THE *WAXY* LOCUS IN MAIZE. II. THE LOCATION OF THE CONTROLLING ELEMENT ALLELES[1]

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THE main barrier in ascertaining whether in higher organisms recombination can occur within the functional unit and subsequently in mapping mutant sites within the gene has been the difficulty of sampling sufficiently large populations to identify the recombinant types. An intensive examination of the *waxy* (*wx*) locus in maize was proposed since the pollen grain could be used as the unit of observation thus readily affording populations of sufficient size to examine the results of events occurring at low frequencies (NELSON 1957). It was subsequently reported that in crosses between certain independently isolated *wx* mutations a characteristic frequency of *Wx* pollen grains could be observed. For one heteroallelic combination, the frequency of *Wx* pollen grains was substantiated in a conventional genetic analysis utilizing somatic tissue (NELSON 1959, 1962). It was concluded that in such favorable situations intragenic recombination could be clearly demonstrated.

Other investigations with the same set of *wx* alleles have confirmed the appearance of *Wx* pollen grains in certain heteroallelic combinations at frequencies far above those attributable to back-mutation (BIANCHI and CONTIN 1963; and BRIGGS and SMITH 1965). At the sugary-1 (*su*) locus in maize, SALAMINI (1967) has observed recombination between different mutants isolated after ethyl methyl sulphonate (EMS) treatment and between various EMS mutants and the standard *su* allele. SALAMINI and BORGHI (1966) have isolated various *glossy-1* (gl_1) alleles from diverse Italian populations that when crossed gave *Gl* gametes at frequencies well above the observed back-mutation frequencies.

In rice, the *glutinous* (*gl*) gene is homologous to the *waxy* gene in maize. LI, WANG, and YEH (1965) have found that in many crosses between different varieties of *glutinous* rice, the frequency of *Gl* pollen grains was far higher than the *Gl* frequency in the parental varieties.

The question of intracistronic recombination in Drosophila has been extensively investigated. The clearest demonstration that it does occur comes from investigations of the *rosy* (*ry*) locus where an ingenious selective system simplified the problem of dealing with large numbers of individuals (CHOVNICK *et al.* 1964). The additional important demonstration has been made that all viable

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ry mutations belong to a single functional unit affecting xanthine dehydrogenase activity (SCHALET, KERNAGHAN, and CHOVNICK 1964). Interallelic recombination has also been shown for alleles at the *miniature* locus (DORN and BURDICK 1962). the *rudimentary wing* locus (GREEN 1963) and the *Notch* locus (WELSHONS 1965).

The purpose of this paper is to summarize the data obtained over the past five years with newly collected *wx* alleles. Of particular interest are those mutations that have arisen by the transposition of a controlling element to the locus. These alleles possess potentially functional *Wx* alleles that are restrained from functioning by the presence of the controlling element. It is of interest to know what recombination studies of such alleles suggest as to the location of such controlling elements at or within the locus. Two mutations, wx^{m-1} and wx^{m-6} of the *Ac-Ds* system and wx^{m-8} of the *Spm* system have been investigated. MCCLINTOCK, who originally detected the existence of such regulatory systems (1951), has recently reviewed the salient features of the *Ac-Ds* and *Spm* systems and their regulation of gene function (1965). For the purposes of discussion, these mutations will be referred to as controlling element mutations.

METHODS AND MUTANT STOCKS

The mutant alleles investigated were received from geneticists and plant breeders in response to a request for *wx* mutations isolated in experimental progenies where contamination by the standard *wx* allele could not explain the occurrence of seeds with the *waxy* phenotype.

The allele designated as wx^C (for Cornell) is the standard *wx* allele received from the Maize Genetics Cooperative when it was located at Cornell University. The allele, previously designated as wx^{Coe} (NELSON 1962), was received from DR. E. H. COE as a tester stock *sh bz wx v* and presumed to have the standard allele. No test here has indicated otherwise, and this stock will henceforth be referred to as wx^C. For the remainder of the paper, the various *wx* alleles will be designated by their superscripts as *C* rather than wx^C or *m-1* rather than wx^{m-1}.

The alleles *H21* and *90* occurred as spontaneous mutations in the inbred lines H21 and 90, respectively. They were isolated by DR. A. M. BRUNSON at Purdue in the 1940's. The allele *R* was also isolated at Purdue by DR. D. L. RICHARDSON.

The alleles *B*, *F*, *H*, *I*, and *J* were furnished by MR. R. P. BEAR of the Bear Hybrid Corn Company, Decatur, Illinois. These were isolated in inbred lines where an accidental outcross by a line that was *wx*/*wx* could have been readily detected.

The alleles *C1*, *C2*, *C3*, *C4*, *C31*, and *C34* were isolated by MR. ALAN CASPER at Blandy Experimental Farm in investigations of the mutagenic effects of γ-rays. The alleles *C1* and *C4* occurred in a control population while the other mutations are presumptive radiation mutations.

The mutations *B1*, *B2*, *B4*, *B6*, *B7*, and *B8* were isolated by DRS. R. A. BRINK and R. B. ASHMAN in an experiment designed to test whether *Mp* as a component of the P^{vv} allele (BRINK and NILAN 1952) increased the frequency of genetic change at selected loci of which *wx* was one, and if it did, to ascertain whether the increased frequency of genetic change was due to transposition of *Mp* from the *P* locus to the affected locus. In the families in which the *wx* mutations arose, the frequency of mutation at the *wx* locus was not higher in plants carrying *Mp* than in the appropriate control population minus *Mp*. Although two unstable *wx* mutations arose in the *Mp* plus plants due to transposition of *Mp* to the *wx* locus, the 6 *wx* mutants listed above were not associated with *Mp* (ASHMAN, personal communication). It should be noted that the *Mp* element associated with P^{vv} is operationally identical to MCCLINTOCK's *Ac* component of the *Ac-Ds* systems (BARCLAY and BRINK 1954).

