EVIDENCE FOR THE INCLUSION OF CONTROLLING ELEMENTS WITHIN THE STRUCTURAL GENE AT THE WAXY LOCUS IN MAIZE

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ABSTRACT

Minimal limits for the structural gene at the waxy locus have been set by investigations of the protein product of the gene. An altered protein is produced by four of the waxy mutants including *B3*, a controlling-element mutation. All are similar to wild type in molecular weight as determined by electrophoresis in SDS acrylamide gels. At least three of the five *wx* controlling-element mutations studied have been shown to lie within the limits of the structural gene.

THE waxy locus on chromosome *9* in maize controls the production of amylose in the endosperm (WEATHERWAX 1922), pollen (DEMEREC 1924) and embryo sac (BRINK 1925). Starch produced in endosperm that carries the *Wx* allele is composed of about 25% amylose and 75% amylopectin, but homozygous *wx* endosperm contains only amylopectin (SPRAGUE, BRIMHALL and HIXON 1943). There are more than 30 *wx* alleles available for analysis, and in an ingenious study, NELSON (1968) prepared a fine-structure map of the locus. A thorough analysis of this locus, however, requires the isolation of the *Wx* gene product and the determination of the effects of the various mutations on *Wx* gene expression.

The effects of the controlling elements at the locus (MCCLINTOCK 1951, 1956, 1965) are of special interest. These are transposable genetic elements that are capable of altering gene expression. Insertion of a controlling element in or near a gene locus can reduce or eliminate expression of that gene. Subsequent transposition or alteration of the element can result in full or partial recovery of gene expression. A thorough review of controlling elements in maize was given by FINCHAM and SASTRY (1974). NELSON demonstrated that recombination occurs between all five of the *wx* controlling-element alleles tested (NELSON 1968, 1976) and questioned their analogy to the operator element of an operon as proposed by MCCLINTOCK (1961), since they are not located at either extreme of the locus. However, he pointed out that, "Probably no decision as to whether the similarities are basic can be made until we examine the product (the protein) produced in the uninhibited state as compared to the product or lack thereof in the controlling element mutants" (NELSON 1968). FINCHAM and SASTRY (1974) also noted the need to determine whether the various *wx* alleles result in qualitative or quanti-

tative changes of the gene product. As they point out, it was not absolutely certain that the locus, as mapped by NELSON, was the structural gene for the *Wx* gene product since all or some of the mutants may lie in an external control region.

The only assay previously employed for the *Wx* gene product has been the measurement of starch-granule-bound nucleoside diphosphate (NDP) sugar-starch glucosyl transferase activity, which is nearly absent in *wx* endosperm (NELSON and RINES 1962; NELSON, CHOUREY and CHANG 1978). Since the transferase activity varies linearly with *Wx* gene dosage (TSAI 1974), it is assumed that the transferase is the *Wx* gene product. Isolation of the transferase and its analysis at the protein level would make it possible to determine whether a certain mutant phenotype results from an absence of the enzyme, decreased synthesis, or production of an altered or inactive form. Also, the potential would exist to determine the limits of the structural gene within the fine-structure map, the precise nature of the various *wx* mutations and the mode of action of the controlling elements in bringing about the mutant phenotype. We report here the isolation of the protein encoded by the *Wx* gene and the characterization of various *wx* alleles with respect to their effects on gene expression.

MATERIALS AND METHODS

Stocks: The conventional alleles tested were: *wx*^{a}, *B*, *B1*, *B2*, *B6*, *B7*, *B8*, *BL2*, *BL3*, *C*, *C1*, *C2*, *C3*, *C4*, *C31*, *C34*, *F*, *H*, *H21*, *I*, *L*, *M*, *P60*, *R*, *STONOR* and *90*. The *C* allele was used as the standard *wx* allele throughout this study. The *wx* controlling-element alleles used were *B3*(*Mp* = *Ac*), *B4*(*Ds*), *m-1*(*Ds*), *m-6*(*Ds*), *m-8*(*Spm*) and two stable *m-1* revertants, *S5* and *S15*. The *wx* alleles used in this study were obtained from OLIVER NELSON and from the Maize Genetics Cooperation Stock Center.

Protein assay and extraction: Presence or absence of the *Wx* protein was determined by electrophoresis of starch-granule-bound proteins on sodium dodecyl sulfate (SDS) polyacrylamide gels (LAEMMLI 1970). The gels in the figures are 10% acrylamide and were stained with Coomassie Blue, except where silver staining (OAKLEY, KIRSCH and MORRIS 1980) is indicated. Densitometric measurements of the stained gels were performed on a Quick Scan R & D densitometer (Helena Laboratories).

