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Intron-mediated enhancement of heterologous gene expression in maize

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Abstract

Chimeric genes containing the coding sequence for bacterial chloramphenicol acetyl transferase (CAT) have been introduced by electroporation into maize protoplasts (Black Mexican Sweet) and transient expression monitored by enzyme assays. Levels of CAT expression were enhanced 12-fold and 20-fold respectively by the inclusion of maize alcohol dehydrogenase-1 introns 2 and 6 in the chimeric construct. This enhancement was seen when the intron was placed within the 5′ translated region but not when it was located upstream of the promoter or within the 3′ untranslated region. Deletion of exon sequences adjacent to intron 2 abolished its ability to mediate enhancement of CAT gene expression. Northern analysis of protoplasts electroporated with intron constructs revealed elevated levels of CAT mRNA. However, this elevation was insufficient to account for the increased enzyme activity. One explanation of these results is that splicing affects both the quantity and quality of mRNA.

Introduction

The role played by introns in the expression of eukaryotic genes *in vivo* remains poorly defined. *In vitro* splicing systems have generated a wealth of information on the biochemical details of the splicing process (see [16] for a review) and an exon-shuffling role in evolution has been proposed [7]. But a housekeeping function in gene expression in keeping with the ubiquitous occurrence of introns within eukaryotic genes has yet to be elucidated. Some genes appear to require introns for normal expression [10, 11] and some do not [3, 17].

Normal expression of the maize alcohol dehydrogenase-1 (*Adh1*) gene [5] is intron-dependent. The absence of all nine introns results in a 100-fold drop in expression, but the presence of intron 1 alone has been reported to restore expression to near wild-type levels [2]. Moreover, this intron can increase expression of unrelated genes if placed at the 5′ end of the transcribed region.

In this report we show that two other introns of *Adh1* are also capable of enhancing heterologous gene expression in maize and that adjacent exon sequences are needed for optimal enhancement.

Materials and methods

DNA constructs

pUC12BG is a modified version of pUC12 in which the unique *Aat*II site in the vector has been

converted to a *Bgl*II site and the multilinker cloning site (MCS) has been modified to contain restriction sites in the following order: 5′...*Eco*RI-*Sac*I-*Sma*I-*Bam*HI-*Sma*I-*Sac*I-*Eco*RI...3′. All DNA expression cassettes have been inserted into the unique *Bam*HI site of this MCS, with the direction of transcription of each cassette being the opposite of *lacZ* (alpha) on the vector.

The CaMV35S promoter fragment present on each cassette represents nucleotides (nt) 7014–7565 of the published sequence [13] and includes 131 nt of 5′ untranslated sequence (5′ UTS) except in the case of the plasmids pDH934, pDH4414, PDH4415, and pDH4416 where the 5′ UTS is reduced to 3 nt (for the effect of these sequences on gene expression, see [14]). In all cases, an *Xba*I linker was added to the 3′ end of the natural CaMV 35S promoter sequence.

The bacterial chloramphenicol acetyl transferase (CAT) gene was originally isolated as a 0.8 kb *Taq*I fragment from Tn9 [9, 14]. Unique sites for *Xba*I and *Hin*dIII were placed 5′ and 3′ (respectively) of this CAT sequence by linker addition.

The 3′ region of the octopine synthetase (OCS) gene, including polyadenylation sequences, was obtained as a 707 bp *Pvu*II fragment from an octopine Ti plasmid [1, 4]. By linker addition, *Hin*dIII and *Bam*HI sites were placed 5′ and 3′ to this sequence, respectively.

The basic CAT gene expression cassette (CaMV 35S – CAT – OCS; pDH4408) was modified by the insertion of maize *Adh1* intron sequences at one of the following locations in the CAT cassette: upstream of the CaMV 35S promoter sequence (*Bam*HI site); between the promoter and CAT (*Xba*I site); or between the CAT and OCS sequences (*Hin*dIII site). To facilitate cloning at the *Xba*I site, the synthetic linker 5′...CTAGCGAAGATCTCGAGTACTGC-TAG...3′ was inserted at this site, adding *Bgl*II, *Xho*I, *Sac*I and *Nhe*I sites for subsequent manipulation. The total 5′ UTS (excluding intron flanking sequences) is 195 nt. There are no 'ATG' triplets in the linker sequences or in the exons of IVS2 or IVS6 but there are two 'ATG' triplets in the 5′ exon fragment of IVS9. IVS2, 6 and 9 comprise, respectively, nt 654–830, 1860–2329 and 2739–2912 of the genomic sequence for *Adh1* [5] with numbering beginning at the 'A' of the initiation codon for translation. Each of the sequences includes the complete intron and part of the flanking exons (see Fig. 1). pDH4426

