribution of *Ac* reinsertions.

The target sites chosen more than once by *Ac* may be hot spots for insertion. These sites show some similarity in DNA sequence to one another and some similarity to the sequence of the ends of *Ac*. Short matches between the ends of a transposon and its target sites also have been noted for bacterial transposons such as Tn*10* (HALLING and KLECKNER 1982). In the case of *P-VV:168* and *P-VV:240*, both insertions are in the identical target sequence but in opposite orientations. They are derived from independent UR chromosomes containing insertions of *Ac* at opposite ends of the *P* gene. The fact that these elements are inserted into the same target site in the opposite orientation suggests that if target site preference is determined in any way by DNA sequence, this information does not determine the orientation of *Ac* insertion. Whether DNA sequence is a significant factor in choosing target sites or promoting transposition remains uncertain.

Variegation patterns and genetic fine structure: A combination of position and orientation of *Ac* results in the distinctive pattern of pericarp variegation conditioned by reconstituted alleles. This variegation pattern can be divided into two components: (1) the striping phenotype which is a function of transposition and (2) the background level of *P* expression, a function of *P* expression in the presence of the *Ac* insertion. These two aspects of variegation appear to be independently determined. For instance, the striping phenotype does not correlate with background *P* expression and vice versa (see Table 5 in the APPENDIX). Furthermore, in an instance in which *Ac* inserted into the identical target site in opposite orientations, the striping phenotype conditioned by these two alleles is nearly identical while the background level of *P* expression is entirely different (Figure 11, C and D).

The differences in striping phenotypes that occur independently of *Ac* dosage do not necessarily reflect a change in the developmental timing of transposition. Red striping is determined by whether the DNA footprint left after imprecise excision restores *P* function. Only excision events that restore full *P* action are red, others may be colorless or dilute red (orange). Differential sensitivity of the target sequences to modifications caused by insertion and imprecise excision may be the major determinant of the striping phenotype. The actual germinal excision rates for reconstituted *P-VV* alleles (measured by the frequency of stable germinal red or colorless derivatives) are relatively constant regardless of *Ac*'s position within *P* (M. MORENO, unpublished results). Hence, while the rate of transposition per se is unaltered, the apparent striping frequency may be profoundly affected. This implies that the striping phenotype can be used to interpret which regions of the *P* gene are most tolerant to sequence modifications. For instance, only a subset of excision events would restore the proper reading frame if the insertion lies within coding sequences. Therefore, imprecise excision from exon sequences should result in less red stripes than insertions in less essential sequences.

This prediction is supported by the data on *Ac* distribution and variegation phenotypes. For instance, all insertions in the 1220-nt exon show less striping compared to most insertions in the introns in this region (Figure 10). Yet there are insertions in noncoding regions that do show reduced striping phenotypes. For instance, *P-VV:160*, *P-VV:413* and *P-VV:380* contain *Ac* insertions in the large 4.9-kb *P* intron and show a VLV phenotype. Insertions which cause a reduced striping phenotype may also define critical noncoding sequences required for gene function. It will be interesting to investigate these sites and their role in gene structure-function.

Differences in striping phenotypes from LV to VLV were detected for insertions within the 1220-nt exon. Assuming a constant transposition frequency for *Ac*, these differences may reflect structural information on the *P* product. Nucleotide modifications caused by insertion and imprecise excision of *Ds* from the *wx-m1* allele lead to amino acid substitutions in the *Waxy* protein associated with kinetic changes in *Wx*-encoded enzyme activity (WESSLER *et al.* 1986). It is likely that imprecise excisions of *Ac* from *P* coding sequence results in similar amino acid modifications. The differences we observed in striping phenotypes among insertions in exons may reflect the sensitivity of certain protein domains to modifications. Minor amino acid substitutions or additions in critical domains may disrupt function and lead to a further reduction in striping rates. Critical protein domains may correspond to insertion sites associated with VLV phenotypes. Therefore, variegation patterns may provide a means to locate functional domains and to modify proteins by imprecise excision of *Ac* from coding sequences.

