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The *Ds1* transposable element acts as an intron in the mutant allele *Adh1-Fm335* and is spliced from the message

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ABSTRACT

The Ds-induced maize Adh1 allele Adh1-Fm335 retains its anaerobic regulation and normal transcription start site despite the presence of the 405 bp Ds element in the 5′ untranslated leader region of the gene. The steady state level of Adh1-specific transcript is reduced to about 1% that of the progenitor or revertant alleles. Run-on transcription studies show that the reduced level of Adh1 specific mRNA is not attributable to a decreased transcription rate. S1 mapping indicates that the Ds element is spliced from the Adh1-Fm335 transcript using a donor site 14 bp into the Ds element and an acceptor site at the 3′ junction of the Ds element with the flanking genome DNA.

INTRODUCTION

The alcohol dehydrogenase 1 (Adh1) gene of maize is one of a specific set of about 20 polypeptides synthesized in response to anaerobic conditions (1). The Adh1 gene has been cloned and sequenced and the coding region, transcription start site and promoter elements defined (2,3).

In the Adh1 allele, Adh1-Fm335 (4), the 405 bp Ds1 controlling element is inserted in the 5′ transcribed leader region of the gene 55 bp upstream from the ATG translation start site (5). This allele is unstable in the presence of the Ac controlling element and is excised from the gene at a low frequency. In the absence of Ac the level of ADH1 enzyme activity is decreased to about 10% that of the progenitor (Adh1-FPR) allele but the specific activity of the enzyme coded from the mutant allele remains the same as that of the progenitor allele (4). The Adh1-Fm335 mutation was originally reported to be thermolabile (4) but this has now been shown to be due to a genetic background effect in the line (Sachs and Schwartz, unpublished). The Adh1-specific mRNA level of the mutant is decreased to about 1% that of the progenitor allele (5). We have characterized the transcriptional activity of the mutant allele further in an attempt to understand how the Ds1 element is acting to cause the mutant phenotype of

low enzyme level. In this report we demonstrate that the Ds element acts as an additional intron in the gene and is spliced from the transcript. The altered processing of the Adh1 transcript results in a lower steady state level of mRNA and hence of ADH1 enzyme, suggesting a decreased stability of the primary or spliced transcript.

MATERIALS AND METHODS

1. Stocks

The Ds1 mutant Adh1-Fm335 together with its progenitor stock (Adh1-FPR) and a revertant allele (Adh1-PRV) were obtained from Dr. Drew Schwartz, Indiana University. The line carrying the standard Adh1-1F allele, BkF (Berkeley Fast) has been described (1). None of these stocks contains the Ac controlling element.

2. RNA extraction and Northern analysis

RNA was prepared as described previously from 3 day old seedlings (6). For anaerobic samples the seedlings were immersed in water for 6 hr prior to extraction of RNA.

For Northern analysis, RNA was electrophoresed on formaldehyde-agarose gels, transferred to nitrocellulose and hybridized to nick-translated plasmid pZML793 (6) which is a cDNA clone of Adh1.

3. S1 mapping

Single strand probes were uniformly labelled by annealing an oligonucleotide primer to the relevant single- stranded DNA clone in M13 mp8 or 9 in suitable orientation. The primer was then extended using the Klenow fragment of DNA polymerase in the presence of α ^{32}P dCTP (7,8). The double-stranded DNA was cleaved with a restriction enzyme which cut at the distal end of the insert and the labelled strand purified on a denaturing 6% polyacrylamide gel. The single stranded probe was extracted, precipitated with 50 μg of the RNA to be mapped, redissolved and hybridized overnight at 49°C as described previously (2). The hybrids were treated with S1 nuclease and the protected DNA fragment visualized on a denaturing acrylamide gel (9). Fragments were sized using a sequencing run of the same fragment or end-labelled pBR322 cut with Hpa II.