Starch granules from immature endosperm were isolated from 20-day-old (20d) kernels that had been frozen and stored at —20°. For SDS extraction, the pericarp and embryo were removed and the endosperm was homogenized in buffer A (0.055 M Tris, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol (BME), 10% glycerol) with a mortar and pestle. The suspension was filtered through a layer of Miracloth and centrifuged at 15,600 × g for one min. The pellet was washed by resuspension and centrifugation 3 times with buffer A, twice with distilled water and twice with acetone. The starch granules were then dried *in vacuo*. 0.01 gm of the granules was mixed with 0.1 ml of buffer A and heated in a boiling water bath for one min. The gelled solution was cooled and an additional 0.2 ml of buffer A was added with stirring. The slurry was centrifuged and the supernatant used for electrophoresis.

Molecular weight determination was performed in a SDS-urea-phosphate polyacrylamide gel system (STRAUSS and KAESBERG 1970), as modified by using 10% acrylamide and 7 M urea. The upper and lower resevoir buffers were 0.1 M sodium phosphate, pH 7.6, with 0.1% SDS.

Isoelectric focusing: Denaturing isoelectric focusing was performed in slab gels by the method of O'FARREL (1975), using pH 3.5–10 and pH 5–8 ampholines (LKB) mixed in equal parts. Immature starch granules were prepared by first removing the pericarp and embryo from the kernel. The endosperm was homogenized in a glass homogenizer with 0.055 M Tris, pH 6.8, containing 10% glycerol. After filtration and centrifugation, as described above, the cohesive

colored layer, which overlays the starch granule pellet, was removed. The starch granules were resuspended and washed 3 times with distilled water, twice with acetone and then dried *in vacuo*. 0.04 gm of starch granules was suspended in 0.3 ml of a solution containing 9.5 M urea, 5% BME and 2% Nonidet P40 (Particle Data Laboratories). After about one min, when the starch had gelled, 0.6 ml of a cold solution of 1% ampholine carriers (pH 3.5–10, LKB) was admixed. The slurry was centrifuged and the supernatant used for isoelectric focusing.

RESULTS

The Wx protein: SDS extracts of starch granules from 20d *Wx/Wx/Wx* endosperm contain only four proteins as visualized on polyacrylamide gels (Figure 1). There is a single major protein and three minor proteins of higher molecular weight. The major protein, termed the Wx protein, comprises about 85% of the total heat-extractable, starch-granule-bound proteins as determined by densitometry. SDS extracts of starch granules from 20d waxy endosperm have the same three minor proteins, but the Wx protein is completely missing (Figure 1) in 25 of the 29 *wx* mutants that produce no amylose. This difference between *Wx* and *wx* endosperm was seen in all the developmental stages analyzed, from 12 days to maturity, as well as between *Wx* and *wx* pollen. By mobility comparisons with molecular weight markers, the size of the Wx protein was estimated to be about 60,000 daltons (Figure 2). SDS extracts of whole endosperm of 20d *Wx*

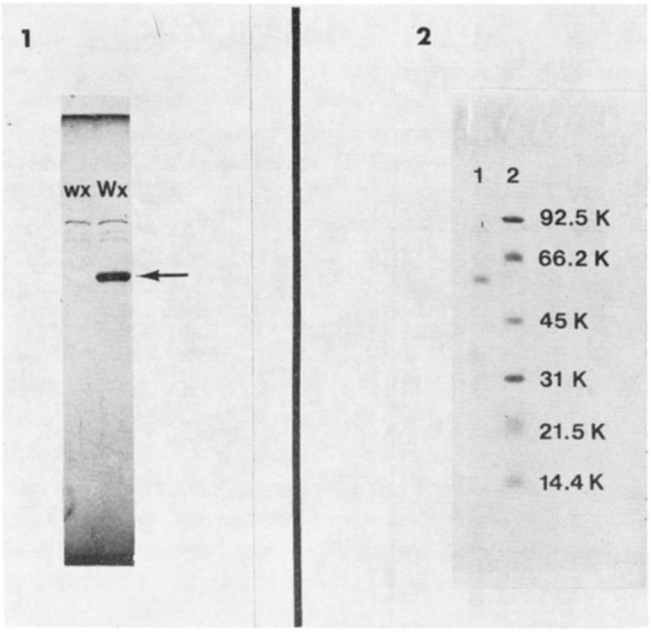

FIGURE 1.—Proteins extracted from starch granules of 20d *Wx* and *wx* endosperm. Arrow indicates the Wx protein. The SDS acrylamide gel was silver stained. (Left.)

