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Genetics

Molecular basis of mutations at the waxy locus of maize: Correlation with the fine structure genetic map

(spontaneous mutation/DNA insertions/polymorphism)

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ABSTRACT **More than 40 mutant alleles of the waxy (*Wx*) locus of maize are available for molecular analysis. Previous studies have examined the nature of phenotypically unstable *Wx* mutant alleles caused by insertion of the maize transposable activator (*Ac*) and dissociation (*Ds*) elements. In this study we have used Southern blot analysis to characterize the locus in 22 strains harboring *wx* alleles with stable mutant phenotypes. Of these mutations, 17 are of spontaneous origin, 4 were induced by γ rays, and 1 was induced by ethyl methanesulfonate. Of these 22 alleles, we find that 13 have either insertions or deletions within the *Wx* transcription unit. The insertions range in size from 150 base pairs to 6.1 kilobases. For 4 of the 6 deletions identified, the two breakpoints are within the *Wx* gene. For 9 other alleles we can detect no obvious lesions within or around the transcription unit. Evidence is presented that the insertions and deletions result in the mutant phenotype and are not polymorphisms. This conclusion is based on two findings: (*i*) a survey of inbred lines revealed only a single instance of polymorphism within the transcription unit, whereas all of the lesions described alter the transcription unit; and (*ii*) there is an excellent correlation between the position of these lesions on the physical map and their relative position on a fine structure genetic map of the locus.**

The *Wx* (waxy) locus of *Zea mays* encodes a starch granule-bound glucosyl transferase involved in starch biosynthesis (1). It is expressed in the endosperm of the developing kernel and in the pollen grain (2, 3). The locus derives its name from the waxy appearance of mutant kernels, the phenotype reflecting an alteration in the underlying starch composition. Because of this easily identifiable nonlethal phenotype, the locus has been the subject of genetic analysis for over 60 yr (3, 4). There are >40 *Wx* mutants of both spontaneous and induced origins available for analysis. Some are stably mutant (i.e., the mutant phenotype remains unchanged) both somatically and germinally in all genetic backgrounds tested. Other alleles display an unstable phenotype because of the insertion of transposable elements (5–8). All *Wx* mutations map to the short arm of chromosome 9. To understand the relationship between the various *Wx* mutants, Nelson constructed a fine-structure map of the locus (9, 10). To date this represents the most rigorous genetic study of a plant gene. This ingenious analysis was accomplished by determining whether various *wx* heteroalleles exhibit recombination. The restoration of *Wx* expression via recombination was scored by staining the pollen grains of these heteroalleles with KI/I_2 and counting the rare black-staining recombinants that contain amylose.

In order to determine the molecular basis of normal and mutant *Wx* expression, the locus was cloned and its gene product was characterized (11). It was found that the *Wx* locus encodes a 58-kDa starch granule-bound protein that is altered in some strains harboring *Wx* mutant alleles (*wx*) and missing entirely from others (11, 12). Of interest to us was the finding of Echt and Schwartz (12) that three *Wx* mutants (strains harboring *wx* alleles *R, C31* and *90*; see Table 1) that produced altered granule-bound proteins mapped to the extremities of Nelson's genetic map. This result suggested that all or most of the *Wx* mutations could be found within the structural gene of the *Wx* protein.

Recent studies have focused on a molecular description of the transposable elements responsible for unstable phenotypes in plants (11, 13–16). However, virtually nothing is known about the molecular basis of stable mutant phenotypes in plants, especially those of spontaneous origin. The *Wx* locus and its genetically characterized mutant alleles provides a unique opportunity to address this question. To this end we analyzed the lesions responsible for 22 mutant *Wx* alleles, 17 of which are of spontaneous origin. We found that 13 of these alleles have either insertions or deletions within or including the *Wx* transcription unit. Furthermore, we found that there is an excellent correlation between the position of an insertion or deletion on the physical map and the relative position of these mutations on Nelson's genetic map. As the genetic map is a functional map, this correlation suggests that the insertions and deletions found associated with mutant alleles are in fact the molecular lesions responsible for the mutant phenotype.