4. Isolation of nuclei and run-on transcription

Seed was germinated for 4-5 days in the dark at 28°C. The coleoptiles were cut from the seedlings and minced with a razor blade in chopping buffer consisting of 2.5% Ficoll 400 (Pharmacia), 5% Dextran T40 (Pharmacia), 0.44 M Sucrose, 0.025 M Tris-HCl, pH 7.8, 0.01 M $MgCl_2$, 0.1 M

β-mercaptoethanol (1 ml/g of tissue) at 4°C. The tissue was transferred to a prechilled mortar, more chopping buffer added (2 ml/g of tissue) and ground gently with a cold pestle. The mixture was filtered through 150 μm and 63 μm Nitex filters. Triton (20%) was added to 1:40 (v/v) of chopping buffer and mixed. The preparation was centrifuged at 1000 g for 10 min. (0°C) and the supernatant discarded. The nuclei were washed by resuspending the pellet in chopping buffer (3 ml/g of tissue) and centrifuging as above. The pellet was resuspended in chopping buffer (60 μl/g of tissue) and the nuclei were allowed to incorporate ^{32}P UTP (500 uCi, 800 Ci/mmole) in a transcription mix that included 0.7 times chopping buffer, ATP, CTP, GTP (each 350 mM), UTP (5.6 μM) $(NH_4)_2SO_4$ (100 mM), glycerol (10%), 0.6 units/μl RNAsin (Promega Biotech) and incubated 30 min at 30°C.

Radioactive RNA was extracted by the addition of 2.15 ml of 5M guanidine isothiocyanate, 50 mM Tris-HCl, pH 8, 50 mM EDTA, 2% Sarkosyl, 5 mM β-mercaptoethanol, 0.7 mg/ml carrier E. coli RNA. The nuclear lysate was warmed to 65°C, for 2 min. and the DNA was sheared by passing rapidly 5 times through an 18 gauge needle. The lysate was mixed with 1.1 g CsCl and layered over a 1.5 ml pad of 5.7 M CsCl, 100 mM EDTA and centrifuged at 243,000 g for 36 hr., at 20°C. The RNA pellet was dissolved in distilled H_2O and ethanol precipitated in the presence of 0.133 M sodium acetate. The RNA was resuspended, extracted with phenol, and reprecipitated. The radioactive nuclear RNA was incubated with nitrocellulose discs containing either the Adh1 or Adh2 cDNA clones in 0.1 ml of 33% deionized formamide, 0.55 M NaCl, 0.05 M Pipes pH7.2, 100 μg/ml tRNA, 100 μg/ml poly(A) 0.45% SDS, 2 mM EDTA overlaid with parafin oil at 43°C for 36 to 48 hr (10). The filters were then washed, dried and counted in liquid scintillant. Hybridization reactions were done in triplicate for each RNA sample. Each hybridization reaction included a disc containing pBR322 DNA to determine background which was then subtracted from all other values.

RESULTS

1. Northern analysis shows the length of the Adh1-specific mRNA from the Ds mutant (Adh1-Fm335) is approximately the same as that in a standard Adh1 allele

RNA was extracted from anaerobically induced maize seedlings homozygous for either the Ds-induced mutant (Adh1-Fm355) allele, or the standard Adh1-1F allele. Poly A+ RNA of the Ds-induced mutant was

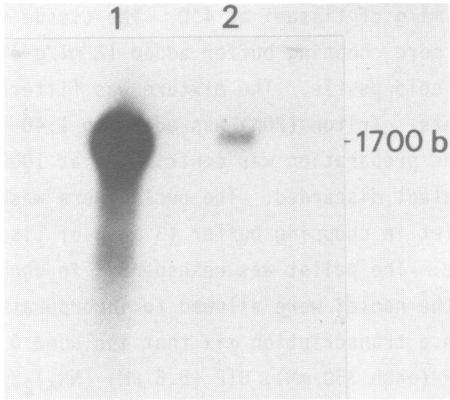


Figure 1: Northern analysis of RNAs from the Ds-induced Adh1-Fm335 and standard Adh1-1F alleles. Seedlings were grown under aerobic conditions and placed under anaerobic conditions for 6 hr. RNA was prepared as described in materials. A. Lane 1: 2 μg poly (A)+ RNA from BkF containing the Adh1-1F wild type allele. Lane 2: 2 μg poly (A)+ RNA from the Ds1-induced Adh1-Fm335 allele. Probe is the Adh1 cDNA clone pZML793 (2).