The genetic fine structure mapping strategies outlined in this paper have uncovered essential regions of *P* that have gone undetected by conventional molecular studies. A major unresolved issue concerning *P* gene structure concerns the function of sequences defined by several reconstituted alleles located in the 5′ 1.2-kb *Sal*I fragment (Figure 6). These insertions are located 5 to 6 kb upstream from the reported start of transcription (GROTEWOLD, ATHMA and PETERSON 1991). The phenotypes of three insertions in the 1.2-

kb *Sal*I fragment (*P-VV:R174*, *P-VV:230* and *P-VV:350*) are light or very light variegated (Table 5) suggesting the presence of critical sequences for *P* function. The interpretation of these variegated phenotypes poses some intriguing possibilities. If the only transcriptional start site for *P* RNA is actually 5 kb downstream from these insertions, these mutations are localizing *cis*-acting sequences such as enhancer elements required for *P* activity. Enhancer elements have been reported to affect gene expression over 30-kb intervals (QIAN, CAPOVILLA and PIROTTA 1991). If such distal enhancers exist, the *P* promoter would represent the largest plant promoter reported to date. However, an alternative explanation exists. The 1.2-kb insertions may be defining additional transcribed sequences, possibly coding regions, required for *P* function that have gone undetected in previous molecular studies. In either case, the insertion site of *P-VV:424* defines the minimum 5′ limits of *P*. We have only detected *Ac* insertions in the 3.0-kb *Sal*I fragment that condition unstable red phenotypes (Figure 2B). Yet, as shown by the insertion in UR280, it is possible that insertions within certain regions of the gene are phenotypically silent raising the possibility that regions further upstream of the 1.2-kb *Sa*l fragment may be required for *P* function. At minimum, our studies define a 12.3-kb region that contains sequences essential for *P* activity. It will be important to examine these upstream regions further, determine the nature of insertion sites and the molecular consequences of excision events that restore *P* function from these alleles. These experiments should help redefine the nature of the *P* promoter region or its product(s).

***Ac* orientation and *P* expression:** The second feature of reconstituted phenotypes is the level of basal *P* expression. In two instances we can attribute this expression solely to *Ac* orientation. The first instance is *P-VV:168* and *P-VV:240*. These alleles contain *Ac* insertions into the same target site but the *Ac* elements are in opposite orientation with respect to *P* transcription. *P-VV:168* conditions a colorless background while *P-VV:240* conditions an orange background. A second example is *P-VV:360* , a colorless background allele which is inserted at the same target site but in opposite orientation as an orange background allele, *P-OVOV*, previously reported (PETERSON 1990). These differences can be explained by differences in splicing patterns. The 5′ terminal repeat region of *Ds* is known to contain several splice donor sites that can serve to remove *Ds* sequences from a primary transcript (WESSLER, GARAN and VARAGONA 1987). Both *P-VV:360* and *P-OVOV* contain insertions of *Ac* oriented with this same 5′ terminal sequence in the proper orientation to serve as a splice donor site (*e.g.*, in opposite orientation with respect to *P* transcription). Normal-sized *P* transcripts have been observed from *P-OVOV* which do not contain *Ac* sequences (PETERSON 1990). The differences in basal *P* expression associated with orientation are consistent with the splicing of *Ac* from the primary *P* transcript in one orientation only that in which *Ac* and *P* are transcribed in opposite orientation. We refer to this orientation as the "read-through" orientation of *Ac*. When *Ac* and *P* are in the same orientation, the lack of basal *P* expression may be the result of premature transcription termination of *P* in *Ac* sequences or the failure of *Ac* to be spliced efficiently from the primary *P* transcript. Consistent with this interpretation is the finding that several truncated chimeric transcripts between *P* and *Ac* are observed in *P-VV:E* when *Ac* is inserted in this orientation (LECHELT *et al.* 1989).

Several reconstituted alleles containing *Ac* in the read-through orientation in the 1220-nt exon showed no basal *P* expression (see Figure 10 and Table 5 in the Appendix). Therefore, the position of *Ac* may determine whether its 5′ terminal region serves as an efficient splice donor or whether the spliced product is functional. Also, basal *P* expression can be observed in *P-VV:352* even though *Ac* is not in a read-through orientation. This insertion may represent instances in which the truncated product is functional or transcription is not terminated within *Ac* and splicing of *P* and *Ac* sequences occurs to give a functional transcript. In the reported promoter region of *P*, several insertions showed background pigmentation levels (*e.g.*, *P-VV:153* and *P-VV:172*). Since these insertion reside 5′ to the reported transcription initiation site of *P* (GROTEWOLD, ATHMA and PETERSON 1991), they may be affecting the level of *P* transcription. Therefore, orientation alone cannot explain all instances of basal *P* expression and further studies are needed to resolve these cases.

In summary, two aspects of reconstitution phenotypes can be explained by the position and orientation of *Ac* in the *P* gene. Striping phenotypes caused by excision of *Ac* from the *P* gene appear to be affected by the position of *Ac* while basal expression levels may be determined mainly by events (*e.g.*, splicing and transcription rates) occurring at the RNA level. A combination of these factors is likely responsible for the overall reconstitution phenotype.