MATERIALS AND METHODS

Maize Strains. The following inbred lines carrying the *Wx* allele were obtained for this study: W23 × K55 from E. Coe; W23 from G. Neuffer; W22 from J. Kermicle; 38-11 from M. Zuber; HY, Krug, and Oh45 from B. Bear; and Ga 209, Ga 211, and Ga 219 from A. Flemming. The origins of the *Wx* mutant alleles are described in Table 1. Strains carrying the *wx* alleles *B5, G, K*, and *M* were obtained from O. Nelson, strains carrying *wx* alleles *I, C2* and *BL2* were obtained from C. Echt, and the remaining *wx* allele-carrying strains came from the Maize Genetics Cooperation Stock Center (Urbana, IL). The strains examined in this study were all homozygous for the designated *Wx* or *wx* alleles.

Reagents. Restriction enzymes, ligase, and DNA polymerase I were from either Bethesda Research Laboratories or from New England Biolabs. Radioactive dATP and dCTP were from Amersham (specific activity, 400 Ci/mmol; 1 Ci = 37 GBq).

Recombinant Plasmids. The construction of plasmid pWx5, which contains the *Wx* transcription unit on a 10.5-kilobase (kb) *Eco*RI fragment, has been described (11). Subclones containing probes 1, 2, and 3 (see Fig. 1*D*) were constructed

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Abbreviations: *Wx*, waxy gene; *wx*, mutant *Wx* allele; bp, base pair(s); kb, kilobase(s); RFLP, restriction fragment length polymorphism.

Table 1. Strains used in this study

wx allele	Nature of mutation	Lesion*	Molecular progenitor *Wx* allele†	Origin‡
90	Spontaneous	ND	*HY*	Brunson
R	Spontaneous	ND	*HY*	Richardson
B	Spontaneous	Deletion	*HY*	Bear hybrid
G	Spontaneous	Insertion	*W23*	Bear hybrid
I	Spontaneous	Insertion	*HY*	Bear hybrid
K	Spontaneous	Insertion	*HY*	Bear hybrid
M	Spontaneous	Insertion	*HY*	Bear hybrid
C1	Spontaneous	ND	*HY*	Blandy farms
C2	γ rays	ND	*HY*	Blandy farms
C3	γ rays	ND	*HY*	Blandy farms
C4	Spontaneous	Deletion	*HY*	Blandy farms
C31	γ rays	ND	*HY*	Blandy farms
C34	γ rays	Deletion	Unknown	Blandy farms
B1	Spontaneous	Deletion	*W23*	Ashman and Brink
B2	Spontaneous	Insertion	*W23*	Ashman and Brink
B5	Spontaneous	Insertion	*W23*	Ashman and Brink
B6	Spontaneous	Deletion	*HY*	Ashman and Brink
B7	Spontaneous	Deletion	*HY*	Ashman and Brink
B8	Spontaneous	ND	*W23*	Ashman and Brink
c	Spontaneous	ND	*HY*	Collins
Stoner	Spontaneous	Insertion	*HY*	From Assam
BL2	EMS	ND	*HY*	Briggs

EMS, ethyl methanesulfonate.
*ND = not determined, a lesion of less than 50 bp.
†This refers to one of the four *Wx* alleles described in Fig. 1*D*. Few of the direct progenitors are actually known.
‡From O. Nelson (9).

by digestion of pWx5 with *Sal* I and subsequent ligation of the resulting fragments into the *Sal* I site of pUC9 (17).

Preparation of Maize Genomic DNA and Filter Hybridization. DNA was purified from 2- to 4-week-old plantlets by the method of Shure *et al.* (11). To rapidly survey many maize strains, genomic DNA was isolated from 1 g of leaf tissue by the "miniprep" protocol of Dellaporta (18). Restricted DNA was electrophoresed through 0.8–1.0% agarose and blotted by the method of Southern (19) as modified by Fedoroff *et al.* (20). Blots were hybridized with plasmids containing probes 1, 2, or 3 (Fig. 1*D*) that were labeled by nick-translation (21) to a specific activity of 1×10^8 to 5×10^8 cpm/μg by using radioactive dATP and dCTP. Autoradiography was for 16–48 hr with an intensifying screen.

