ANALYSIS OF A CONTROLLING-ELEMENT MUTATION AT THE *Adh* LOCUS OF MAIZE

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ABSTRACT

A *Ds*-suppressed *Adh* mutant was isolated by the allyl alcohol pollen selection technique. The mutant produces a reduced level of an altered thermolabile enzyme suggesting that the *Ds* element is inserted in the *Adh* structural gene. The mutant protein is enzymatically active and does not differ detectably in size from the progenitor protein. A number of possible explanations for the data are presented.

THE controlling elements of maize are transposable genetic units (McCLINTOCK 1951, 1956, 1965). They are detected by their effect on gene activity or chromosome organization. Insertion of a controlling element at a gene locus can block gene activity, thus giving rise to the recessive phenotype. Subsequent transpositions of the element away from the locus can restore gene activity.

Most of the available information on the function and behavior of controlling elements has been deduced from genetic analyses of phenotypic expression. In some cases, gene expression was monitored at the level of protein synthesis and enzymatic activity (SCHWARTZ 1960; HANNAH and NELSON 1976; DOONER and NELSON 1977). Little is known about the structure and behavior of the controlling elements at the molecular level. Does the element have to be inserted within the limits of the structural gene to suppress gene action? When the element is located in the structural gene, is it transcribed and translated? In an attempt to answer such qustions, we have selected for controlling-element mutations at the *Adh* locus, which specifies alcohol dehydrogenase, an enzyme readily amenable to biochemical analyses and characterization (SCHWARTZ 1971, 1973). This paper deals with the analysis of one such *Adh* mutation. It is stable in the absence of *Ac*, but produces a much reduced amount of enzyme. Reversion to the active form occurs only in the presence of *Ac*. The results reported in this paper suggest that a *Ds* element is inserted within the *Adh* structural gene. Although the enzyme specified by the mutant is not detectably different in size or charge, it does exhibit an altered thermostability.

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MATERIALS AND METHODS

The allyl alcohol pollen selection technique (SCHWARTZ and OSTERMAN 1976) was used to recover a *Ds*-suppressed *Adh* gene. Plants homozygous for *bz2-m* and *Adh-F* were used as the pollen source. *bz2-m*, located on chromosome *1* about 20 map units proximal to *Adh*, is a *Ds*-suppressed *Bz2* gene (NUFFER 1955). These plants also carried *Ac*, as they were grown from kernels that exhibited reversions of *bz2-m* in the endosperm in the form of purple sectors on a bronze background. The allyl-alcohol-treated pollen was applied to silks of *Adh-S/S* homozygotes. Progeny kernels were grown, and the plants were self-pollinated and out-crossed to various testers.

Samples were prepared for electrophoresis by soaking kernels overnight and excising a portion of or the entire scutellum. The scutella were squashed on Whatman #3 filter paper inserts wetted with 5 mM sodium phosphate buffer, pH 7.5. Starch gel electrophoresis was carried out at 4°, and the gels were stained for ADH activity as previously described (SCHWARTZ and ENDO 1966).

Two-dimensional native-SDS polyacrylamide gel electrophoresis was done, following the procedure of FERL, DLOUHY and SCHWARTZ (1979). For sample preparation, kernels were soaked overnight and scutella homogenized in sample buffer (62.5 mM Tris, *pH* 6.8, 10% glycerol and 5% β-mercaptoethanol; 1 ml/scutellum) and centrifuged at 45,000 $\times$ g for 15 min. Forty μl of the supernatant was loaded per channel on an 8.75% native (non-SDS) polyacrylamide slab gel. Electrophoresis was carried out at 25 ma constant current at 4°. At the completion of the run, individual channels were cut from the gel and boiled for 2 min in sample buffer containing 1% SDS. The channels were horizontally applied to SDS 10% polyacrylamide slab gels for the second-dimension run. Electrophoresis was performed at 20 ma constant current at room temperature. The electrophoretograms were stained for proteins with 1% Coomassie Brilliant Blue in 50% methanol and 10% acetic acid. Gels were destained by immersion in a solution of 10% acetic acid and 5% methanol with repeated changes.