FIGURE 2.—Molecular weight determination of the Wx protein in an SDS-urea-phosphate gel system. The standards are in lane 2 with their molecular weights listed. The Wx protein, estimated to be 60,100 daltons, is in lane 1. (Right.)

and *wx* kernels were compared to see whether the Wx protein was present elsewhere in the cell and not starch-granule bound in *wx* endosperm and whether the *wx* mutation affected any other endosperm proteins. No differences between *Wx* and *wx* endosperm proteins are observed unless the extracts are heated to swell the starch granules and release the Wx protein (Figure 3). Thus, the Wx allele is associated with a major starch-granule-bound protein.

The amount of Wx protein is correlated with the dosage of the Wx allele. One, two and three doses of *Wx* in the triploid endosperm show a progressive increase in Wx protein levels (Figure 4). The minor proteins do not appear to vary with the dosage.

The wx *alleles*: Thirty-three *wx* alleles were screened for the presence of the Wx protein. Of the 26 conventional *wx* allelic mutants, where there is no evidence for the involvement of transposable controlling elements, four (*R, C31, 90* and wx^a) have a Wx protein (Figure 5) and the rest completely lack the protein. The amount of Wx protein in *90* and wx^a is strikingly reduced. The conventional mutants all lack amylose and stain red with iodine-potassium iodide (IKI), except

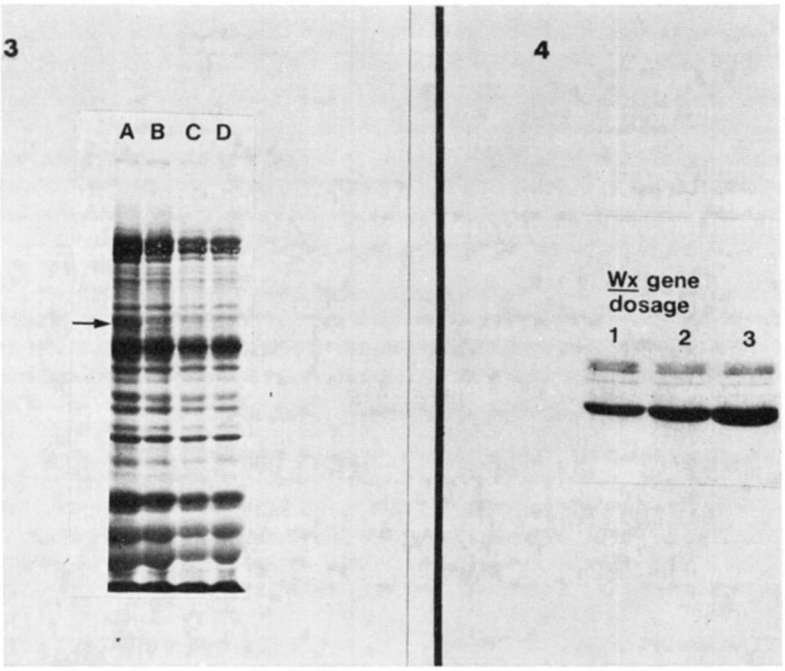

FIGURE 3.—Whole endosperm proteins and the effect of heating on extraction of the Wx protein from starch granules. Lanes A and C are from *Wx* and lanes B and D are from *wx* endosperm. 20d endosperms were mascerated in buffer A (MATERIALS AND METHODS). The preparations loaded in lanes A and B were heated in a boiling water bath to swell the starch granules. The preparations loaded in lanes C and D were not heated. Note that the Wx protein is observed only in the heated *Wx* sample (arrow). (Left.)

FIGURE 4.—Correlation of Wx protein level with *Wx* gene dosage. Note that the minor bands do not vary with the dosage of the *Wx* gene (Right.)