RESULTS

Polymorphisms Within and Around the *Wx* Gene. The mutant *Wx* alleles examined in this study are described in Table 1. Many of these mutants were isolated over the past 60 yr, and direct progenitors are no longer available. For these alleles it is important to know if changes in the size of restriction fragments reflect a mutation or a polymorphism. This is of particular importance when analyzing plant genes which, in the few cases examined, exhibit a high degree of restriction fragment length polymorphism (RFLP) (22, 23). To determine the extent of RFLP within and around the *Wx* gene, we examined several inbred lines by Southern blot analysis with *Wx*-specific probes. The results of our analysis of 12 inbred lines are summarized in Fig. 1. The cloned *Wx* gene has been described (11) and is displayed in Fig. 1*D* along with some relevant restriction sites. We designated this allele *HY* after an inbred line we have examined that is indistinguishable from the cloned locus. By hybridizing labeled restriction fragments to dot blots (24) containing endosperm poly(A), we and others have delimited the *Wx* transcription unit to the region indicated by the 5'-to-3' arrow (unpublished result; ref. 15). The direction of transcription is inferred by the position of homology with the cDNA clone pcWx0.4 (ref. 11; Fig. 1*D*).

Among the inbred lines examined there are four different *Wx* alleles. The names given these alleles (*HY, W22, 38-11,* and *W23*) represent one of the lines that harbors that particular *Wx* gene and flanking sequences. The modified restriction maps shown in Fig. 1*D* were generated by digesting genomic DNA with either *Sal* I/*Sst* I, *Sal* I, or *Sal* I/*Pst* I and hybridizing with a combination of probes 1, 2, or 3 (Fig. 1*D*) or subclones of these probes. Part of this analysis is shown in Fig. 1 *A, B,* and *C*. We found that the DNA corresponding to probe 1 is highly polymorphic. When genomic DNA was digested with *Sal* I/*Sst* I and probed sequentially with the two *Sal* I–*Sst* I fragments that comprise probe 1, we found that both halves reveal RFLP. The 3' *Sst* I–*Sal* I fragment (with respect to the direction of *Wx* transcription) is conserved in alleles *HY, W22* and *38-11* but is larger in *W23* (Fig. 1*A*, in which 3' fragments are noted by small circles). The additional 250 base pairs (bp) in allele *W23* was localized to the *Sst* I–*Eco*RV fragment (Fig. 1*D*). The 5' half of probe 1 hybridized to the second band present in each digest of Fig. 1*A*. We interpreted these RFLPs as insertions or deletions (Fig. 1*D*) within this fragment because the results of additional digests with *Hin*dIII/*Sst* I or *Sal* I alone were consistent with this interpretation (data not shown). It should be noted that these RFLPs are outside of the region believed to be the *Wx* transcription unit.

Among the inbred lines, only one RFLP was found within the transcription unit. Digestion with *Sal* I revealed that the *Sal* I fragments represented by probes 2 and 3 are essentially conserved in all strains examined (Fig. 1*B*). However, when the double digest of *Sal* I/*Pst* I was probed with the 5' and 3' *Sal* I–*Pst* I fragments that comprise probe 2, a small RFLP was detected in the 3' half in allele *W23* (Fig. 1 *C* and *D*). This small deletion eliminated the *Sst* I site in this strain (Fig. 1*D*). Additional blots indicated that the sites shown at the 3' end