compared with poly A+ RNA from the Adh1-1F stock by Northern analysis, using an Adh1 cDNA clone, pZML793 as the probe. The results showed that, within the limits of resolution of the gel system, the length of the Adh1-Fm335 (Ds) mRNA was the same as that from the Adh1-1F (Fig. 1). The amount of Adh1-specific poly A+ RNA in the mutant was about 1% of the level in the Adh1-1F line.

2. Expression of RNA from the Ds1 mutant is anaerobically induced

The maize Adh1 gene is induced in seedling roots by anaerobic conditions such as flooding (11), with an increase in ADH1 enzyme activity of about 20 times the level found in aerobic conditions (12). Adh1-specific RNA increases approximately 50 fold following anaerobic treatment (6), with the time course of mRNA accumulation preceding that of the enzyme. In order to determine whether the Ds1 mutant allele exhibited the same regulation as the Adh1-1F allele, 3 day old seedlings bearing the mutant Adh1-Fm335 allele were anaerobically induced by flooding and RNA was extracted after various time intervals. The RNAs were subjected to Northern analysis and hybridized to the Adh1 specific probe, pZML793.

The results (Fig. 2) showed that the steady state level of Adh1 RNA in the Ds1 mutant line is subject to anaerobic control and the time course of anaerobic induction resembles that of the standard Adh1-1F allele (2).

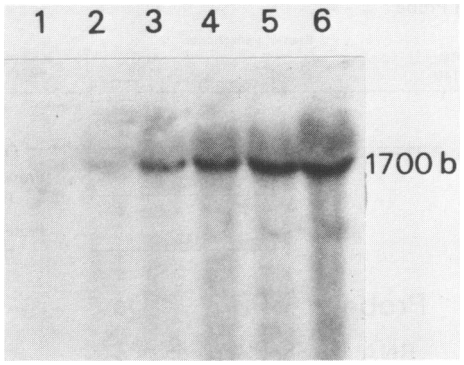


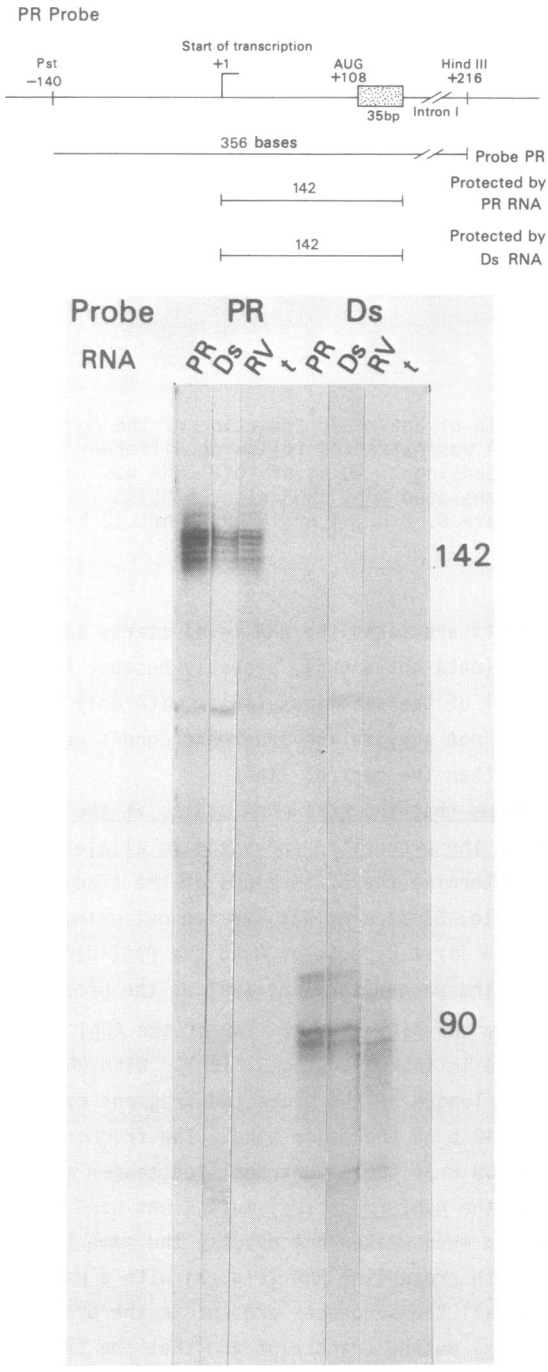
Figure 2: Time course of anaerobic induction of the Adh1-Fm335 mRNA. For Northern analysis RNA was extracted following different times of anaerobic treatment of maize seedlings. 40 μg of total RNA was loaded in each track. Probe is the nick translated Adh1 cDNA clone pZML793 (2). The times of anaerobic treatment are 0, 2 hr, 4 hr, 6 hr, 8 hr, 12 hr for tracks 1-6 respectively.

