GENETIC STUDIES ON MUTANT ENZYMES IN MAIZE. III. CONTROL OF GENE ACTION IN THE SYNTHESIS OF pH 7.5 ESTERASE

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ONE of the basic problems in differentiation concerns the control systems that govern the turning on and off of genes during development. JACOB and MONOD (1961) have shown that the regulatory system in *Escherichia coli* which controls the synthesis of β -galactosidase by the structural gene involves two genetic elements, an operator and a regulator. The structural gene, which is responsible for the production of the enzyme and its specificity, is activated by the closely linked operator element whose activity, in turn, may be blocked by a repressor substance produced by a regulator gene. The structural gene is activated by the operator under those conditions where (a) an inducer substance is added which somehow blocks the action of the repressor substance, or (b) where the regulator gene is mutant in that it fails to produce the repressor substance. McCLINTOCK (1961) has recently pointed out the parallels existing between the bacterial system and the two-element control systems described earlier in maize, dissociator-activator, suppressor-mutator (McCLINTOCK 1956a, b, 1958).

This paper is concerned with the regulatory system of the *E* gene, which controls the specificity of the pH 7.5 esterase in maize. The timing of the action of this gene is controlled by some element at or very close to the site of the structural gene. In heterozygous endosperms the activity of two allelic structural genes may be turned off at different stages in development.

METHODS AND MATERIALS

The procedure followed in handling the material and for starch gel electrophoresis and development of zymograms is similar to that described earlier (SCHWARTZ 1960). Enzyme level was determined by visual examination of esterase bands in starch gel after staining for activity. The electrophoretic analysis was carried out with extracts from washed endosperm tissue that had been separated from the embryo and maternal tissue.

RESULTS

The pH 7.5 esterase specified by the *E* gene in maize is synthesized in the triploid endosperm as well as diploid plant (pericarp, developing embryo, seed-

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ling, etc.) tissue. Three alleles of this gene designated E^S , E^N , and E^F have been found in maize (SCHWARTZ 1960). The isozymes specified by these alleles are easily distinguishable in starch gel electrophoresis. In homozygous condition each allele produces a single isozyme shown by a single band with characteristic electrophoretic migration rate. Heterozygotes between any two of these alleles contain the two isozymes produced by the alleles when in homozygous condition, and in addition a third "hybrid" enzyme type with an intermediate migration rate. Thus, it is easy to distinguish the action of each of the two alleles in heterozygous material.

We have found an altered form of the E^F allele, designated $E^{F'}$, which differs from E^F in the timing of gene action. The isozyme formed by $E^{F'}$ shows exactly the same electrophoretic migration rate as that of E^F , and there is no evidence suggesting any difference between the two enzyme types. The $E^{F'}$ allele was first found in the inbred line M14. A large number of M14 plants were tested and all were found to be homozygous for $E^{F'}$. Two other genetic marker stocks of maize were also found to carry $E^{F'}$, but it is impossible to determine if these similarly behaving alleles are of common or independent origin.

In diploid plant tissue, $E^{F'}$ cannot be distinguished from E^F . It shows normal activity and the concentration of the isozyme specified by $E^{F'}$ in various diploid tissues parallels that of any of the other alleles. This is true even in seedlings, in which enzyme level increases and then decreases with time after germination. The abnormal behavior of the $E^{F'}$ alleles is apparent only in the developing endosperm. In young endosperm tissue (up to about 14 days after fertilization in the winter greenhouse) the $E^{F'}$ allele is fully active, producing the normal amount of enzyme. However, by 19 days (16 days in the field during the summer where development is somewhat faster) the $E^{F'}$ allele is completely inactive. $E^{F'}/E^{F'}/E^{F'}$ endosperm at this stage of development has a very small amount of enzyme, but probably represents persistent enzyme that had been synthesized in the young endosperm tissue rather than incomplete blockage of gene activity. Endosperms carrying the other alleles contain a high level of enzyme concentration throughout development. The E^N allele was found to still be active in endosperm tissue culture (kindly supplied by DR. J. STRAUSS) that had been maintained by subculture for eight years.