Spectrophotometric assays were performed by the method of EFRON and SCHWARTZ (1968). The rate of reduction of NAD+, monitored by the increased absorbancy at 340 nm, was used as a measure of ADH activity. A change of 0.001 in absorbancy is equal to 1 unit of enzyme activity. Protein determination was made following the method of BRADFORD (1976). Crude enzyme extracts for assay were prepared from dry kernels by grinding in a Wiley Mill with a #20 mesh screen. The meal was incubated with a 5 mM sodium phosphate buffer, pH 7.5, in a ratio of 1 gram of meal to 1 ml of buffer. The slurry was incubated for 15 min at room temperature, centrifuged at 45,000 $\times$ g for 15 min, and the pellet was discarded.

RESULTS AND DISCUSSION

The *Ds* mutant *Adh* allele (*Adh-Fm335*), selected by the allyl alcohol pollen treatment, shows strikingly reduced enzyme activity. Starch gel electrophoretograms of homozygotes show only a trace of activity at the FF homodimer position, as illustrated in Figure 1. In heterozygotes with active *Adh* electrophoretic variants, the active homodimer and heterodimer isozyme bands are distinctly visible in the zymograms, with little or no Fm·Fm homodimer band apparent. As is seen in Figure 1, the active homodimer band is considerably more intense than the heterodimer band.

By spectrophotometric analysis, ADH activity in the *Adh-Fm335* homozygote is reduced about seven-fold as compared to siblings homozygous for *Adh-W*, a standard active allele. In crude extracts of pooled F_2 scutella, the average specific activities of *Adh-W* and *Adh-FM335* homozygotes were 6.51 u/mg and 0.99 u/mg, respectively. The value for the *Adh-Fm335/W* heterozygote was 3.3 u/mg.

The three dimeric isozyme bands produced in heterozygotes of two normally active ADH electrophoretic variants occur in a ratio of 1:2:1, with the heterodimer being twice as intense as either homodimer band. ADH dimers composed

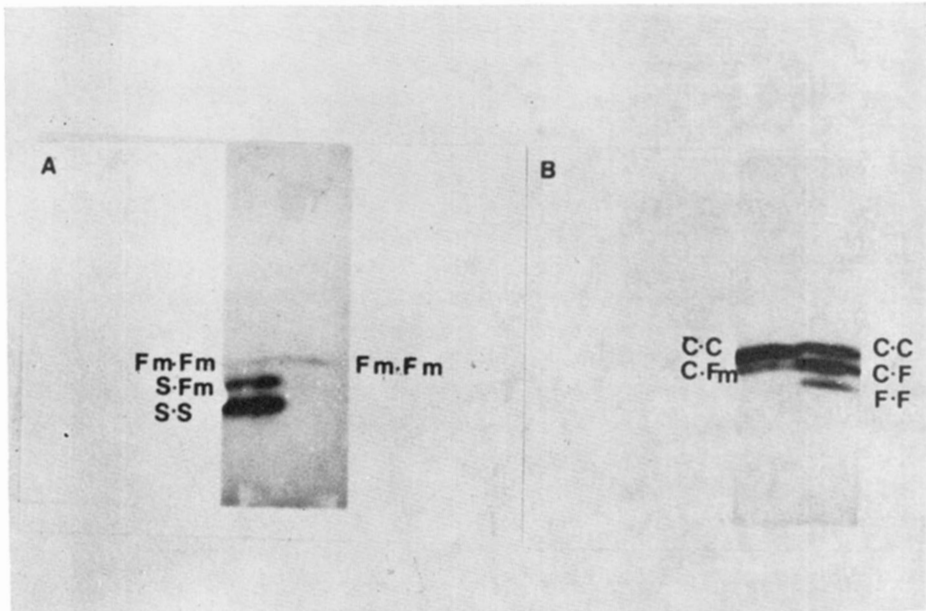

FIGURE 1.—Zymograms illustrating mutant *Adh-Fm335* activity. (A) Comparison of *Adh-Fm335/S* on the left of the zymogram and the homozygous *Adh-Fm335* on the right, showing the reduced activity of the mutant allele. (B) Comparison of *Adh-C/Fm335* on the left and *AdhC/F* on the right.