When later time points are taken the RNA level starts to decrease earlier than in the control (data not shown), probably because ADH1 enzyme is required for survival of the seedlings (13); with only a low level of ADH1 enzyme, seedlings do not survive the anaerobic conditions and begin to deteriorate earlier than the control line.

3. S1 mapping shows that the Adh1 mRNA starts at the same nucleotide in the Ds1 mutant as in the progenitor and standard alleles

In order to determine the 5′ terminus of the transcript from the Adh1-Fm335 (Ds) allele, S1 mapping was carried out using specific single stranded probes. The first experiment used the PstI-HindIII A (2) fragment of the Adh1 gene of the progenitor line (PR) as the probe (Fig. 3). This fragment extends from the PstI site at -140 of the Adh1 gene to the HindIII site at position +216 in intron 1 (Fig.3, Top). With RNA from the progenitor line, the length of the protected fragment covered the range 139-145 bases with 142 b as the major band. The fragment consists of the first exon and the 108 b of the 5′ untranslated leader region.

When RNA from the Adh1-Fm335 (Ds) mutant was used to protect the probe, the signal was much weaker but exactly the same length fragments were protected as with progenitor RNA (Fig. 3) with a major band at 142 b. This indicates that all the sequences present in the progenitor transcript are present in the Ds1 mutant transcript and that the transcription start


PR Probe
Start of transcription
Pst
−140
+1
AUG
+108
Hind III
+216
35bp
Intron I
356 bases
Probe PR
142
Protected by
PR RNA
142
Protected by
Ds RNA
Probe
PR
Ds
RNA
PR
Ds
RV
t
PR
Ds
RV
t
142
90

site is exactly the same in the two stocks. A similar result with revertant RNA was obtained.

When the probe is made from the A fragment of the mutant allele containing the Ds element (the A fragment in this allele is 800 bp long) two fragments are protected by the progenitor RNA (Figs. 3, 4A). The first is a range of bands from 87-92 b but with a major band at 90 b and is the region which extends from the site of insertion of the Ds element to the exon 1/intron 1 boundary (Fig. 4, Top). The second fragment is 52-55 b long, corresponding to a fragment which extends from the transcription start to the site of insertion of the Ds element where the sequences diverge.When the Ds RNA is used to protect the Ds probe two fragments are protected; one is the 90 b fragment seen in the progenitor but the other fragment is 68-69 b (Fig. 4A, 4B), 14-16 b longer. This extends from the start of transcription of the mutant allele to the site of insertion of the Ds element and continues 14-16 b into the Ds element.