The experimental system and its utility: We have outlined a genetic strategy in which a transposable element can be used to perform mutagenesis and fine-structure genetic analysis *in vivo*. By transposition of *Ac* from nearby chromosomal sites, a large number of reconstituted alleles can be recovered containing reinsertions of *Ac* dispersed throughout the gene. Moreover, the imprecise excision of *Ac* from each new insertion site can be used to generate a series of alleles with stable nucleotide modifications in coding and

noncoding sequences. Such modifications may prove valuable in structure-function studies. In the absence of efficient homologous gene replacement methods, the advantage of reconstitutional mutagenesis is that the genetic background remains isogenic and the mutant alleles occupy their normal chromosomal position.

Reconstitutional mutagenesis should be applicable to any *Ac*- or *Ds*-induced mutation and not only to color genes such as *P*. While the *P* gene has the advantage of the resolution of a visual marker, in principle, the technology can be extended to developmental mutations with minor modifications. The general strategy would include the following steps. Several revertant alleles carrying a transposed element would be recovered from an initial *Ac*- or *Ds*-induced mutation. Because of the frequent occurrence of short-range transpositions, approximately 15% of these chromosomes should be unstable. Southern blots or PCR strategies can be used to screen for chromosomes with nearby transposed elements. Lines homozygous for unstable chromosomes would be test-crossed and progeny screened for new mutations that potentially represent reconstituted alleles. Reinsertions would be confirmed molecularly. A *Ds* reporter allele, such as *r-sc:m3*, can be incorporated into these lines and used to monitor *Ac* dosage. In the absence of somatic variegation, the tolerance of insertion sites to imprecise excision can be determined by the frequency of germinal reversion of reconstituted alleles.

The utility of this strategy is greatly extended by the ability of *Ac* to function in heterologous plant systems such as tobacco (BAKER *et al.* 1986), Arabidopsis and carrot (VAN SLUYS, TEMPÉ and FEDOROFF 1987), tomato (YODER *et al.* 1988), potato (KNAPP *et al.* 1988), and petunia (HARING *et al.* 1989). In the tobacco system, tight clustering of tr-*Ac* elements around the donor locus has been observed (DOONER *et al.* 1991) indicating a reconstitutional mutagenesis approach would be feasible as genes are tagged in these systems. Beyond plants, elements that transpose via a DNA intermediate, such as *P* elements of Drosophila, may be potential systems for development of similar strategies.

We thank LISA SHUSTER for technical assistance, TIM NELSON, ALISON DELONG, ELSBETH WALKER and THOMAS BRUTNELL for helpful discussions and critical reading of this manuscript. We also thank TOM PETERSON for sharing unpublished sequence data. The Connecticut Agricultural Experiment Station for their generous provision of field facilities. This work was supported by a National Institutes of Health grant (RO1 GM38146) to S.L.D. M.A.M. was supported by a McKnight Foundation award to the Plant Science Group at Yale.

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Communicating editor: W. F. SHERIDAN

APPENDIX

The reconstituted *P* alleles are given in Table 5.