Analysis of $E^{F'}$ heterozygotes revealed that the blockage of gene action is limited to the $E^{F'}$ allele. $E^{F'}/E^{F'}/E^F$ heterozygotes contain a concentration of enzyme expected from one dose of E^F in older kernels. Heterozygotes of $E^{F'}$ with E^S or E^N show only a single enzyme band in electrophoresis of extracts from older endosperm. Only the SS band is found in $E^{F'}/E^{F'}/E^S$ or $E^{F'}/E^S/E^S$ genotypes, whereas only the NN band is observed in $E^{F'}/E^{F'}/E^N$ or $E^{F'}/E^N/E^N$ genotypes. In these the FF band as well as the hybrid bands are absent. In young endosperm the normal zymogram is obtained so that, for example, $E^{F'}/E^{F'}/E^S$ genotypes show intense FF and FS hybrid bands as well as a weak SS band. This is illustrated in Figure 1, which shows that by 19 days after pollination the more intense FF and FS isozyme bands of 14-day endosperm have disappeared leaving only the SS band. This situation is also found when we test young and old endosperm

FIGURE 1.—Comparison of zymograms of $E^{F'}/E^{F'}/E^S$ endosperm with time after fertilization. Left, 14-day-old endosperm from winter plant with full activity of the $E^{F'}$ allele showing FF, FS (hybrid), and SS isozyme bands. Right, 19-day-old endosperm showing only the SS isozyme, a situation caused by blockage of the action of the $E^{F'}$ allele.

FIGURE 2.—Comparison of zymograms of $E^{F'}/E^{F'}/E^N$ and $E^S/E^S/E^N$ endosperm from summer plants harvested 16 days after pollination. The intensity of the NN isozyme band of $E^{F'}/E^{F'}/E^N$ endosperm with inactive $E^{F'}$ allele is considerably greater than the corresponding isozyme band in the $E^S/E^S/E^N$ endosperm. The band seen at the SS position in the $E^{F'}/E^{F'}/E^S$ zymogram represents another unrelated slow-moving esterase enzyme (SCHWARTZ 1960).

tissue from the same ear, harvesting half of the ear 14 days after pollination and the other half 19 days after pollination in the greenhouse.

The activities of the $E^{F'}$ and E^S or E^N alleles in heterozygotes appear to be completely independent. If one compares the relative intensities of the NN isozyme band in older endosperm of $E^{F'}/E^{F'}/E^N$ versus $E^S/E^S/E^N$ genotypes, in which the same E^N/E^N plant was used as the male parent in both crosses, it is found that the NN band in the former is in fact considerably more intense than in the latter (Figure 2). Since only the E^N allele is active in $E^{F'}/E^{F'}/E^N$ endosperm, all of the product of the E^N allele is utilized in the synthesis of the NN isozyme, whereas in the $E^S/E^S/E^N$ endosperm a large part of the product of the E^N gene interacts with the product of the E^S allele to form the hybrid NS isozyme.

The immature maize kernel is composed of three distinct tissues: maternal tissue made up of the pericarp and the degenerating nucellus, endosperm, and embryo. The pH 7.5 esterase is found in all three. The enzyme is synthesized independently in each of these tissues. There is no diffusion throughout the

kernel from a localized site of synthesis. From appropriate crosses, as for example, $E^{F'}/E^{F'} \times E^N/E^N \delta$, extracts from the three tissues of older kernels can show completely different zymograms. The pericarp, being maternal, contains only FF-type isozyme; the endosperm shows only the NN-type enzyme since the $E^{F'}$ allele is already inactive; and the embryo shows three isozymes, NN, FF, and the hybrid NF. The level of esterase activity is sufficiently high to allow electrophoretic analyses of pericarp, endosperm, and embryo separately from single, immature kernels. Thus, in the same kernel where the $E^{F'}$ allele has become inactive in the endosperm, full activity is found in the embryo.