The two unstable *wx* mutations from BRINK resulting from the transposition of *Mp* to the *wx* locus are autonomously unstable since the regulating element is present at the affected locus. It is not clear whether mutations such as these result from the presence of *Mp* alone at the locus or whether both *Ds* and *Mp (Ac)* elements are present. MCCLINTOCK (1965a) has reported that for 4 originally autonomous mutable loci alleles were obtained that no longer show evidence of the regulator at the locus but respond to the regulator when it is located elsewhere in the chromosome complement. One of the BRINK *wx* mutations is quite autonomously unstable with a high frequency of reversion from *wx* to *Wx* in both somatic and germinal tissue. Therefore, it was not possible to use this mutation in investigations of the type described here. The second autonomously unstable *wx* mutation, designated as *B3*, has such a low rate of germinal reversion that it can be used in such studies, but investigations are incomplete. The results will not be presented in this paper in detail.

The mutable *wx* alleles, *m-1*, *m-6*, and *m-8* were received from DR. BARBARA MCCLINTOCK. Action of the alleles *m-1* and *m-6* is regulated by the *Ac* (Activator) system whereas that of *m-8* is regulated by the *Spm* (Suppressor-mutator) system. In the absence of an active *Ac*, *m-1* and *m-6* behave as stable *wx* alleles. Similarly, in the absence of an active *Spm*, *m-8* behaves as a stable *wx* allele. Should an active *Ac* be introduced into a genome with either *m-1* or *m-6*, reversion to functional alleles of *Wx* will occur at high rates in somatic and germline cells. A similar response to an introduced active *Spm* is given by *m-8*. The response of each allele to its regulator is specific; *m-1* and *m-6* respond to *Ac* but not to *Spm*. Conversely, *m-8* responds to *Spm* but not to *Ac*. This specificity indicates the presence at the locus of each of these alleles of some responding component. One purpose of this study has been to determine not only the location of the component but also the effects of such components on intralocus crossing over. All tests for this purpose were conducted with plants in which no active regulator was present.

In order to minimize the differences in genetic background between the various alleles, all have been crossed and backcrossed several times to the dent inbred M14. Since the various alleles became available over a period of some years, the stocks used have different numbers of backcrosses to M14. When the alleles were made available to me as heteroallelic combinations *(wx^x/wx^C)* or *(wx^x/wx^C)* ⊗ as were the *B1–B8* alleles and the *C1–C4*, *C31* and *C34* alleles, the combinations were outcrossed immediately to M14 *(Wx/Wx)*. A number of isolates were then tested to ascertain which had recombination properties different from *C*. One isolate from each allele was then selected for further backcrossing to M14.

The *m-1*, *m-6*, and *m-8* alleles were also received as heterozygotes with *C*. These stocks were outcrossed both to recover the desired allele and to derive stocks without the pertinent regulatory element which was present in each stock as received from MCCLINTOCK. The desired allele in the isolates was then identified by its ability to respond to the pertinent regulatory element in test crosses.

The unit of observation in these analyses is the pollen grain which allows large populations to be scored. The unique features of this system have been discussed (NELSON 1957). It should suffice to note here that *Wx* pollen grains are stained a deep blue by an I_2 — KI solution while *wx* pollen grains are stained tan to light brown. The phenotype (staining reaction of a pollen grain) is governed by its own genotype at the *wx* locus and not by that of the plant producing it (BRINK and MACGILLIVRAY 1924; and DEMEREC 1924).

The pollen grains are collected before they are shed by fixing segments of the tassel in 70 percent ethanol. A "curing" period of several weeks is desirable since newly collected microspores do not stain as readily with a standard strength stain as do those that have been immersed in the fixative for a longer period. The solution used to stain the pollen is a modification of that suggested by KONZAK (1952). The formulation for the stain is 25 ml H_2O, 250 mg KI and 45 mg I_2. The KI is dissolved in the minimal quantity of water necessary and then the I_2 added before diluting to total volume. One drop of "Tween 80" is added and then 0.5 grams Baker's gelatin. The gelatin is solubilized by heating on a hot plate for 5 minutes. The use of a Bunsen burner or high temperatures on the hot plate tends to decolorize the solution. The stain is prepared each

day for use. At room temperature, it remains liquid for a full day in a flask but will gelatinize in 0.5—1 hr when spread thinly over the surface of a slide.

In certain instances, it may be desirable to vary the strength of the stain by increasing or decreasing the amount of I_2. For example, in working with inbred material where the number of pollen grains per anther is often low, there is more iodine available for absorption per pollen grain, and *wx* pollen grains may tend to stain more deeply than desirable. This can be compensated for by increasing the number of anthers taken or decreasing the iodine content (to 35 mg) or both. If more anthers are taken from vigorous field-grown material in order to increase the population per slide, the iodine content may need to be increased.

In preparing the slide, 24 anthers—3 anthers from each of 8 florets are selected. Greatest differentiation between *Wx* and *wx* is obtained when the anthers are taken from the less mature floret in glumes that are ready to open, but this is not necessary. The anthers are placed in the small stainless steel cup of a Virtis Microhomogenizer, cut apart with scissors, and homogenized for 2 minutes after the addition of 0.75 ml stain. The homogenate is then strained through 2 layers of cheesecloth onto the surface of a 80 × 100 mm slide. One drop of stain is added if necessary, and the microspores distributed evenly over the area that will be covered by a 50 × 75 mm cover slip. After the mixture has set, the edges of the cover slip are coated with colorless nail polish. Such preparations will keep for several days and can be scored and counted at any time in that period. Maximum differentiation between *Wx* and *wx* pollen grains is usually found 24 hours after a slide has been prepared.

Such preparations are best viewed with a combination of transmitted and reflected light as obtained with an AO Cycloptic Binocular with a substage base mirror when illumination is from a lamp inserted in the illumination port behind the objective. An estimate of the total population on the slide is obtained by multiplying the sum of 15 counts through a grid over the surface times a constant. The slide is then scanned and each *Wx* pollen grain marked with a drop of Kodak Opaque.

RESULTS

The location of mutant sites relative to outside markers: A cross between two *wx* heteroalleles can also be subjected to a conventional genetic analysis. In such cases, the pattern of segregation for outside markers in the *Wx* gametes can be used to locate the mutant alleles relative to the markers. Such a test has been reported for the heterozygote *Bz 90 V/bz C v* (NELSON 1962). The majority of the *Wx* gametes were carrying the markers *Bz v* thus providing evidence that the order of the mutant sites is *(Bz)—C—90—(V)*.