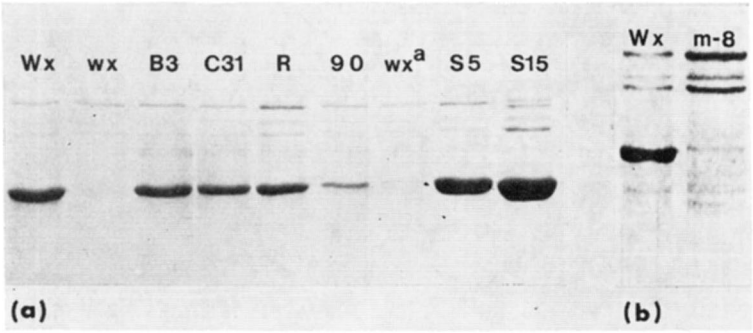

FIGURE 5.—Electrophoretogram of allelic forms of the Wx protein. (a) The alleles included are *Wx*, *wx*, *B3*, *C31*, *R*, *90*, wx^a, *S5* and *S15*. The *wx* allele, which does not specify a Wx protein, is included for comparison. Alleles *Wx*, *wx*, *B3*, *C31* and *90* are in a W22 genetic background; alleles *R* and wx^a are in an M14 genetic background. *S15* is homozygous for *ae* (amylose extender), which is responsible for the difference in the minor protein banding and for the increased amount of the Wx protein. All samples are from 20d endosperms. The gel was stained with Coomassie Blue. (b) Silver-stained electrophoretogram of Wx proteins specified by *Wx* and *m-8*. Both alleles are in the W22 background. The low level of Wx protein in *m-8* requires the highly sensitive silver stain for detection. Six times the volume of sample was loaded for *m-8* as for *Wx*.

for wx^a, which has about 9% of the normal amylose levels (BRIMHALL, SPRAGUE and SASS 1945) and stains a lavender color with IKI.

Among the five *wx* controlling-element alleles mapped by NELSON (1968, 1976) *B3*, the *Mp* mutant, produces about the normal level of Wx protein and, in the *Spm* mutant *m-8*, the protein is greatly reduced (Figure 5). The *m-8* kernels analyzed lacked the *Spm* regulator element that causes transposition. In these kernels, a very low level of amylose is produced by the endosperm (MCCLINTOCK 1961). No Wx protein was seen in *m-1*, *m-6* and *B4*, which are *Ds*-controlled mutants. Along with *B3*, these also lack amylose starch. Interestingly, *S5* and *S15*, both stable waxy revertants that were derived from *m-1* and no longer respond to *Ac* (NELSON, personal communication), resemble wx^a in having low amylose levels, as determined by IKI staining, but seem to have full levels of Wx protein (Figure 5).

Electrophoretic characterization: No differences could be detected between the molecular weights of any of the allelic Wx proteins, as they all migrated to the same position in SDS acrylamide gels (Figure 5). However, differences between the allelic Wx proteins were revealed by isoelectric focusing. The Wx proteins from *B3*, *R*, *C31* and *90* differ in their migrations in isoelectric focusing from the Wx protein produced by the *Wx* allele and from each other (Figure 6). The Wx proteins from wx^a, *S5* and *S15* have the same migrations in isoelectric focusing as does the protein from *Wx*. It has not yet been possible to obtain isoelectric focusing data for *m-8*.

DISCUSSION

The data presented provide evidence that the structural gene for the Wx protein is at the *wx* locus. The protein is present in *Wx* and absent in 25 of 29 *wx*

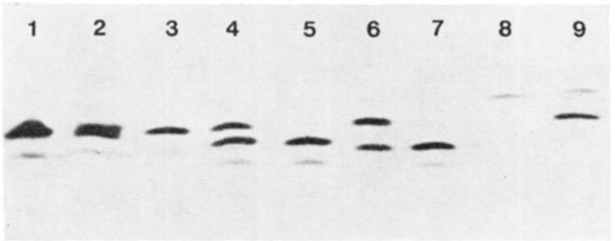

FIGURE 6.—Isoelectric focusing gel of Wx proteins specified by various alleles of the *Wx* gene. The basic end is toward the top of the gel. Lane 1, *Wx*; lane 2, an equal mixture of samples from *Wx* and *B3* (the *B3* Wx protein is slightly more acidic than the *Wx* one); lane 3, *B3*; lane 4, an equal mixture of samples from *Wx* and *R*; lane 5, *R*; lane 6, *Wx/C31* heterozygote; lane 7, *C31*; lane 8, *90*; lane 9, an equal mixture of samples from *Wx* and *90*. The faint band below the Wx protein seems to be a modified form of the protein since it is missing in *wx* and its migration is correlated with that of the major form of the Wx protein in the various alleles.

mutants that do not produce any amylose in their endosperm or pollen. The level of Wx protein progressively increases within increasing dosage of the *Wx* allele, while the other starch-bound proteins remain unchanged. Some *wx* alleles specify Wx proteins that differ in charge from that specified by the *Wx* allele.