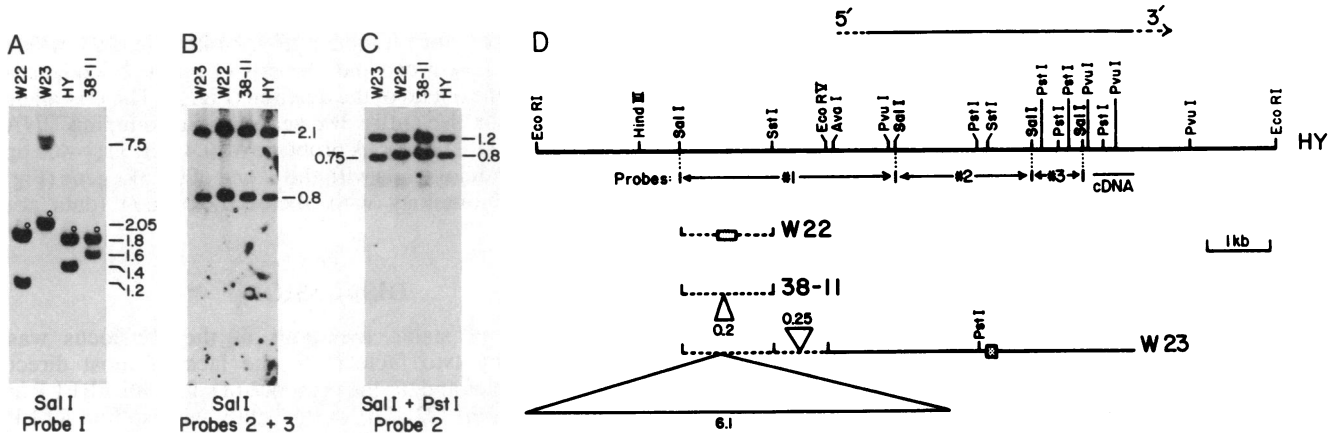

FIG. 1. Southern blot analysis of the *Wx* locus in various inbred lines. (*A–C*) Genomic DNA was digested with the enzyme noted, electrophoresed through agarose, blotted as described, and hybridized with the probe(s) noted and displayed in *D*. The size of the fragments are in kb. For each gel, λ and pBR322 restriction fragments were transferred and probed simultaneously as molecular weight markers. (*D*) Selected restriction sites within the cloned *Wx* allele in strain HY (11), the position of probes 1, 2, and 3, and a summary of RFLPs found in strains W22, 38-11, and W23. The 5'-to-3' arrow above the HY map represents the direction and approximate limits of the *Wx* transcription unit. Dotted lines indicate the extent of uncertainty.

of allele *HY* are conserved in the other inbred lines. However, there were extensive RFLP outside of this region in all strains examined.

These data demonstrate that the *Wx* transcription unit is almost perfectly conserved for the restriction sites examined. Since most, if not all of the mutations fall within the *Wx* structural gene (ref. 12; as mentioned in the Introduction), observed differences between the restriction maps of mutant and wild-type alleles should represent the molecular lesions.

Molecular Lesions Associated with *Wx* Mutant Alleles. Genomic DNA was isolated from strains harboring the *wx* alleles described in Table 1. All were digested with *Sal* I, blotted, and hybridized with probes 1, 2, or 3. Of the 22 alleles examined in this way, 9 showed no detectable alterations. That is, probes 2 and 3 hybridized with fragments of 2.1 kb and 0.8 kb, respectively, while probe 1 hybridized with one of the four polymorphic fragments depicted in Fig. 1*D*. In Table 1 we have classified these lesions as "not determined" (ND). It should be noted that this method of analysis could not detect lesions that altered restriction fragments by <50 bp.

We found that seven alleles have insertions within the *Wx* transcription unit. This conclusion is based on the following evidence. Four alleles (*Stoner, B5, G* and *M*) have large *Sal* I fragments homologous to probe 2 (Fig. 2*A*). In addition, probe 2 is weakly homologous to a 1.0-kb *Sal* I fragment of