In order to confirm this result an oligonucleotide corresponding to the first 20 bases of the non-coding ('bottom') strand within the 5' end of the Ds element (Fig. 5, Top) was synthesized. The oligonucleotide was used as a primer on the single stranded A fragment of the mutant Ds-containing allele to synthesize a probe which extended to the Pst site at -140 bp. When this probe was used for S1 mapping with Ds RNA a fragment of 68-69 b was protected (Fig. 5).

S1 mapping shows that the Adh1 specific transcript from the mutant allele is 14-16 b longer than would be expected from the length between the start of transcription and the site of insertion of Ds. This extra length is presumably due to the inclusion of 14-16 b of the sequence of the Ds1 element in the mRNA in addition to all the sequence present in the progenitor alleles. Thus the 405 bp Ds1 element in Adh1-Fm335 resembles an intron. It is present in the gene sequence but the bulk of it is spliced

Figure 3: S1 mapping to determine the nature of the mRNA from the Ds-induced mutant allele Adh1-Fm335. 40 μg of total RNA of progenitor (PR), Ds, or revertant (RV) lines was used for each S1 mapping experiment. Probes were radioactively labelled single stranded copies of the A (PstI-HindIII) fragment of the progenitor line (PR) and the Ds mutant line (Ds). tRNA was used as a control (t). The 52-55 b fragment is not obvious in this experiment. Two separate exposures have been combined to to enable the Ds tracks to be seen. There are minor bands present in all tracks which are breakdown products of the probes. The diagram shows the PR probe and protected fragments.

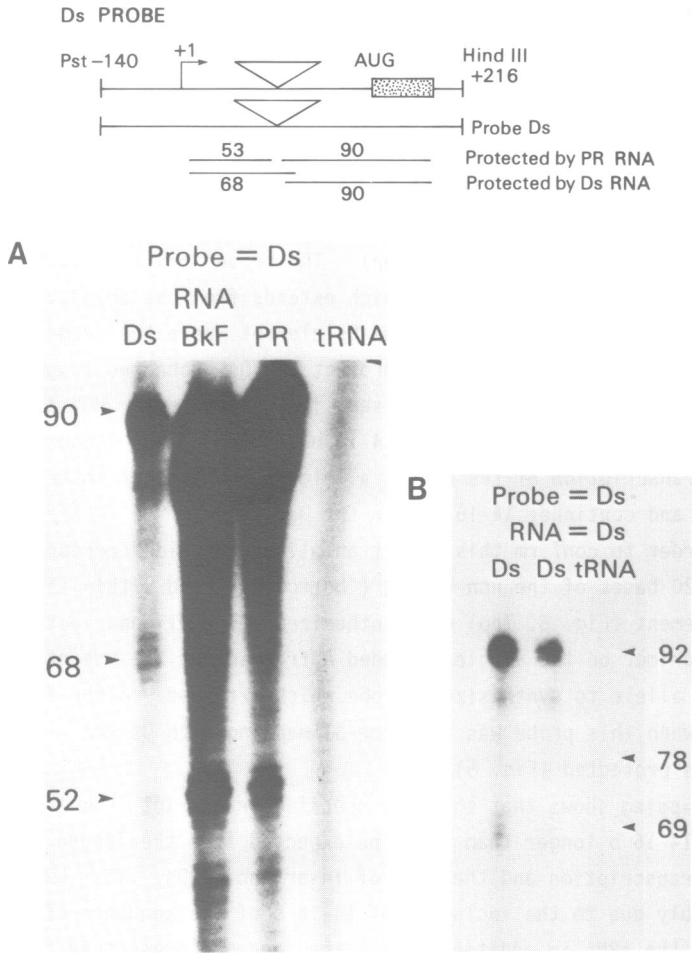


Figure 4: S1 mapping of the 5′ end of the transcript from the Adh1-Fm335 (= Ds) allele. Conditions were as in Fig. 3. A) 3 μg of poly (A)+ RNA from the Ds line was used and 10 μg of total RNA from the progenitor (PR) and standard (BkF) lines, tRNA is transfer RNA. B) Same as A except only the Ds tracks were loaded. The 68-69 bp band is arrowed. Diagram shows the Ds probe and protected fragments.

out in mRNA production leaving only 14-16 b of the Ds1 sequence in the mRNA.