The procedures employed for extraction of the Wx protein preclude measurement of enzymatic activity, but the Wx protein is almost certainly the starch-granule-bound NDP sugar-starch glucosyl transferase. Both the transferase activity (TSAI 1974) and the amount of Wx protein increase with increasing dosage of the *Wx* gene. Furthermore, the waxy phenotype is characterized by both a significant reduction of starch-granule-bound transferase activity (NELSON and RINES 1962; NELSON, CHOUREY and CHANG 1978) and by a loss of the Wx protein in the majority of cases. The four cases where no amylose is produced, but the Wx protein is present (*R*, *B3*, *C31* and *90*), are examples of mutants that produce inactive transferases. The residual transferase activity found in waxy starch granules is due to a second bound transferase that has a much lower K_m than that specified by the *Wx* gene (NELSON, CHOUREY and CHANG 1978). This activity may be specified by one of the minor starch-granule-bound proteins that are found in both *Wx* and *wx* endosperm.

Significant reductions of the Wx protein are seen in alleles *90* and wx^a (Figure 5). These reductions cannot be accounted for by differences in genetic background, as *Wx*, *B3*, *C31* and *90* are in the W22 background, but only in *90* is the protein reduced. Both wx^a and *R* are in the M14 background, but *R* does not differ from *Wx*, *B3* and *C31* in the amount of Wx protein present. The reductions observed thus appear to be allele specific. The high level of Wx protein in the *S15;ae* material is probably due to *ae*, since the mutation has been shown to increase the amount of extractable Wx protein (unpublished results).

NELSON used the *wx* alleles to construct a fine-structure map of the locus (NELSON 1968; NELSON 1976), but the map does not delineate the structural and regulatory regions of the gene (Figure 7). As has been pointed out previously, some or even most of these mutants could be in a *cis*-acting regulatory region outside the structural gene. This distinction is of special importance with regard to the

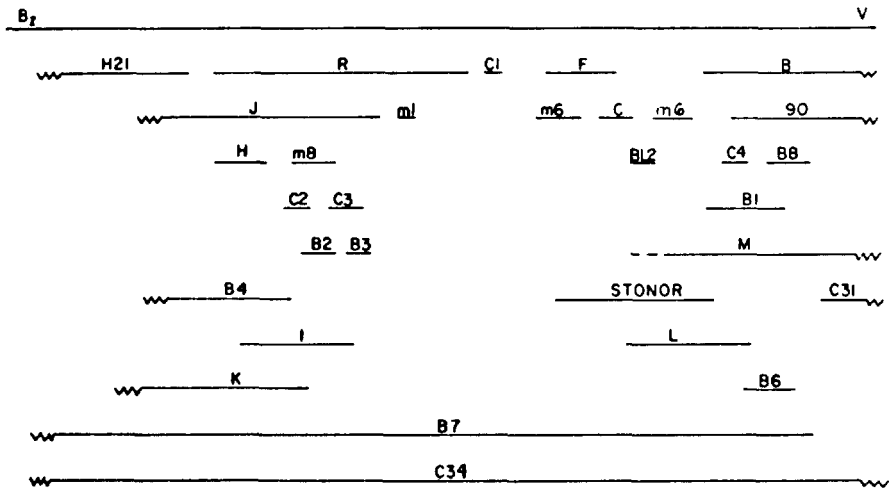

FIGURE 7.—Fine-structure map of *wx* locus, reprinted from NELSON (1976). Overlapping of lines indicates no detectable recombination between the alleles ($< 1.5 \times 10^{-5}$ recombination frequency). *Bz* and *V* are flanking markers on the chromosome.

sites of the controlling-element mutations. Are they in the structural or the external regulatory portions of the locus?

The results presented in this paper establish that the structural gene must extend from the sites of the mutations *90* and *C31* at the proximal end of the locus (relative to the centromere) to *B3* and *R* in the distal half. A mutation is considered to be in the structural gene if it results in the formation of an altered protein. The Wx proteins specified by these four mutants differ from that specified by the *Wx* allele in that all are enzymatically inactive, since no amylose is produced. Also, the Wx proteins from *B3*, *R*, *C31* and *90* differ in charge, as determined by isoelectric focusing. Thus, the mutations must involve the structural gene. Control tests with 10 unrelated inbred lines showed no differences in the isoelectric migration of their Wx proteins, so that it is unlikely that such differences are due to natural polymorphisms.