The $E^{F'}$ allele represents a stable heritable form of the E gene. This condition has persisted throughout three generations of backcrosses to stocks carrying the other alleles. The stability is further substantiated by the fact that this allelic form is found in the large number of inbred M14 plants tested.

The block in enzyme synthesis by the $E^{F'}$ allele appears to be at the gene level rather than in the release of the enzyme from the ribosome. The only particle-bound esterase isozyme (SCHWARTZ 1962) that can be released from the particles by deoxycholate treatment in older endosperms of the genotype $E^{F'}/E^{F'}/E^S$ and $E^{F'}/E^{F'}/E^N$ are SS and NN isozymes, respectively. Linkage studies reveal that the factor responsible for the difference in timing of gene action of the $E^{F'}$ allele, as compared to the other alleles, is situated at or very close to the locus of the structural gene. Postulating a separate regulatory element (R), which is responsible for the turning on and off of the structural E gene, $E^{F'}$ plants would have a normal E^F structural gene but a mutant regulator (R^M) since an apparently normal FF-type isozyme is produced by active $E^{F'}$. The regulatory element must be linked to the structural gene and operate only in the cis condition since in the heterozygotes the activity of the allele on the homologous chromosome is not influenced by $E^{F'}$. The cross used to test for linkage of the regulatory and the structural genes is shown in Figure 3. Since there is no other detectable phenotypic difference between the various alleles, recombination between the regulatory and structural genes is being tested for by electrophoretic analysis on starch gel of individual endosperms of kernels resulting from the backcross shown in Figure 3. The nonrecombinant progeny from this cross are of two types showing light, or heavy NN isozyme bands, depending upon whether the E^N gene is present in one or three doses. One of the two recombinant classes (Figure 3c) could easily be detected since it is the only one which would have three isozyme bands (FF, FN and NN). To date we have tested over 5,000 individual endosperms resulting from these testcrosses and have not found a single recombinant. A total of 2,573 endosperms received the E^F allele from the female parent. Since recombination can be detected only in the endosperms which carry this allele (Figure 3c), the observed rate is 0/2573.

We have recently found an altered form of the E^S allele designated $E^{S'}$. Like $E^{F'}$, this allele does not cause a detectable alteration of the isozyme specified by the structural gene, but alters the timing of gene action. In the $E^{S'}$ complex, the activity of the structural gene is turned off somewhat later than in the $E^{F'}$.

FIGURE 3.—Test for recombination between regulatory and structural gene.

DISCUSSION

The results presented in this paper on the regulatory system of the pH 7.5 esterase indicate that the locus of the structural gene controls the timing of gene action. The results in themselves do not require introducing a separate regulatory element at the locus, such as the JACOB and MONOD "operator." However, the idea of a compound locus consisting of a regulatory element and a structural gene is reasonable since the isozymes specified by the regulatory mutants $E^{F'}$ and $E^{S'}$ are identical in electrophoretic mobility with those specified by E^F and E^S , respectively, suggesting identity in the structural genes. The postulated regulatory element is similar to the operator in the β -galactosidase system (JACOB and MONOD 1961) in that both operate only in the cis condition and are closely linked to the locus of the structural gene. However, in view of the active participation of the various forms of the regulatory element in the control of the timing of gene action, they can best be compared to the states of dissociator in the *Ac-Ds* system (McCLINTOCK 1951, 1956a, b).

It is not maintained that the timing of gene action must be completely under the control of a locus of the structural gene. The possibility of an inducer activating or a repressor substance inhibiting the regulatory element is not ruled out. If we postulated that a certain concentration of repressor substance is required for inactivation, the regulatory mutants could differ simply by having lower thresholds of inactivation by the repressor. According to this scheme, the concentration of repressor increases with time after fertilization in the developing endosperm, and in the older endosperm the repressor level is sufficiently high for inactivation of the $E^{F'}$ and $E^{S'}$ alleles but not high enough for inactivation of

the normal acting E^F , E^N , and E^S alleles. Conversely, the regulatory mutants could have different thresholds of activation by an inducer. In the same older kernels, where $E^{F'}$ is inactive in the endosperm, this allele is still fully active in the developing embryos, which are almost completely embedded in the endosperm. Thus, in this scheme, the level of inducer or repressor in the embryo and endosperm must be different.