4. Run-on transcription studies show that the mutant phenotype is not caused by a deceased rate of transcription

Following anaerobic induction, the steady-state level of Adh1 mRNA in

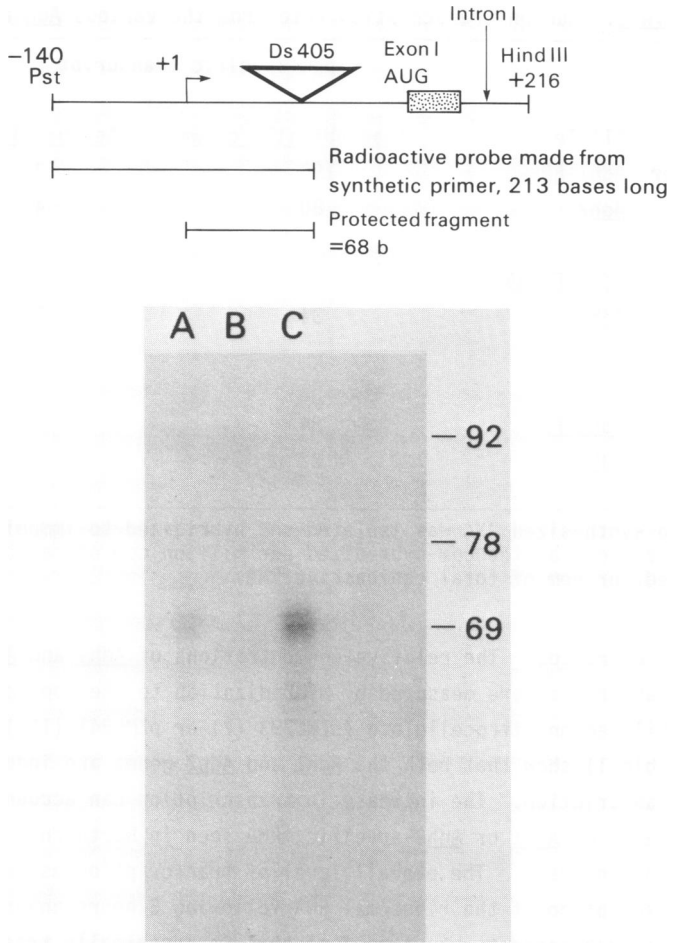


Figure 5: S1 mapping of the 5′ region of the Ds element. The probe is shown in the diagram above the figure and consists of a uniformly labelled probe extended from a primer which consists of the bases complementary to the first 20 bp of the Ds element and continues to the PstI site at -140. Lanes A & C. 1 and 3 μg of poly A+ RNA from the maize lines bearing Adh1-Fm335 allele. B) tRNA.

wild type strains is increased approximately 50 fold as measured by Northern hybridization (6). In order to show that this increased RNA level is due to increased transcription, nuclei were isolated from 3 day old seedlings either with or without a 6 hour anaerobic treatment. Nuclei were allowed to incorporate radioactive nucleotide triphosphate into RNA and

Table 1. Run-on transcription rate from the various Adh alleles

		ppm specific transcript	
Stock	Allele	Anaerobically grown	Aerobically grown
Progenitor	Adh1-FPR	190	2
	Adh2	80	4
Ds	Adh1-Fm335	34	2
	Adh2	34	1
Revertant	Adh1-FRV	84	3
	Adh2	22	3

* In vitro synthesized RNA was isolated and hybridized to immobilized DNA. Results are shown as the cpm hybridized per million cpm of radioactive RNA synthesized, or ppm of total radioactive RNA.