In the above test, it was necessary to cut and stain each kernel to detect the seeds from *Wx* gametes. Further, no test could be made to detect possible contaminants although the majority of the *Wx* gametes were carrying one or both of the recessive markers, and the probability that they could occur as the result of contamination was slight. A superior system was used in all subsequent tests. The mutant *amylose extender* (*ae*), (KRAMER, PFAHLER and WHISTLER 1958) has been incorporated in all stocks utilized. The seeds of the double mutant stocks *wx/wx;ae/ae* have defective endosperms resembling those of the *sugary* mutant of maize. Endosperms that are *Wx/wx/wx;ae/ae/ae* are usually distinguishable from those that are *wx/wx/wx;ae/ae/ae* or *Wx/wx/wx; Ae/ae/ae*. If all stocks are double mutant $wx^z;ae$ in conventional analyses of crosses between two different *wx* alleles, the distinctive phenotypes can be used to detect seeds resulting from fertilization by recombinant *Wx;ae* gametes as well as to eliminate most *Wx;Ae* contaminants.

TABLE 1

The assortment of outside markers in Wx *seeds arising from the* F_1 Bz 90 V/bz C v[1] *in 1960 and 1963*

	1960		1963	
	Number	Percent	Number	Percent
Bz v	63	58	18	62
bz v	27	25	9	31
Bz V	15	14	1	3.4
bz V	3	2.7	1	3.4
	108		29	

[1] The F_1 was used as a male onto *bz C v/bz C v* plants.

This system was used in 1963 to repeat the conventional analysis of the cross between *90* and *C*. The F_1 *Bz 90 V/bz C v;ae/ae* was used to pollinate the tester stock *bz C v/bz C v;ae/ae*. Seeds with putative *Wx/wx/wx;ae/ae/ae* endosperms were identified. From these, 36 plants were obtained that were crossed to the tester stock, *bz C v/bz C v;ae/ae*. Of these, 31 were *Wx/wx;ae/ae* as originally classified; 2 were *Wx/wx;Ae/ae* contaminants; and 3 were *wx/wx;ae/ae*. These latter had either been misclassified or arose by heterofertilization.

Of the 29 *Wx* recombinants from pollinations in which the *90/C* heterozygote was the male parent, 18 were *Bz, v*, 9 *bz v*, 1 *Bz V* and, 1 *bz V*. These data are compared to those of 1960 in Table 1. The ratio of *Bz v* gametes to *bz v* is similar in both tests, but the percentage of *Bz V* gametes was lower in the 1963 test where the contaminant kernels (which would be *Bz V*) could be identified. This suggests that some of the apparent *Bz V* recombinants in the 1960 test were contaminants. The conclusion that the order of the mutant sites is (*Bz*)—*C*—*90*—(*V*) is unaffected.

A similar analysis was made in 1964 for the mutant *H21*. Plants that were *Bz H21 V/bz C v;ae/ae* were used as male parents on the *bz C v/bz C v;ae/ae* tester. Tassel collections were also made for estimates of *Wx* frequency by standard pollen scoring techniques. In a total population of 1,571,000 pollen grains from 9 plants, 776 *Wx* pollen grains were detected or a frequency of 49×10^{-5}. This compares with a frequency of 46×10^{-5} *Wx* pollen grains in the original cross testing *H21/C* recombination in the absence of *ae/ae* (NELSON 1959).

Due to poor germination and dry weather, the conventional analysis yielded a total population of only 21,698. Of these, 8 were verified *Wx,ae* recombinants with 5 being *bz v* and 3 *bz V*. This indicates a location for *H21* distal to *C* contrary to the location suggested earlier on the basis of recombination frequencies alone. The order then of the three mutants within the locus is *bz—H21—C—90—v*.

Frequencies of Wx *pollen grains in cross between* wx *alleles of independent origin:* Since the original report of recombination in crosses between some *wx*

TABLE 2

Results of a diallele set of crosses among wx *alleles*[1]

The $\bar{x}$ Wx *frequencies* $\times 10^{-5}$ *and the* $S\bar{x}$ *are presented in the upper right half. The number of plants and the estimated number of gametes sampled* $\times 10^{5}$ *are given in the lower left half. All data from 1964 field except* m-6 × m-8 *which is from the 1964 greenhouse. The* $\bar{x}$ Wx *frequency for any cross is reduced by the factor* $\bar{x}P_1 + \bar{x}P_2/2$ *to compensate for back mutation and suppressor mutation.*