of one active and one inactive subunit have half of the activity of a dimer with two active subunits, and heterozygotes that carry a "normal" allele and an allele that specifies the full level of an inactive protein show two isozyme bands of equal ADH activity, the "normal" homodimer and the heterodimer (SCHWARTZ and ENDO 1966). The isozyme pattern observed in the *Adh-Fm335* heterozygotes, with the heterodimer band being much less intense than the active homodimer band, indicates that the reduced activity of the mutant results from a striking reduction in the amount of ADH protein produced. This is also clearly shown in the 2-D electrophoretogram of Figure 2, described below.

That *Adh-Fm335* is a *Ds*-suppressed mutant was determined from its response to *Ac*. In the absence of *Ac*, *Adh-Fm335* is a stable, weakly active mutant. However, in the presence of *Ac*, it reverts back to the fully active *Adh-F* form. We have constructed strains homozygous for both *Adh-Fm335* and *bz2-m*. The presence of *Ac* is detected by the color-variegated phenotype of the kernel resulting from the mutability of the *bz2-m* allele. Thus, the association of *Adh-Fm335* mutability and the presence of *Ac* could be examined. The test was accomplished by crossing *Adh-Fm335 bz2-m* plants, with or without *Ac*, by *bz2-m* homozygotes that carry an *Adh* electrophoretic variant other than *Adh-F*. The resulting ears were scored for *bz2-m* mutability to double-check the presence or absence of *Ac*. Kernels from these ears were genotyped by starch gel electrophoresis to check for reversions of *Adh-Fm335* to an active *Adh* allele. For example, in the cross of *Adh-Fm335* to *Adh-C*, kernels exhibiting a 1:2:1 ratio for CC, CF and FF isozyme bands, respectively, are scored as revertants designated *Adh-F'*, which are found

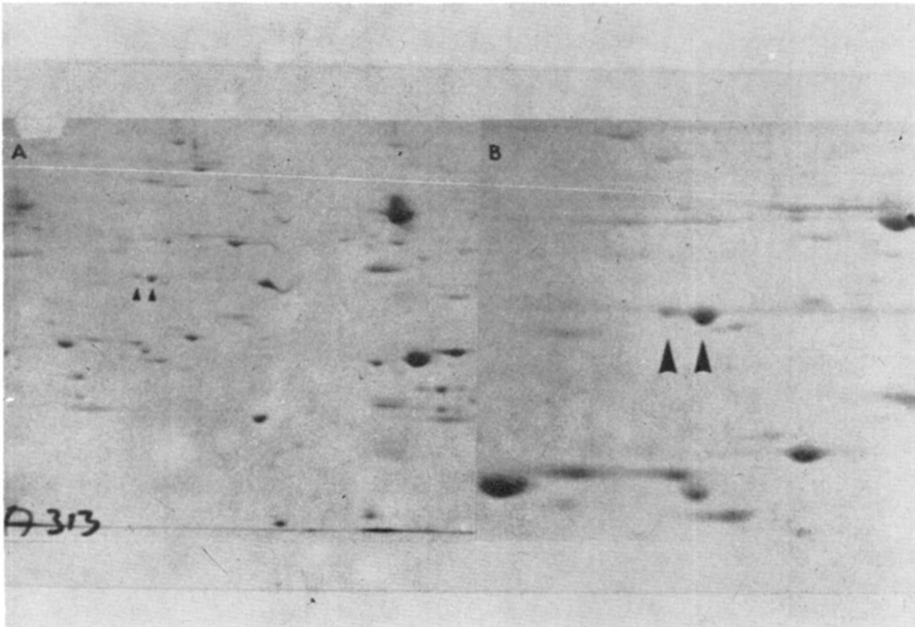

FIGURE 2.—Two-dimensional polyacrylamide electrophoretogram of *Adh-C/Fm335*. Scutella from five kernels were extracted for analysis. (A) shows the entire gel. (B) is a closeup of the ADH region. Arrows indicate the ADH spots. The large spot is composed of C polypeptides and the smaller spot is made up of C and Fm polypeptides representing the heterodimer.

only on ears that carry *Ac*. We genotyped 3,092 kernels by starch gel electrophoresis. There were a total of 48 *Adh-F'* revertants out of 1693 kernels from the *Ac*-containing ears; no revertants were found in the 1,399 kernels from ears that lacked *Ac*.