transcripts extracted. The relative concentrations of Adh1 and Adh2 specific transcripts were measured by hybridization to the appropriate cDNA clone immobilized on nitrocellulose (pZML793 (2) or pZML841 (15)). The results (Table 1) show that both the Adh1 and Adh2 genes are induced at the level of transcription. The increase in transcription can account for the increased level of Adh1 or Adh2-specific mRNA seen in Northern hybridization analyses. The overall level of transcription as measured by run-on transcription of the ribosomal RNA following 6 hours anaerobic conditions is decreased to about half of that in aerobically treated tissue (see also 16). In the Ds1 mutant Adh1-Fm335 there is a large increase in transcription rate of the Adh1 gene following anaerobic induction. The final level of Adh1-specific transcript in the Ds mutant is not identical to that of Adh1-FPR or Adh1-FRV, but is 2.5 to 5.5 times lower. Most of this difference can be accounted for by line specific differences in Adh1 levels. Control hybridization using transcripts of the Adh2 gene showed similar amounts of variability between the lines. Lines of maize tested for the amount of ADH enzyme induction following anaerobic induction can show 5-fold difference in levels of ADH enzyme activity (Pryor, pers. comm.). The decreased level of Adh1 specific mRNA in the mutant is likely

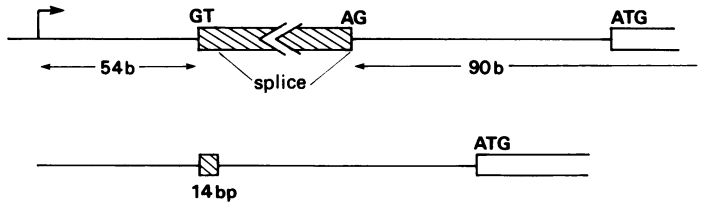


Figure 6: Diagram showing the splicing of the Ds1 element from the transcript of the Adh1-Fm335 (Ds) allele. The top figure is the genomic region showing the transcription start site and the location of the Ds element. The lower diagram shows the mRNA. The Ds1 element sequences are shown in hatched boxes. ATG indicates the start of the coding region of the Adh1 gene and GT and AG the splice junctions.

to be a result of decreased RNA stability, since the level of transcription is not decreased substantially in the mutant.

DISCUSSION

S1 mapping of mRNA of the Ds1 mutant allele, Adh1-Fm335, shows that the site of transcription initiation is at the same nucleotide as in its progenitor allele. Transcription of the mutant gene is still anaerobically inducible as shown both by nuclear run-on experiments and by measurement of the steady-state level of mRNA. The mutant has the same promoter as the progenitor allele and is subject to the same regulation. We have shown the anaerobic regulatory element (ARE) of the Adh1 gene to be located between positions -140 and -99 (3,17) and these sequences are unaltered in the Ds1 mutant.

Although the relative level of transcription of Adh1 in the Ds1 mutant is 2.5 to 5.5 times lower than the revertant and progenitor alleles respectively, these levels do not account for the 100-fold reduction in the Adh1 mRNA seen in the Ds1 mutant. These small differences in transcription rate may be attributable to line specific differences. The reduced level of Adh1 mRNA in the Fm335 line must principally be due to post-transcriptional events.

If the decreased Adh1-mRNA level in the Ds1 mutant is not due to decreased transcription, what is its cause? S1 mapping shows that most of the 405 b from the Ds element is spliced from the transcript, only the 14-16 b located at the 5′ end of the Ds element remaining in the mRNA (Fig. 6) and the 3′ splicing occurring at the 3′ junction of the element with the flanking genome DNA. This suggests that the Ds element is acting as a

Table 2 Splicing signals within the Ds1 element

5′ donor (Ds1)	gggactga TAGGGATGAAAACG*GTCGGAATC
Adh1 consensus	AAG*GTAAG G T
3′ acceptor (Ds1)	TTTCATCCCTAg*ggactga
Adh1 consensus	CCAG*GNNC T
Lariat consensus	Py X Py T Pu A Py
Ds1 lariat	T C C T A A C