	H21		J		H		I		R		m-8		C2		C3		m-1		C1		F		m-6		C		C4		B	
H21			0.4	0.4	1.5	0.8	2.9	1.1	1.8	1.2	5.8	1.5	9.4	2.4	10	3.2	32	1.9	38	5.9	29	3.6	15	3.0	67	4.0	42	6.0	91	13.0
J	3	1.3			0	0	0	0	0.5	0.4	0	0	0	0	0.1	0.1	8.1	2.1	17	4.3	9.5	5.1	5.3	1.0	35	1.0	23	3.6	33	4.0
H	5	2.7	3	1.4			0.4	0.3	0.3	0.4	13	1.5	15	4.8	21	5.9	30	2.5	51	5.1	33	5.6	21	0.9	64	4.4	34	6.2	50	2.1
I	6	3.6	3	1.5	5	2.0			0.1	0.1	0.4	0.5	0	0	0	0	10	3.2	11	2.5	15	2.8	9.4	3.1	50	6.4	35	2.1	58	3.2
R	5	2.0	3	1.9	5	3.2	3	2.1			0	0	0.4	0.3	0	0	0	0	4.4	1.6	6.8	1.7	4.6	2.7	44	4.7	55	13.0	53	2.2
m-8	3	2.5	3	2.8	3	2.6	3	2.2	3	2.4			0.7	0.2	1.0	0.5	5.7	1.2	19	3.4	15	5.2	26	3.5	26	7.0	69	11.4	27	6.6
C2	3	2.1	3	2.3	4	1.5	3	1.7	3	2.1	3	2.87			3.7	1.8	16	1.5	66	13.2	19	3.2	38	11.9	55	7.8	31	4.3	80	15.2
C3	3	1.2	3	1.4	3	1.5	3	1.7	3	.99	3	2.5	3	1.3			20	2.5	62	9.6	12	4.1	31	3.2	44	9.4	29	5.3	73	5.2
m-1	3	1.3	4	.90	4	1.6	4	1.7	3	.88	3	1.7	3	1.6	3	.96			38	7.9	6.2	2.9	24	4.3	15	2.0	47	4.3	41	1.6
C1	5	2.0	3	1.1	3	1.4	3	1.8	5	1.5	6	5.4	5	2.2	5	2.0	4	1.8			7.5	0.9	40	5.6	12	2.7	37	5.4	26	9.2
F	3	1.6	4	1.6	3	1.4	4	2.4	5	2.9	3	2.3	3	1.8	5	2.0	3	.90	3	.89			0	0	0	0	11	2.2	26	6.0
m-6	3	1.7	3	2.1	3	2.1	4	2.5	6	1.5	2	.92	3	1.4	3	2.0	4	1.8	4	3.1	3	1.3			4.4	0.3	14	3.7	12	3.7
C	6	2.6	3	1.6	3	1.4	3	1.8	3	1.3	3	2.7	5	3.0	5	.94	3	1.6	3	1.1	3	1.3	3	1.7			57	3.9	45	10.6
C4	3	.98	3	.92	3	1.5	3	2.0	3	.94	3	2.1	5	1.7	3	2.4	3	1.9	4	2.3	3	1.6	3	2.0	4	3.2			1.2	1.0
B	5	2.7	4	1.1	3	1.9	3	1.8	3	1.5	5	4.0	3	1.5	3	1.0	3	2.0	3	.76	3	2.1	4	1.6	5	2.4	5	1.3		

[1] The number of backcrosses for each allele to M14 is given in Table 3.

alleles (NELSON 1959) 24 *wx* alleles of independent origin have been investigated to various extents. The most complete test comprised all possible crosses between the *wx* alleles *C*, *B*, *H21*, *C1*, *C2*, *C3*, *C4*, *R*, *I*, *H*, *J*, *F*, *m-1*, *m-6*, and *m-8*. This series of crosses was intended to locate as precisely as possible the component (controlling element) responsible for the origin and behavior of *m-1*, *m-6*, and *m-8*. The *m-1* and *m-8* alleles had been found not to recombine with *R*, and *m-6* × *R* gave only a low rate of recombination. Accordingly a group of alleles known not to recombine with *R* constitute the majority of the group. The data for these crosses are given in Table 2. The estimate of frequency of *Wx* pollen grains for any cross has been reduced by the mean frequency of the two parental lines as a correction for gametes expected to arise through back mutation or suppressor mutation. The data for the parental lines are given in Table 3. The rates of reversion for the various *wx* alleles range from 0 to 2.7×10^{-5}. BIANCHI and TOMASSINI (1965) have reported higher reversion rates for *C* in detailed investigations of the *Wx* frequency in different side branches of the tassel. In our investigations where the main spike is sampled, only relatively low rates of reversion are observed. The regulatory element alleles are as stable as the other alleles and possibly more so.

The data from the conventional analyses involving *90* × *C* and *H21* × *C* are the basis for the conclusion that *H21* is distal to *C* and *90* proximal to *C*. It has been shown previously that *B* and *90* do not recombine, but *B* and *C* do (NELSON 1959). The location of *B* is, therefore, proximal to *C*. The *R* allele shows a very low rate of recombination with *H21* (Table 2). Neither allele will recombine

TABLE 3

The Wx *frequencies of homoallelic stocks*[1]
1964 field-grown plants unless otherwise specified[2]

Allele	Number of plants	Estimated number of gametes	$\bar{x}$ Wx × 10^{-5}	$S\bar{x}$
C(M14[6]) ('66 GH)	4	242,360	0.5	0.5
B(M14[5])	2	176,100	0.7	0.7
H21 (M14[6])	3	254,000	0.3	0.3
R(M14[4]) ('64F + '66GH)	4	224,500	0.6	0.4
C1(M14[3])	4	186,500	0.7	0.4
C2(M14[3])	3	314,100	1.0	0.6
C3(M14[3])	3	149,600	2.7	1.7
C4(M14[3])	5	215,700	1.5	0.8
I(M14[3])	4	427,500	1.3	0.5
H(M14[5])	3	372,100	1.5	0.5
J(M14[5])	5	103,400	0.7	0.4
F(M14[5])	4	216,000	2.3	1.3
m-1(M14[4]) ('66GH + '66F)	8	522,800	0.1	0.1
m-6(M14[2]) ('66F)	13	971,100	0	0
m-8(M14[1]) ('66GH + '66F)	9	529,300	0.1	0.03

[1] The superscript for M14 indicates the number of backcrosses to that inbred.
[2] GH indicates greenhouse-grown plants and F field-grown plants.

with *J*. All three alleles, *H21*, *J*, and *R*, have relatively high rates of recombination with *C*. Thus the three alleles define a continuous segment of genetic material distal to *C*.

The majority of alleles in this test were chosen because in previous tests in heterozygous combination with *R* they had not given a *Wx* frequency above that anticipated by back-mutation. These alleles non-recombinant with *R* may or may not give *Wx* recombinants in crosses with each other depending on the cross. All but one recombine with all alleles with which *R* gives recombinants. The exception is *J* which does not give recombinants with either *R* or *H21*, two alleles that recombine with a low frequency. The *R* allele includes an extended segment of genetic material covering a number of alleles that are capable of recombining with each other.

Still another segment of the genetic material is defined by the *F* allele which does not recombine with *C* and *m-6*. The latter two alleles do give a low level of recombination. A third segment is defined by the *B* allele as shown by the data presented in Tables 2 and 4.