To test the possibility that the *Adh-Fm335* mutation is not in the *Ac-Ds* system and that reversion to the fully active allele is in response to another activator linked to *Ac*, a similar (but smaller) test was conducted for the response of *Adh-Fm335* to *Mp*. *Mp* and *Ac* arose independently, and both transpose frequently and cause the transposition of *Ds* (BARCLAY and BRINK 1954). The presence of *Mp* at the *P* locus (P^{vv}) causes pericarp-color variegation and affords an unequivocal identification of *Mp*-carrying plants. In crosses of *Adh-Fm335/Adh-C,Mp* × *Adh-W/Adh-W*♂, we scored 17 revertant *Adh-F'/W* kernels and 509 nonrevertant *Adh-Fm335/W*.

The presence of other dehydrogenases that act on endogenous substrates prevents the *in situ* testing for alcohol dehydrogenase activity in the maize endosperm without prior extraction of the enzyme. However, this is possible in pollen, where the endogenous substrates can be leached out by repeated freezing and thawing (FREELING 1976). Pollen grains that carry a null or inactive *Adh* allele fail to show the color associated with reduced nitroblue tetrazolium when stained for ADH activity; whereas, pollen grains with an active allele stain deep blue. *Adh-Fm335* pollen stains faintly. We found a complete correlation between the presence of *Ac* and the occurrence of intensely staining grains in pollen from

Adh-Fm335 homozygotes. Ten plants with intensely staining pollen grains, ranging from 2 to 8%, were all shown to carry *Ac* by crosses to *bz2-m* tester plants. The three plants scored that lacked *Ac* produced only light staining pollen. Thus *Adh-Fm335*, which is stable and reverts only in the presence of *Ac*, is clearly a *Ds*-suppressed gene.

In addition to causing a reduction in the amount of enzyme produced, the presence of the *Ds* element also results in the formation of an altered polypeptide, since the Fm335 enzyme has reduced thermostability. Extracts were prepared from *Adh-Fm335/Fm335*, the progenitor *Adh-F/F* and revertant *Adh-F'/F'* mealed kernels. The Fm335 enzyme differed from the other two in the degree of inactivation by heating. Since the homozygous mutant kernels have a much reduced amount of ADH enzyme, this variable was eliminated by adjusting the extracts to equal ADH activity by the addition of a similarly prepared extract from *Adh*-null homozygous kernels having no detectable ADH activity. The ADH levels in *Adh-F/F* and *Adh-F'/F'* samples were thus reduced without diluting the extracts. The samples were divided into aliquots that were incubated at 50° or 55° for varying periods of time and their ADH activity assayed spectrophotometrically.

The results are presented in Table 1. The enzymes specified by the progenitor *Adh-F* allele and the *Adh-F'* revertant are similar in thermostability and are considerably more stable at the increased temperature than the Fm335 enzyme. The alteration in the thermostability of the enzyme points to a change in the primary structure of the protein. This reflects a change in the DNA segment that is responsible for the amino acid sequence of the ADH protein and suggests that *Ds* is located within the limits of the *Adh* structural gene. The fact that the thermostability of the enzyme specified by the revertant *Adh-F'* allele is similar to that of the normal enzyme supports the conclusion that the *Ds* suppression is responsible for the decreased thermostability of Fm335.