The flanking genomic DNA which is duplicated upon Ds1 insertion is shown in lower case, the Ds element in capitals. The inverted repeats present in the Ds1 element are underlined. The presumed splicing site is indicated by an asterisk.

tenth intron in the gene. The position at which the S1 mapping shows splicing occurs is close to a presumptive 5′ splice donor site in the Ds1 element. This sequence (Table 2) compares with the consensus sequence of splice sites from the Adh1-1F gene (7) (Table 2). The 3′ acceptor sequence of the Ds1 element (Table 2) is a good fit to the acceptor consensus sequence (Table 2). The apparent poor fit of the 5′ donor site to the consensus is in fact no worse than some of the other intron donor sites in the Adh1-1F gene (7). A sequence located about 30 bp before the 5′ splice site has been identified as being important for lariat formation during splicing of an intron (18, Table 2), and the Ds element has a sequence TCCTAAC, in perfect agreement with this lariat sequence and located at the correct position (Table 2).

We conclude that the Ds element in the Adh1-Fm335 gene has all the necessary sequence attributes of an intron and that it is spliced. One possible reason for the lower level of Adh1 mRNA in the Ds-induced mutant is that transcripts are less stable. This could be a consequence of the extra sequences present in the primary transcript or, more likely, inefficient splicing of the additional intron. If there is inefficient splicing the unspliced transcript must be unstable as we have no evidence for a longer transcript. Although there is a lower level of Adh1-mRNA, it

does code for the normal polypeptide (Sachs and Schwartz, unpublished observation).

During anaerobic induction of maize seedlings there is preferential translation of anaerobic mRNAs including that of Adh1 mRNA. In the Drosophila heat shock response (19) preferential translation is dependent upon the 5′ leader sequences of the mRNA. It is possible that similar controls may function during anaerobic induction in maize. The alteration of the 5′ leader in the Ds mutant might affect preferential translation, however the ratio of mRNA to enzyme appears normal or low in the Ds mutant so it is likely that preferential translation of Adh1 mRNA is also operative in this stock.

In general, introns are lost over evolutionary time (e.g. in preproinsulin, (20)). We have described here how a transposable element can generate a new intron in a gene. The insertion of the Ds1 element in other genes could also result in it being spliced out as an intron. The donor sequence and the lariat sequence are present in the element but the acceptor site is dependent upon the sequence at the point of insertion. The Ds1 elements sequenced so far (21,22, Schifelbein et al., unpublished) contain both lariat and donor sequences and may function as introns. Ac and its deletion Ds derivatives contain the donor site but lack a good lariat site in the orientation in which Ac is normally seen in genes, so may be dependent upon acceptor sites in genomic DNA.

Recently, Wessler et al. (23) have examined transcripts of Ds induced wx mutants in maize. The Ds elements inserted in these alleles wx-m9 and wxB4 are Ds elements which are deletion derivatives of the autonomous controlling element Ac and share only the sequences of terminal inverted repeats with the Ds1 family. Wessler et al. also find that the Ds element is spliced out of the message using variable 5′ donor sites in the element and 3′ accepter sites in the flanking sequence. In another Ds induced Adh1 mutant allele, Adh1-2F11, the presence of the Ds2 element allows use of a cryptic splice signal in the Adh1 gene (24). The allele bz-m13csq of the bronze gene contains a 902 bp defective Spm transposable element in the second exon. However 40-50% of the wild-type enzymatic activity is present; analysis of cDNAs shows that this occurs through splicing, using the donor site of the normal bz-1 intron and an acceptor site within the terminal inverted repeat of the defective Spm element (25).

This paper shows that a transposable element can affect gene expression not only by inactivating genes. In the case of the Ds1 element

in Adh1-Fm335 the Ds1 element is acting as an intron and gene expression is reduced to about 10% of the progenitor allele. For this to occur, the Ds1 element has to insert in the correct orientation and adjacent to sequences which can provide a 3' acceptor splice site.

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