Figure 1 depicts the order of the alleles within the *wx* locus. The map is drawn as a complementation map to emphasize the fact that many alleles appear as

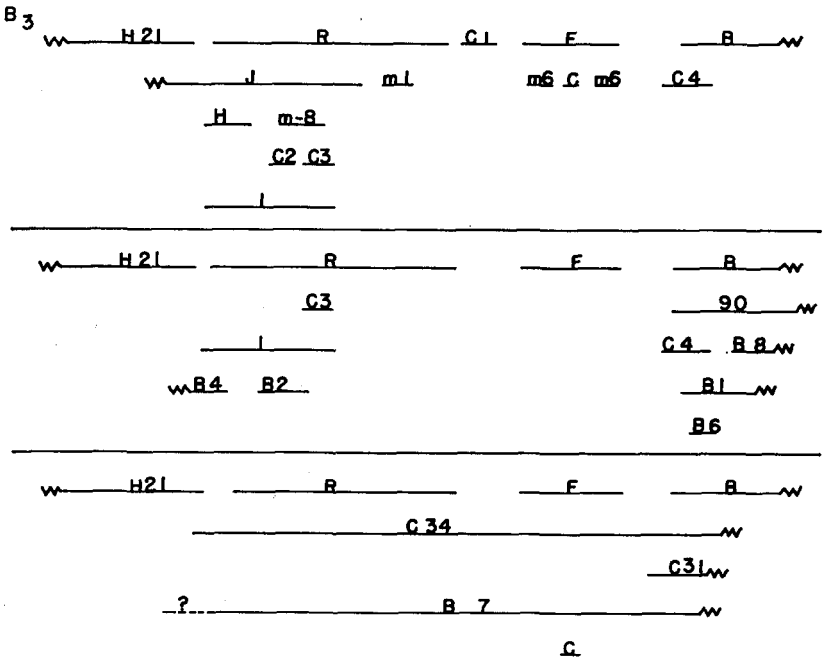

FIGURE 1.—The location of the *wx* alleles within the locus. The three horizontal divisions of the figure depict the location of alleles that have been tested in crosses among themselves. If the lines representing two mutants overlap, the mutants do not recombine. If the lines do not overlap, the mutants do recombine. The serration terminating the lines representing some mutants indicates that there is no proximal (or distal) mutant with which the mutant recombines. Thus there is no means of delimiting the mutant on that side.

TABLE 4

The Wx *frequency in various crosses of* wx *heteroalleles from the listed years*[1]

Cross	Year	$Wx \times 10^{-5}$ (Individual plants)	$\tilde{x}$ $Wx \times 10^{-5}$
F(M14[5]) × C31	'67F	31, 63, 42	45
F(M14[5]) × C34	'67F	0, 0, 0, 0	0
m-6 (M14[3]) (Resp.) × m6 (M14[2]) (Non-Resp.)	'67F	0, 0, 0, 0	0
C31 × H21(M14[6])	'67F	25, 45, 29, 29	32
C31 × R(M14[4])	'67F	12, 26, 20, 16	19
C31 × B(M14[5])	'67F	0, 0, 0, 0	0
C34 × H21(M14[6])	'67F	0, 0, 0, 0	0
C34 × R(M14[5])	'67F	0, 2, 0, 0	0.5
C34 × B(M14[5])	'67F	0, 0, 0, 1.7	0.4
B7(M14[1]) × R(M14[4])	'67F	1.3, 0, 0, 0	0.3
B7(M14[1]) × F(M14[5])	'67F	0, 0, 0, 0	0
B7(M14[1]) × m-6(M14[2]) (NR)	'67F	0, 0, 0, 0	0
B7(M14[1]) × B(M14[5])	'67F	0, 0, 0, 0	0
C34 × H21(M14[6])	'67GH	2.3, 0, 2.1	1.5
C31 × R(M14[4])	'67GH	29, 13, 23, 19	20
F(M14[5]) × C31	'67GH	28, 37, 30	32
C31 × B(M14[5])	'67GH	0, 0, 0, 1	0.5
C31 × H21(M14[6])	'67GH	29, 38, 49, 58	40
C34 × R(M14[4])	'67GH	0, 0, 0, 0	0
F(M14[5]) × C34	'67GH	0, 0, 1.9	0.6
C34 × B(M14[5])	'67GH	0, 0, 0	0
C(M14[6]) × C31	'66F	25, 21, 36, 31	28
C(M14[6]) × C34	'66F	0, 0, 0, 0	0
m-1(M14[3]) × m-6(M14[2]) (NR)	'64GH	23, 28	26
90(M14[5]) × B1(M14[3])	'64GH	0, 0, 0, 0	0
90(M14[5]) × B6(M14[3])	'64GH	0, 0	0
B1(M14[3]) × B6(M14[3])	'64GH	0, 0	0
B1(M14[3]) × B8(M14[1])	'64GH	2.7, 0	1.4
B6(M14[3]) × B8(M14[1])	'64GH	1.9, 4.3, 3	3.1
B6(M14[3]) × C4(M14[2])	'64GH	0, 0	0
B8(M14[1]) × C4(M14[2])	'64GH	3, 10, 2.4, 12	6.8
C4(M14[2]) × B1(M14[3])	'64GH	0, 0	0
I(M14[1]) × B2(M14[3])	'64GH	0, 2.1	1.1
I(M14[1]) × B4(M14[3])	'64GH	2	2
B2(M14[3]) × B4(M14[3])	'64GH	13, 22	18
B2(M14[3]) × H21(M14[6])	'64GH	2.2, 2.2	2.2
B4(M14[1]) × H21(M14[6])	'64GH	0, 0	0
C3(M14[1]) × B2(M14[3])	'64GH	0, 2.1	1.1
B1 × B2	'61GH	70, 65	68
B1 × B4	'61GH	122, 95	109
B1 × C	'61GH	25, 18	22
B1 × R	'61GH	32, 37	35
B2 × B4	'61GH	82, 41	62
B2 × B4	'61GH	20, 22	21

TABLE 4 (Continued)