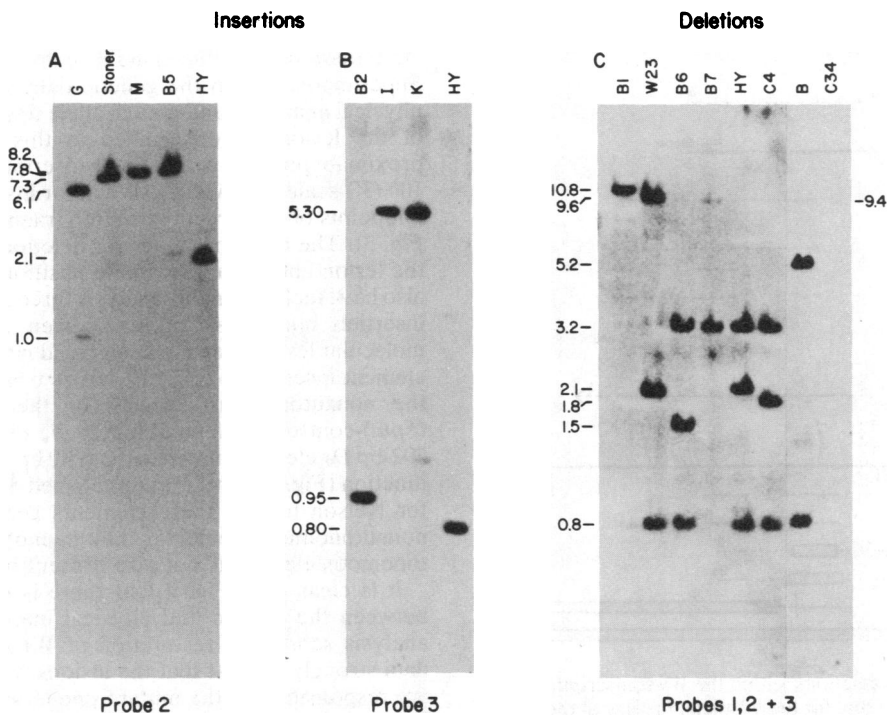

FIG. 2. Southern blot analysis of *Wx* mutant alleles. DNA from 13 homozygous *wx* alleles were digested with *Sal* I, electrophoresed through 0.8% agarose, blotted, and hybridized with the labeled probe shown in the figure. The wild-type control included on each blot is DNA from strain HY for *A* and *B* and strains W23 and HY for *C*. The restriction fragment lengths displayed represent average values of many gels. In *C*, the 0.8-kb fragment of the *B1* allele did not transfer well. Similar blots of *B1* DNA clearly reveal this fragment.

the *G* allele. Three other mutant alleles (*I, K* and *B2*) possess large *Sal* I fragments that are homologous to probe 3 (Fig. 2*B*). For each allele, multiple restriction digests were performed to insure that the altered fragment reflected an insertion into an existing fragment rather than a deletion or a rearrangement that might fuse two fragments into one new one. For the probe 2 insertions (Fig. 2*A*), only one *Sal* I fragment was altered [i.e., probe 3 hybridized with a 0.8-kb fragment, and probe 1 was homologous in each case to a fragment that corresponded in size with one of the molecular progenitors (Table 1)]. Furthermore, digestion with *Sal* I/*Pst* I localized each insertion to either the 5′ or 3′ side of probe 2 (Fig. 3). The probe 3 insertions (Fig. 2*B*) have a 2.1-kb *Sal* I fragment homologous to probe 2, and the size of DNA fragments hybridizing with probe 1 is the same as one of the molecular progenitors (Table 1). In addition, *B2, I* and *K* each possess a *Pst* I fragment homologous with probe 3 (Fig. 1*D*) that is larger than its *Wx* counterpart by the size of the insertion (data not shown). These data are summarized in Fig. 3.

Six alleles have deletions within or including the *Wx* transcription unit. To make this determination, *Sal* I digests of genomic DNA isolated from strains harboring the *wx* alleles *B, B1, B6, B7, C4*, and *C34* were blotted and hybridized with either probes 1, 2, or 3. The blot in Fig. 2*C* was hybridized with all three probes. For four of the alleles, we can identify both breakpoints (*B, B1, B6*, and *C4*). Both probes 1 and 2 hybridized to the 5.2-kb band of *B* and the 10.8-kb band of *B1*. When a *Sal* I/*Sst* I digest was hybridized with probe 1, we found that the 5′ *Sal* I–*Sst* I fragments generated were intact and corresponded with a particular molecular progenitor (Table 1) and that the 3′ *Sal* I–*Sst* I fragment was fused with probe 2 sequences. The size of the fused fragment permitted an estimate of the deletion size for both *B* and *B1* (Fig. 3). The *B6* and *C4* deletions have both breakpoints in the 2.1-kb *Sal* I fragment. A *Sal* I/*Pst* I digest of *B, B1, B6*, and *C4* hybridized with probe 2 delimited the deletions to the regions shown in Fig. 3. Only the 5′ breakpoint of the *B7* allele could be identified. Both *Sal* I (Fig. 2*C*) and *Sal* I/*Pst* I digests of this allele indicated that the *Sal* I site between probes 1 and 2 is intact. Probe 2 had weak homology with a *Sal* I fragment of ≈9.4 kb (Fig. 2*C*), which is probably a fusion of what remains of probe 2 sequences with DNA at the 3′ end of the deletion (Fig. 3). The *C34* allele was deleted for the entire *Wx* gene and surrounding DNA (Figs. 2*C* and 3). The cDNA probe pcWx0.4 [ref. 11; ≈400 bp in length and homologous with the 3′ end of the *Wx* gene (Fig. 1*D*)] had no homology with alleles *B7* or *C34* (data not shown).