TABLE 5

Reconstituted *P* alleles

Reconstituted allele	UR chromosome	Original *Ac* dosage	One-*Ac* phenotype[a]	
			Background phenotype	Striping phenotype
P-VV:130	UR49	1	O	MLV
P-VV:131	UR49	ND[b]	C	MV
P-VV:132	UR49	2	C	MV
P-VV:133	UR49	1	C	VLV
P-VV:134	UR49	2	C	MV
P-VV:135	UR49	2	C	MV
P-VV:140	UR50	1	C	VLV
P-VV:141	UR50	1	C	MV
P-VV:142	UR50	1	C	MV
P-VV:143	UR50	1	C	LV
P-VV:144	UR50	ND	C	LV
P-VV:145	UR50	1	C	MV
P-VV:146	UR50	1	C	LV
P-VV:147	UR50	ND	O	MLV
P-VV:148	UR50	1	C	MLV
P-VV:149	UR50	ND	LO	MV
P-VV:150	UR1	ND	C	MLV
P-VV:151	UR1	1	C	MLV
P-VV:152	UR1	1	C	MV
P-VV:153	UR1	1	O	MV
P-VV:160	UR2	2	C	VLV
P-VV:161	UR2	1	C	MV
P-VV:162	UR2	ND	C	LV
P-VV:163	UR2	1	C	LV
P-VV:164	UR2	2	C	MV
P-VV:165	UR2	2	C	MV
P-VV:166	UR2	2	C	LV
P-VV:167	UR2	1	C	MV
P-VV:168	UR2	1	C	MLV
P-VV:169	UR2	2	C	MLV
P-VV:170	UR2	1	C	MV
P-VV:171	UR2	1	C	MV
P-VV:172	UR2	1	VLO	MV
P-VV:173	UR2	2	O	LV
P-VV:174	UR2	2	LO	LV
P-VV:175	UR2	1	C	LV
P-VV:176	UR2	2	C	LV
P-VV:177	UR2	1	C	MLV
P-VV:178	UR2	2	O	VLV
P-VV:181	UR2	2	O	LV
P-VV:190	UR3	1	C	VLV
P-VV:191	UR3	1	C	LV
P-VV:200	UR4	1	C	MLV
P-VV:201	UR4	1	C	VLV
P-VV:202	UR4	1	O	MLV
P-VV:203	UR4	ND	C	MLV
P-VV:205	UR4	ND	O	MV
P-VV:210	UR6	2	C	VLV
P-VV:211	UR6	2	C	VLV
P-VV:212	UR6	2	C	MLV
P-VV:213	UR6	2	C	MLV
P-VV:214	UR6	1	C	MV
P-VV:216	UR6	ND	DO	MLV
P-VV:217	UR6	2	DO	LV
P-VV:218	UR6	ND	O	MV
P-VV:220	UR10	1	C	MV
P-VV:221	UR10	2	C	LV
P-VV:222	UR10	2	C	MV
P-VV:223	UR10	1	C	MLV
P-VV:224	UR10	ND	C	VLV
P-VV:225	UR10	2	C	MV
P-VV:226	UR10	2	C	MV
P-VV:227	UR10	1	C	MLV
P-VV:228	UR10	1	LO	MV
P-VV:230	UR11	2	LO	VLV
P-VV:231	UR11	1	C	LV
P-VV:232	UR11	ND	O	MLV
P-VV:233	UR11	1	C	MV
P-VV:240	UR13	2	O	MLV
P-VV:241	UR13	1	C	VLV
P-VV:242	UR13	1	C	MLV
P-VV:250	UR16	1	C	LV
P-VV:251	UR16	1	C	LV
P-VV:252	UR16	1	C	MLV
P-VV:253	UR16	ND	O	MLV
P-VV:260	UR17	1	C	MV
P-VV:261	UR17	1	C	MLV
P-VV:262	UR17	1	C	MV
P-VV:263	UR17	ND	C	VLV
P-VV:264	UR17	2	C	MLV
P-VV:270	UR18	1	C	MV
P-VV:271	UR18	ND	C	MV
P-VV:281	UR21	1	DO	MLV
P-VV:290	UR22	2	C	MLV
P-VV:300	UR23	ND	C	MLV
P-VV:301	UR23	1	C	VLV
P-VV:302	UR23	1	C	MV
P-VV:303	UR23	1	C	MLV
P-VV:304	UR23	1	C	MLV
P-VV:305	UR23	1	C	MV
P-VV:310	UR30	1	C	MV
P-VV:320	UR32	1	C	MLV
P-VV:321	UR32	1	C	MV
P-VV:322	UR32	ND	C	MLV
P-VV:331	UR33	ND	C	MV
P-VV:340	UR34	1	C	MV
P-VV:350	UR35	2	C	LV
P-VV:351	UR35	1	O	MV
P-VV:352	UR35	2	DO	MV
P-VV:353	UR35	1	O	LV
P-VV:354	UR35	2	C	MLV
P-VV:355	UR35	1	C	MV
P-VV:360	UR36	2	C	MV
P-VV:380	UR38	2	C	VLV
P-VV:381	UR38	ND	C	MV
P-VV:390	UR39	1	C	MV
P-VV:391	UR39	1	C	MV
P-VV:400	UR41	1	C	VLV
P-VV:401	UR41	2	C	LV
P-VV:402	UR41	2	C	LV
P-VV:403	UR41	2	O	MV
P-VV:404	UR41	2	DO	MLV
P-VV:410	UR42	2	C	MLV
P-VV:411	UR42	ND	C	MLV
P-VV:413	UR42	2	C	VLV
P-VV:420	UR45	1	C	MV
P-VV:421	UR45	ND	O	MV
P-VV:422	UR45	1	O	MLV
P-VV:423	UR45	ND	O	MV
P-VV:424	UR45	1	O	MV
P-VV:425	UR45	1	O	MV

[a] Abbreviations: C = colorless; VLO = very light orange; LO = light orange; O = orange; DO = dark orange; VLV = very light variegated; LV = light variegated; MLV = medium light variegated; MV = medium variegated.

[b] ND = not determined. Kernel progeny from original reconstituted ear were too few to determine *Ac* copy number with certainty.