Cross	Year	$Wx \times 10^{-5}$ (Individual plants)	$\bar{x}$ $Wx \times 10^{-5}$
B2 × C	'61GH	30, 36	33
B2 × R	'61GH	0, 1.5	0.8
B4 × B6	'61GH	80, 28	54
B4 × B8	'61GH	83, 141	112
B4 × C	'61GH	29, 70	50
B4 × R	'61GH	3, 0	1.5
B6 × B1	'61GH	7, 5	6
B6 × B8	'61GH	0	0
B6 × C	'61GH	65, 41	53
H21 × B4	'61GH	1.8	1.8
H21 × B6	'61GH	71, 35	53
B8 × B2	'61GH	97, 152	125
B8 × C	'61GH	36, 58	47
a × B1	'61GH	2.4, 0	1.2
a × B2	'61GH	59, 26	43
a × B4	'61GH	50	50
a × B6	'61GH	2.8, 0	1.4
90 × B1	'61GH	0, 0	0
90 × B2	'61GH	59, 58	59
90 × B4	'61GH	43, 45	39
90 × B6	'61GH	3, 0	1.5
90 × B8	'61GH	12, 4	8
H21 × B1	'61GH	34, 34	34
H21 × B2	'61GH	2.2, 13	7.6
H21 × B8	'61GH	31, 40	36
B6 × R	'61GH	68, 53	61
B8 × R	'61GH	45, 37	41
H21 × R	'61GH	5(1+), 0	2.5
B × B1	'61GH	2.1, 1.3	1.7
B × B2	'61GH	29	29
B × B4	'61GH	28, 62	45
B × B6	'61GH	0, 2	1
B × B8	'61GH	1, 5.4	3.2

[1] GH indicates greenhouse-grown plants and F field-grown plants.

other than a point on the genetic map. The segment defined by *R* is placed between *H21* and *C* since the recombination frequency of *C* × *H21* is greater than that of *C* × *R* or those of *C* times most alleles non-recombinant with *R*. Except for *m-1*, the alleles covered by *R* have been ordered using the overlapping deletion method of BENZER (1959). The arrangement of the data in Table 2 shows that for the alleles covered by *R*, there is an order such that all the non-recombinant crosses of an allele can be arrayed in a set uninterrupted by a cross in which recombination takes place. This is possible since most of the alleles do appear to occupy a segment of greater or lesser size. The allele *m-1* must then be located closer to *C* than the other alleles covered by *R*. The allele *m-6* is tentatively depicted on both sides of *C* since no decision can be made as to whether this allele

is proximal or distal to *C*. A comparison between the data in Table 2 and the position of the alleles in Figure 1 shows that recombination frequencies are not additive across the locus, and no rearrangement of alleles makes for substantially better additivity considering the group of alleles together. The primary criteria in deriving the proposed map have been the data from the conventional genetic analyses demonstrating that *H21* is located distal to *C* and *90* proximal to *C* and whether or not recombination could be detected in the F_1 between any two alleles. The assumption has been made that any cross with a *Wx* frequency less than 1×10^{-5} after correction for parental *Wx* frequencies does not show recombination between the *wx* alleles.

Other *wx* alleles of independent origin have been analyzed genetically. The crosses were made and the F_1 progenies grown in various years. The results are presented in Table 4. The investigations yielding the data in Table 4 have been designed primarily to locate approximately the alleles within the locus, and the number of plants scored per cross have been fewer than for the crosses reported in Table 2. The reversion frequencies of the parents were not estimated so that the *Wx* frequency of an F_1 cross is not corrected on this basis. This is particularly important where the cross between two alleles gives a low frequency of *Wx* pollen grains ($2–3 \times 10^{-5}$). In such an instance, it is not possible to decide whether two alleles can be considered to recombine at a low frequency or not. Any attempt to map these alleles definitively is premature pending an all-combination series of crosses between those alleles that give zero or low recombination with an allele such as *B* or *R* defining a segment of the locus, but the general location of the alleles reported in Table 4 is indicated in Figure 1 in the two lower sections of the figure.

Several of the crosses have been made more than once, and the crosses grown at different times. In general, the results agree for a given cross, but several exceptions should be noted. The crosses of *B2* × *B4* and *B2* × *H21* give values that are lower in the 1964 GH than in the 1961 GH. These discrepancies could be due to the difference in genetic background (the 1964 stocks had been backcrossed 3 times to the inbred M14 while the 1961 stocks had been crossed only once), but this seems unlikely. It is possible that errors in pollinating or labelling led to some cross other than the presumed cross being sampled in either 1961 or 1964.

With these reservations, it is possible to place the majority of these alleles approximately. The *B7* and *C34* alleles are unique in that they do not recombine with *R*, *F*, or *B* and hence cover larger segments of the locus than any other alleles. It is not clear whether *C34* recombines with *H21* at a low rate or not (see '67 GH and '67 F results). The alleles *B1*, *B6*, *B8*, and *C31* either do not recombine with *B* (*C4*, *90*) or do so at a very low frequency. In crosses among themselves, they either do not recombine or do so at a low rate depending on the cross. They do recombine with all other alleles investigated. The alleles *B2* and *B4* do not recombine with *R* or recombine at a very low frequency. They recombine with each other at low frequencies and will recombine with all alleles that are recombinant with *R* with the exception of *B4* × *H21*. These latter alleles must be

investigated by a set of all-combination crosses with the other alleles that do not recombine with *R* (*I, H, J, C2, C3, m-8,* and *m-1*). Where such crosses have been made and scored, the *Wx* frequencies have ranged from zero to rather low depending on the cross.

Only one allele, *C1*, recombines with all alleles with which it has been tested. It obviously, therefore, is not located in one of the segments defined by *R, F,* or *B*. The most likely location for *C1* judging by the frequency of *Wx* pollen grains in crosses with other alleles is between *R* and *F*.

It has been noted that all three alleles *m-1, m-6,* and *m-8* used in the all-combination set of crosses were responsive to the pertinent regulatory element. In the process of recovering the responsive *m-6* allele, a non-responsive derivative of *m-6* was also recovered from the same *Wx/m-6;Ac* plant. The non-responsive (NR) *m-6* allele behaves as a perfectly stable *wx* allele no longer responding to the presence of *Ac* in the genome by somatic and germinal reversions to functionality. In Table 4, the data are given for several crosses involving the non-responsive *m-6*. In the 1964 greenhouse, two plants of the cross *m-6 NR* (*M14*^{2}) $\times$ *C*(*M14*^{6}) gave *Wx* frequencies of 4 and 8 $\times$ 10^{-5}. The cross *m-6 R* (*M14*^{2}) $\times$ *C*(*M14*^{6}) in the 1965 field gave 4, 5, and 5 *Wx* $\times$ 10^{-5} for the 3 plants sampled. The cross between *m-6 NR* (*M14*^{2}) and *m-6 R* (*M14*^{2}) gave 0, 0, 0 *Wx* $\times$ 10^{-5} for the plants sampled. These data suggest that the modification of *m-6* which altered its ability to produce *Wx* alleles in the presence of *Ac*, occurred at the *m-6* specific site in the locus.