As an alternative it could be postulated that all alleles are turned off at the same time during development but that the isozyme specified by the $E^{F'}$ allele is more labile than those specified by the other alleles and does not persist as long after cessation of gene action. This alternative is rendered unlikely by the following observations: (a) in embryo and diploid plant tissue the stability of the isozyme specified by the $E^{F'}$ allele parallels that of the other isozymes, (b) the hybrid isozyme (FN or FS) as well as the FF isozyme is lacking in older $E^{F'}$ heterozygous endosperms, and (c) the concentration of NN isozyme produced in older endosperms of the genotype $E^{F'}/E^{F'}/E^N$ is considerably higher than the NN isozyme concentration in $E^S/E^S/E^N$ endosperm (Figure 2).

There appears to be a fairly sharp cutoff of the action of the $E^{F'}$ allele in maturing endosperms. This cannot be determined directly since the sole criterion for gene action is the presence of the allele-specific isozyme, and the enzyme persists in active form in the tissue for some time after it is synthesized. However, this can be deduced from the relative concentrations of isozyme bands in electrophoresis of maturing $E^{F'}$ heterozygous endosperms that still show some of the product of the $E^{F'}$ allele. In such endosperms of the genotype $E^{F'}/E^{F'}/E^S$, the FF and hybrid FS isozymes are low but almost equal in concentration (Figure 4). Such a condition would exist if up to the cutoff time the $E^{F'}$ allele was as active as the E^S allele (with random association of gene products four ninths of the enzyme would be FF, four ninths FS, and only one ninth SS), and following the cutoff only SS isozyme was synthesized. If, on the other hand, the inactivation of the $E^{F'}$ allele were gradual, these endosperms would show only the hybrid FS isozyme in addition to the NN with only very little, if any, FF isozyme. With

FIGURE 4.—Zymogram of maturing $E^{F'}/E^{F'}/E^S$ endosperm with low concentration of FF and FS (hybrid) isozymes. Note relative intensities of these bands.

random association, most of the sparse product of the less-active $E^{F'}$ allele would associate with the more abundant product of the still-active E^S allele to form hybrid enzyme, as is the case with the product of the single paternal allele in heterozygous, triploid endosperm tissue (SCHWARTZ 1960).

SUMMARY

In maize, the timing of the activity of the E gene, which specifies the pH 7.5 esterase enzyme, is postulated to be under the control of an element situated at or very close to the site of the structural gene. The behavior of the regulatory mutants, $E^{F'}$ and $E^{S'}$, is described. These mutants cause a precocious cessation of gene action in the developing endosperm but not in the developing embryo or seedling. In heterozygotes for a regulatory mutant and a normally active allele, the activity of the two allelic structural genes are turned off at different stages of endosperm development.

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LITERATURE CITED

- JACOB, F., and J. MONOD, 1961 Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* **3**: 318-356.
- McCLINTOCK, B., 1951 Chromosome organization and genic expression. Cold Spring Harbor Symp. Quant. Biol. **16**: 13-47.
- 1956a Internuclear systems controlling gene action and mutation. Brookhaven Symp. Biol. **8**: 58-74.
- 1956b Controlling elements and the gene. Cold Spring Harbor Symp. Quant. Biol. **21**: 197-216.
- 1958 The suppressor-mutator system of control of gene action in maize. Carnegie Inst. Wash. Yearbook **57**: 415-429.
- 1961 Some parallels between gene control systems in maize and in bacteria. *Am. Naturalist* **95**: 265-277.
- SCHWARTZ, D., 1960 Genetic studies on mutant enzymes in maize. Synthesis of hybrid enzymes by heterozygotes. *Proc. Natl. Acad. Sci. U. S.* **46**: 1210-1216.
- 1962 Genetic studies on mutant enzymes in maize. II. On the mode of synthesis of the hybrid enzymes. *Proc. Natl. Acad. Sci. U. S.* **48**: 750-756.