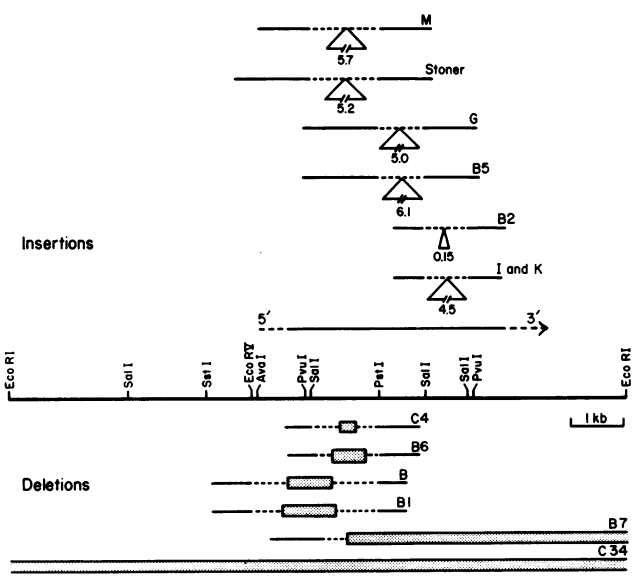

FIG. 3. Insertions and deletions within the *Wx* transcription unit in 13 mutant *Wx* alleles. Except for the *B2* allele, the size of the DNA insertions are not drawn to scale. The actual length of these inserts are shown in kb. The extent of deletions are depicted by stippled regions. Dotted lines indicate uncertainty in the precise position of insertions and deletions with respect to the restriction sites found within the *Wx* transcription unit.

DISCUSSION

Our analysis of stable mutations at the *Wx* locus was complicated by two factors: (*i*) the lack of most direct progenitor alleles and (*ii*) the presence of substantial RFLP in the maize genome (22, 23). In this study we also find RFLP when the *Wx* locus is examined in many inbred lines. However, this polymorphism is largely confined to regions outside of the transcription unit. This finding was essential for the success of this type of analysis because previous genetic and biochemical studies (11, 12) indicated that most mutations were within the *Wx* transcription unit. It is for these reasons that we feel confident that the seven insertions and six deletions found associated with mutant *Wx* alleles represent molecular lesions rather than polymorphisms.

Is it possible to state with equal confidence that the lesions detected represent the primary event responsible for the loss of *Wx* expression? Since *Wx* revertant alleles cannot be isolated from these strains [Nelson (9) estimates that the rate of reversion for the various *wx* alleles ranges from 0 to 2.7×10^{-5}], it is conceivable that other, less obvious alterations are also present at the locus. To address this question we have compared the position of deletions and insertions found in this study with the relative position of these alleles on the fine-structure genetic map of the locus (9, 10). Construction of the genetic map was primarily based on a determination of whether the F_1s of *wx* heteroalleles exhibit recombination, thus producing a functional *Wx* gene. This was scored by determining the frequency of *Wx* pollen grains produced by each particular *wx* heteroallele. The relative positions of the *Wx* mutations as deduced in this manner are shown in Fig. 4. Superimposed upon the genetic data are the results of our physical analysis. Under each allele designation is the nature of the lesion (as determined in this study) and the approximate position within the transcription unit on a 1 (5′) to 100 (3′) scale. The values shown for insertions represent the midpoints of the target restriction fragments (as displayed in Fig. 3). The range of values for deletions reflects the size of the lesion and the approximate position of breakpoints. We also have included in this analysis three transposable-element insertion mutations that have been characterized at the molecular level. They are designated *m6* [*wx-m6*, a 2.1-kb *Ds* element insertion (6, 11, 13)], *m8* [*wx-m8*, a 2-kb insertion of the nonautonomous element of the suppressor–mutator (*Spm*)-controlling element family (7, 15)], and *m1* [*wx-m1*, a 392-bp *Ds* element inserted about 40 bp 5′ from the probe 2–3 junction (Fig. 1*D*) (ref. 5; unpublished data)]. It was possible for Nelson to map these elements genetically because, as nonautonomous elements, they cannot transpose if an autonomous element is not also present in the genome (9).

It is clear from Fig. 4 that there is excellent correlation between the genetic and physical maps. Since the genetic analysis scores the restoration of *Wx* gene function, these data strongly suggest that the lesions we have characterized are responsible for the mutant phenotypes.