The effect of genetic background and environment on intralocus recombination: Previous evidence has indicated that the recombination value characteristic of a cross between two *wx* heteroalleles may be influenced by genetic background (NELSON 1962). The same result has been found in a test designated to investigate this specific point. Four of the original set of *wx* alleles, (*C, H21, 90,* and *B*) were placed in a similar genetic background by 5 backcrosses to the dent inbred, M14. The recovered *wx* alleles were then crossed in all possible combinations.

TABLE 5

The $\bar{x}$ Wx *frequencies*[1] *in heteroallleic crosses where the* wx *alleles were in their original background or recovered in an M14 background*

	1958 Field		1964 Greenhouse				1965 Field	
	Original		Original		Recovered		Recovered	
Crosses	$Wx \times 10^{-5}$	$S\bar{x}$	$Wx \times 10^{-5}$	$S\bar{x}$	$Wx \times 10^{-5}$	$S\bar{x}$	$Wx \times 10^{-5}$	$S\bar{x}$
H21 $\times$ B	28	2.2	48	4.0	84	4.5	91	13.0
C $\times$ H21	46	2.7	75	2.6	92	11.5	67	4.0
C $\times$ B	30	2.9	29	3.4	70	4.9	47	10.6
H21 $\times$ 90	32	2.7	80	5.0	80	6.1	..	..
90 $\times$ B	1.4	0.6	0.7	0.3	0.5	0.5	..	..
90 $\times$ C	88	5.7	131	8.2	129	4.6	..	..

[1] Not corrected for *Wx* frequencies of parental stocks.

The alleles in their original backgrounds were also again crossed in all combinations. Both sets of crosses were grown in the greenhouse in the winter of 1964–65. The resulting data are given in Table 5 together with the data from the original set of crosses grown in the field in 1958. The frequency of *Wx* recombinants for a given cross is apparently influenced both by the environment (1958 field data *vs* 1964 greenhouse data for the original crosses and 1964 greenhouse *vs* 1965 field data for recovered alleles) and by the genetic background (1964 greenhouse data, original alleles *vs* the recovered alleles). On the whole, the crosses between heteroalleles in a similar genetic background tend to give higher frequencies of *Wx* gametes than crosses between heteroalleles in dissimilar backgrounds. This is not true, however, of the cross *90* × *B*, where no recombination is found in either background if cognizance is taken of the reversion frequencies of the parents.

There is a noticeable tendency for frequencies of *Wx* gametes in a given cross to be higher in the greenhouse than in the field as shown in the two pertinent sets of comparisons.

The frequency of *Wx* pollen grains in the combination *C* × *m-1* has been checked at several steps during the incorporation of *m-1* into the M14 background. The results are given in Table 6. It is apparent that the three isolates of *m-1* and the subsequent conversions of *m-1* to the M14 background give similar frequencies of *Wx* pollen grains when crossed by *C*.

Tests for complementation: The substitution of a *wx* allele for *Wx* results in the formation of starch lacking amylose (the straight chain component) in the endosperm and gametophytic tissue. Complementation, whether inter- or intracistronic, should result in the synthesis of some amylose in the endosperms of seeds heterozygous for two complementing alleles. All crosses between *wx* alleles have been phenotypically *waxy*. Our experience with intermediate *wx* alleles has shown, however, that endosperms with 6–7% amylose are still phenotypically *waxy* visually. The intercrosses between the first group of *wx* alleles analyzed were assayed for amylose content with negative results (NELSON 1959). The F_1 seeds of the all-combination set of crosses reported in Table 2 have been checked for possible complementation in the following manner. The endosperm

TABLE 6

The Wx *frequencies in different crosses of the alleles* C *and* m-1 *during conversion to a common background*

Cross	Year	Indiv. plant values[1]	$\bar{x}$
C × *m-1*	'62GH	16, 25, 19[2]	20
m-1(M14³) × *C(M14⁶)*	'64GH	15, 19	17
m-1(M14⁴) × *C(M14⁶)*	'65F	16, 19, 12	16

[1] $Wx \times 10^{-5}$.

[2] These values are for different isolates of *m-1* from plants that were *C/m-1*; *Ac* as obtained from MCCLINTOCK.

tissue of a few seeds from each cross was stained with a standard strength KI—I_2 solution. If the stained endosperms appeared more blue than those of the *C* allele, the starch was isolated and the amylose content assayed by the method of ULMANN and AUGUSTAT (1958). In all crosses, the assays were negative: i.e., no amylose was present. Therefore, there is no complementation among the alleles tested.

DISCUSSION

The 24 *wx* alleles investigated can be ordered within the locus as shown in Figure 1. The initial consideration in placing the mutational sites within the locus is the data from conventional genetic analyses of the F_1's *bz C v/Bz H21 V;ae/ae* and *bz C v/Bz 90 V; ae/ae*. The assortment of outside markers in *Wx* recombinants from these crosses indicate that the mutational site in *H21* is distal to *C* and the mutational site in *90* is proximal to *C*. Therefore, other *wx* alleles that recombine with *C* but not with *H21* (*J*, for example) or that recombine with *C* but not with *J*(*H, I, R, m-8, C2* and *C3*) are distal to *C*. A location proximal to *C* is similarly established for *B* and *C4* which do not recombine with *90*.

For the alleles, *J, H, I, m-8, C2, C3* and *m-1*, which do not recombine with *R*, a linear order is established using the overlapping method of BENZER (1959). This is possible since a number of the alleles appear to occupy a segment of the locus. Such mutations do not recombine with two other mutations which are themselves capable of recombining. The alleles *B7, C34, R, F*, and *B* are the most obvious examples, but *m-8, I, J, 90*, and *B1* fall into the same category.