The controlling elements associated with the *m-1*, *m-6* and *B3* mutations are clearly situated within the limits of the structural gene. *B3* specifies an altered protein, and *m-1* and *m-6* map between *B3* on the left and *C31* and *90* on the right. The situation with regard to *m-8* and *B4*, which are closely linked to but lie to the left of *B3*, is not quite so certain. Neither recombine with the *R* mutation, which makes an altered Wx protein, but the position of *R* is not precisely mapped (Figure 7).

Of special interest, and under intense investigation, is the finding that the Wx protein specified by the *B3* mutant, which has a foreign *Mp* (=*Ac*) controlling element inserted within the limits of the structural gene, is not detectably different in size from the Wx protein specified by the wild-type *Wx* allele. A similar situation has been reported for the *Ds*-suppressed *Adh* locus (OSTERMAN 1979; OSTERMAN and SCHWARTZ 1979).

Reversion by transposition of *Ds* presumably involves excision and removal of the controlling element from the affected locus. However, the excision of *Ds* is not always precise and can leave the gene in a stable, but altered, form such that the protein product of the reverted gene differs from that produced prior to the *Ds* insertion into the locus (DOONER and NELSON 1979). This appears to be the case with the two stable revertants of *m-1*, *S5* and *S15*. They are only partially revertant in that they make reduced amounts of amylose. The fact that they show the full amount of Wx protein is further evidence that the *Ds* element of the *m-1* mutation is located within the structural gene. If the alteration in *m-1* occurred in a regulatory portion of the locus and repressed gene activity so that no Wx protein was produced, the release of repression by transposition would be expected to result in the synthesis of a fully active protein and *Wx* amylose levels.

The mutant phenotypes produced by *B4*, *m-1*, *m-6* and *m-8* are the result of the absence or drastic reduction of the amount of the Wx protein and are not due to the production of an inactive protein, as is the case for *B3*. In the mutants where the Wx protein is absent or reduced, the transcription, translation or RNA processing may be defective so that no or little protein is synthesized. Alternately, a mutant protein may be formed that cannot enter the amyloplast or bind to the starch granule, or does so inefficiently. The latter alternative would require that the unbound protein be unstable and readily degraded since no proteins are observed in whole endosperm SDS extracts of the mutants that are not also seen in similar extracts from *Wx* endosperm.

The NDP sugar-starch glucosyl tranferase is tightly bound to the starch granule, and the question can be raised as to whether the enzyme is bound to the amylose or amylopectin moiety. FRYDMAN and CARDINI (1967) presented evidence for the binding of the enzyme to amylopectin in potato and pea. The results reported in this paper indicate that, in maize, the enzyme specified by the *Wx* gene can also bind to the amylopectin of the starch granule even though this gene appears to be involved only in the synthesis of amylose, and amylopectin production is not affected in mutants that lack the active enzyme. Mutants *B3*, *90*, *C31* and *R* all lack amylose, but there is a strong binding of the Wx protein to the starch granules.

By identifying those *wx* alleles that produce structurally altered proteins it has been possible to set minimal limits to the structural gene on the fine-structure map. With this information, it can be seen that at least three of the *wx* controlling-element mutations, *B3*, *m-1* and *m-6*, certainly lie within the limits of the structural gene. Further biochemical studies with the Wx proteins associated with the *wx* controlling-element mutants should be important in the analysis of the action of eukaryotic transposable elements and gene action in general.

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Note Added in Proof:

The *B3* allele that we received from OLIVER NELSON and reported on in this paper differs from the *B3* allele that NELSON used in his fine-structure mapping of the *wx* gene. The mapped allele was in an M14 background; whereas, the allele we studied was in W22. Subsequent analysis

showed that it behaves as a stable waxy mutant resulting from transposition of the *Mp* element away from the *wx* locus. In this respect it is similar to the *S5* and *S15* stable mutants recovered from *m-1*. In contrast to the *B3* (M14) allele, which produces no Wx protein, possesses activator activity and shows a low frequency of reversion to *Wx* in the endosperm, *B3* (W22) produces the normal amount of an altered, inactive Wx protein and exhibits neither activator activity nor any sign of reversion. A recombinational analysis, performed according to the method of NELSON (1968), reveals no detectable recombination between the two *B3* alleles. These results support the proposition that the *Mp* element of *B3* is located within the limits of the *wx* structural gene. If its site were outside the coding region of the *wx* gene, the Wx protein of *B3* (W22) produced after transposition of the *Mp* element away from the *wx* locus should be enzymatically active and synthesize amylose.

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