For one allele, *R*, the results of our study are inconsistent with the extent of this mutation on the genetic map. Although Nelson found that *R* failed to recombine with many *wx* alleles (Fig. 4), we cannot detect any obvious lesion for this allele. Our results are in agreement with the findings of Echt and

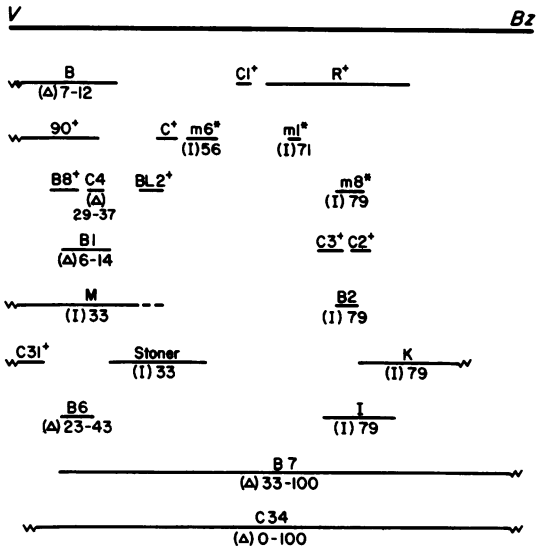

FIG. 4. Correlation between the genetic map and the physical map of the *Wx* locus. The fine structure map of the locus is that of Nelson (9, 10). It is constructed as a complementation map such that any two mutants do not recombine if their horizontal lines overlap. Lines that terminate in serations indicate that there is no mutant more distal (or proximal) with which that mutant recombines. Superimposed on these horizontal lines are the molecular nature of each lesion (I, insertion; Δ, deletion) and the relative positions of the lesions found associated with each allele. The method by which a number value was assigned to each is described in the text. *V* (virescent) and *Bz* (bronze) are genetic loci that flank the *Wx* locus. +, Alleles that are indistinguishable from the progenitor alleles by Southern analysis; *, mutations caused by the insertion of maize transposable elements.

Schwartz (12), who analyzed the starch granule-bound proteins produced by this strain. This allele programs the synthesis of an inactive *Wx* protein of correct molecular weight (58 kDa) but altered mobility on isoelectric-focusing gels. The amount of protein synthesized is comparable with nonmutant strains. Although a large inversion would fail to recombine with many *wx* alleles and might be undetectable by our Southern analysis, this type of lesion is inconsistent with the biochemical data.

Two of the *wx* alleles that we have characterized were not included in the genetic analysis because they have a leaky phenotype. It is interesting to note that these alleles, *G* and *B5*, have large insertions within the *Wx* gene (Fig. 3). We have determined recently that strains harboring the *B5* allele make a reduced amount of a wild-type-sized transcript (2.5 kb; unpublished data). It remains to be determined whether this element is in intron or exon sequences.

Spontaneous mutations in *Escherichia coli* (25, 26), yeast (27) and *Drosophila melanogaster* (28, 29) are frequently caused by DNA insertions. The involvement of DNA insertions in unstable plant phenotypes has been well documented (11, 13–15) and recently reviewed (16). Far less is known about the molecular lesions responsible for stable mutant phenotypes of spontaneous origin in plants. To our knowledge in only one instance has a mutation of this type been associated with DNA insertions or rearrangements. A naturally occurring mutant allele of the soybean lectin gene contains a 3.4-kb insertion that has the structure of a transposable element (30). Of the 17 mutant alleles of spontaneous origin examined in this study, 7 are associated with insertions and 5 with deletions. It is possible that 2 of the insertions, *I* and *K*, are the same—both have 4.5-kb insertions into the same region (Fig. 3) and both have the same molecular progenitor (Table 1). Only 5 alleles have lesions that must be smaller than our limit of resolution (50 bp). These results suggest that gross chromosomal changes make up a large proportion of spontaneous mutations in maize.

The existence of nonautonomous transposable elements in maize poses the interesting possibility that some of the insertions described in this study might be capable of transposition when crossed with a strain harboring the appropriate autonomous element. The *Wx* insertion mutations have been crossed with strains harboring the autonomous elements of four maize controlling element families: *Ac-Ds*, *Spm*, *Bg*, and *Uq*. None of these crosses resulted in an unstable *Wx* phenotype. This does not, of course, rule out the possibility that these elements are capable of transposition. After all, they did at one time transpose into the *Wx* locus. They may represent a class of elements activated not by an autonomous element but possibly by stress in the corn field (31, 32). In this regard they may have a similar origin to insertions we find associated with RFLP outside the *Wx* transcription unit in alleles *38-11* and *W23* (Fig. 1*D*). Finally, these insertions may be defective controlling elements no longer able to transpose. The answers to these questions await the cloning and detailed characterization of these mutations.

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