The physical basis for the apparent size of these mutations could be different in different instances. Deficiencies and inversions of varying sizes could account for mutations that appear to cover a segment of the locus. Most of the alleles revert at low rates which would tend to rule out deficiencies unless mutation at another locus to produce a gene epistatic to *wx* is invoked. No such locus has yet been identified.

In any cross between two *wx* alleles, the production of *Wx* pollen grains at a frequency above the mean frequency of the parent alleles is interpreted as an indication that the mutant sites in the two alleles are spatially separated so that recombination between the sites occurs. One of the products of recombination between the mutant sites reconstitutes the normal organization of the locus. In investigations with the *wx* alleles, the frequency of *Wx* pollen grains appears to reflect the distance between the mutant sites only approximately. Therefore, the frequency of *Wx* pollen grains in a cross between two alleles has been given less weight in map construction than the crosses in which two alleles do not recombine. The recombination frequencies across the proposed map of the locus are not additive. No other arrangement than that proposed gives greater additivity considering the entire group of alleles. Apart from the lack of additivity, the data have been internally consistent in that an allele located in a segment defined by *B* or *F* or *R* combines with all alleles located outside of that particular segment.

The exception is *J* which is non-recombinant with both *R* and *H21*. An allele may or may not recombine with another allele located in the same segment.

The non-additivity of recombination frequencies could be caused by one of several factors or a combination of the factors. Except for the mutants *B1* through *B8* (which occurred in a W23 background) and the mutants *C1–C4* (which occurred in another inbred stock), all other mutants occurred in different backgrounds. The *Wx* alleles in these different lines were all capable of supporting amylose synthesis. This does not necessarily imply that all the *Wx* alleles were exactly the same structurally. A locus could conceivably contain a small duplication or deletion that did not lead to a shift in the reading frame and still be functional. Attempts to place all *wx* alleles in a similar genetic background by backcrossing to M14 would not correct such structural differences.

The controlling element mutations have been of particular interest throughout the investigation. These mutations are as stable as any *wx* mutation in the absence of their regulatory element. The data in Table 2 show that recombination does occur between all the controlling element alleles tested and between such alleles and most stable alleles. If the locus is a single cistron as seems indicated, the controlling elements are not located at either extremity of the locus as might be expected by analogy with the operator element of an operon. Recent investigations by IPPEN *et al.* (1968) indicate that the *lac* operator (O) in *E. coli* is not located at an extremity of the operon. There is a promoter (*P*) site at which transcription is initiated mapping externally to the operator. It is possible, however, that all the controlling elements are located in a contiguous segment of genetic material. If *m-6* is located distal to *C* as recombination frequencies suggest, then the elements are located in a relatively limited segment of the locus. Preliminary data suggest also that *B3* (*Wx* plus *Mp* (*Ac*) present at the locus) is located in the same segment. *B3* is covered by *R* and does not recombine with *m-8*. It is not possible to test for recombination with *m-1* and *m-6* since *B3* carries the regulatory element (*Ac*) for these mutants, and in the F_1, considerable germinal reversion is noted. These four controlling element mutations constitute a small sample, and the clustering noted for these alleles may be fortuitous.

MCCLINTOCK (1961) has discussed the considerable similarities between the controlling element systems in maize and the control of transcription of genetic information in bacteria by the operator and regulatory elements of an operon. Probably no decision as to whether the similarities are basic can be made until we can examine the product (the protein) produced in the uninhibited state as compared to the product or the lack thereof in the controlling element mutants. For such an investigation, the *wx* locus offers considerable difficulties. The substitution of a *wx* allele for *Wx* results in the loss of the starch granule-bound (ADPG-starch, UDPG-starch) glucosyl transferase (NELSON and RINES 1962; and NELSON and TSAI 1964). The amount of the starch granule-bound glucosyl transferase activity is linearly proportional to the number of *Wx* alleles in the endosperm for both a diploid series (AKATSUKA and NELSON 1965) and a tetraploid series (TSAI, unpublished data). These data suggest that the *wx* locus is

either the structural gene for the starch granule-bound glucosyl transferase or acts to specify the number of sites at which the enzyme is bound. It has not been possible thus far to release the enzyme from the starch granules in order to investigate its properties as a soluble enzyme or its structure.

The controlling elements in maize have also been compared with bacterial episomes (JACOB 1960 and PETERSON 1967). Since the controlling and regulatory elements can be transposed within the genome occupying a variety of different positions, the analogy with a bacterial episome such as *F* that can occupy any of a number of positions on the chromosome of *E. coli* when integrated (JACOB and WOLLMAN 1957) is striking. However, bacterial episomes can all exist in an autonomous or non-integrated state (see DRISKELL-ZAMENHOF 1964 for a review of bacterial episomes). To date, no condition identifiable as the autonomous state of a regulatory element in maize has been detected, and it might, indeed, be difficult to detect. Bacterial episomes frequently confer new properties on the host cell by virtue of associated genetic material. The controlling elements appear only to regulate the function of the locus to which they are attached. The analogies between the controlling elements and bacterial episomes break down under detailed examination. It is likely that the controlling elements in maize represent elements normally concerned with the regulation of gene function. These are seen in the *Ac-Ds* and *Spm* systems in an abnormal context. In this context, they are simply suppressing gene action having lost the subtleties of regulation that characterize the elements as normally integrated into the genome. It is quite possible that these elements have no exact parallels in the simpler organization of the bacterial chromosome.

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SUMMARY

A revised map of the *wx* locus locating 24 alleles approximately is based on pollen and conventional genetical analyses. Three controlling element alleles, *m-1* and *m-6* of the *Ac-Ds* system and *m-8* of the *Spm* system recombine with each other and with most other *wx* alleles. There are stable *wx* alleles located both distally and proximally to the controlling element alleles.—Recombination frequencies across the locus are not additive, but map construction is possible using overlapping mutations plus the data from conventional analyses.—Many alleles cannot be represented as points on the genetic maps since they do not recombine with two or more alleles that recombine. Two alleles, *B7* and *C34* do not recombine with any of the other *wx* alleles with which they have been tested.

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