Other cases of transposition of *Ds* into a structural gene have been reported. The *Ds* mutants *sh2-m* (HANNAH and NELSON 1976), *Bz-wm* and *bz-m4* (DOONER and NELSON 1977) also produce structurally altered proteins. NELSON

TABLE 1

Thermal stability of F, mutant Fm335 and revertant F' enzymes

Temperature	*Adh* genotype of extract	Minutes of incubation			
		0	10	20	30
50°	F/F	100	97.5	92.92	92.9
	Fm335/Fm335	100	100	86.7	62.3
	F'/F'	100	99.1	94.7	93.8
55°	F/F	100	86.9	73.2	61.3
	Fm335/Fm335	100	76.5	60.0	34.7
	F'/F'	100	76.5	70.0	55.0

Enzyme was extracted from homozygous kernels of each of the tested genotypes. The extracts were adjusted to equal activities by the addition of a similar extract from *Adh*-null homozygous kernels. Extracts were incubated for 0, 10, 20 and 30 min at either 50° or 55°. Activities are expressed as the percent of original activity remaining after incubation. All values are the average of at least three determinations.

(1968) has done fine-structure mapping of the waxy gene and has shown that "there are stable *wx* alleles located both distally and proximally to the controlling element alleles."

The *Adh-Fm335* allele appears to have a *Ds* element inserted in the structural gene, yet specifies an active enzyme, albeit in reduced amount. We predicted that the Fm335 enzyme would differ significantly in size as compared to the enzyme specified by the unsuppressed gene. If the *Ds* element is translated and does not cause a reading-frame shift, the amino acid content of *Fm335* polypeptide should be increased by one third of the number of nucleotides in the *Ds* element. If *Ds* causes a shift in reading frame, *Fm335* could either be larger or smaller depending upon the position of the next termination codon. Similarly, transcription may be blocked at *Ds*, resulting in a smaller polypeptide.

The effect of the *Ds* insertion on the size of ADH protein was examined by native-SDS two-dimensional polyacrylamide gel electrophoresis. In the first dimension, the proteins are separated mainly on the basis of charge, and the dimeric structure of the enzyme is maintained. The SDS second dimension separates largely on the basis of molecular weight. The ADH protein spots had been unequivocally identified in the two dimensional gels by the use of *Adh* variants that alter the migration of the proteins in both the first and second dimensions (FERL, DLOUHY and SCHWARTZ 1979).

Scutella from *Adh-C/Adh-Fm335* heterozygous kernels were extracted for electrophoresis. The 2-D electrophoretogram is shown in Figure 2. The large spot (indicated by the right arrow) is at the position of the CC homodimer and is composed solely of C polypeptides. The smaller spot (indicated by the left arrow) is at the heterodimer position and is composed of C and Fm335 polypeptides. No spot is evident at the F·F homodimer position. This confirms the conclusion drawn from the starch gel zymograms that there is a much reduced synthesis of the Fm335 mutant protein. At the position of the C·Fm335 heterodimer, only a single protein spot is observed. If the molecular weight of the Fm335 polypeptide was altered and differed significantly from that of the C polypeptide, the protein would appear as an elongated or double spot of equal intensity. We have estimated that a change of 500 daltons should be detectable. This is equivalent to about 5 amino acids or 15 base pairs of DNA.

There are a number of possible explanations for these data:

(a) The *Ds* element is transcribed and translated, but is less than 15 bases in a multiple of 3.

(b) *Ds* is located near the terminus of the *Adh* structural gene and reduces the size of the polypeptide by a few amino acids by blocking elongation of the transcript or translation of the 3′ terminal end of the message.

(c) The *Ds* element may have been transposed into an intervening sequence in the *Adh* gene. This scheme would require that the *Ds* insertion modify the intron excision such that the processed *Adh* message is altered, as well as reduce the efficiency of mRNA processing, to account for the quantitative and qualitative changes in the protein.

(d) The element may itself act as an intervening sequence and be excised from both the DNA and the RNA transcript. The DNA excision mediated by *Ac*

would produce an *Adh-F′* revertant, and the RNA excision, which would occur even in the absence of *Ac*, would lead to the formation of a small amount of active ADH enzyme. The processing of the RNA transcript would have to be such that the segment of RNA excised was either smaller or larger, by a few codons, than the nucleotide sequence transcribed from the *Ds* insertions, or else the Fm335 protein would not differ from that of F or F′.

Similar studies with controlling-element mutations at the waxy locus provide support for the hypothesis that the controlling elements may act as introns (SCHWARTZ and ECHT, manuscript in preparation).

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