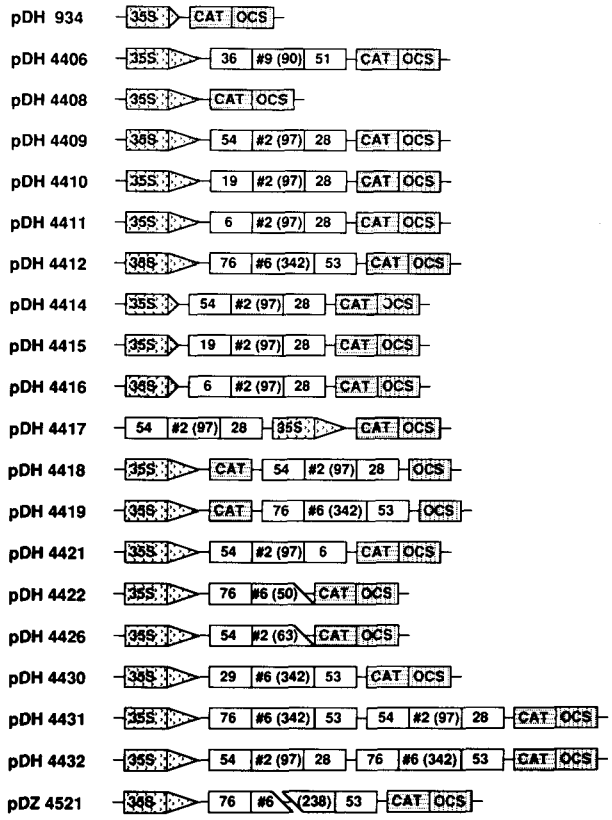

Fig. 1. Diagram of tested constructs. All cassettes are flanked by *Bam* HI restriction sites and located within the MCS of pUC12BG such that the direction of transcription is the opposite of *LacZ* (alpha) in the vector. 35S = cauliflower mosaic virus 35S promoter fragment with either 131 nt of 5′ untranslated sequence (long triangle) or 3 nt of 5′ untranslated sequence (short triangle). CAT = fragment containing coding sequence for bacterial chloramphenicol acetyl transferase from Tn9. OCS = 3′ untranslated region and polyadenylation sequences from octopine synthetase gene of Ti plasmid. Relative position of intron fragments (non-stippled boxes) as indicated. The '#' symbol identifies the maize *Adh1* intron used. Numbers in the intron boxes are lengths in nucleotides of 5′ exon/(intron)/3′ exon sequences within the intron fragment. Truncated intron boxes show position of deletions affecting sequences within the intron. Fragment and gene sequences not to scale.

contains a truncated IVS2 fragment in which the 3′ splice site-proximal 34 nt of the intron plus the entire 3′ flanking exon have been deleted. pDH4422 contains a similar deletion of IVS6 in which the 3′ 292 nt of the intron and the 3′ exon have been deleted. pDZ4521 contains an IVS6 fragment from which the 104 bp *Dde* I fragment internal to the intron has been deleted. The effect of this deletion is to remove one iteration of a 104 bp repeat present in 3 tandem copies in IVS6.

Transient gene expression assays

Protoplasts were prepared from the maize suspension cell culture line Black Mexican Sweet (BMS). Approximately 1×10^6 protoplasts were mixed with fresh MS medium (450 mOsm with mannitol) adjusted to pH 8.0, 20 μg of supercoiled plasmid DNA, 150 mM KCl, and transferred to sterile 0.4 cm plastic cuvettes fitted with aluminum foil electrodes [15] or to 24-well plastic culture plate and a Hoeffer Scientific ring electrode. An electric field of 200 V (500 V/cm) was applied at room temperature by discharge of a 1200 μF capacitor. Protoplasts were then diluted with 9 volumes of fresh MS medium and incubated in the dark for 1–2 days until they were assayed for gene expression. Normally, 1/10 of the treated protoplasts (ca. 1×10^5) were extracted for analysis of CAT activity. Non-radioactive, acetylated products were separated and analyzed by HPLC essentially as described [18]. The HPLC technique is linear over 3 orders of magnitude, and in our hands is more reliable and less tedious than the more commom radioactive TLC methods. Using these methods, the 35S promoter construct pDH4408 produced a basal level of CAT activity resulting in 10–30% acetylation of substrate. The more active intron constructs required further extract dilution. Due to variation in the absolute level of gene expression obtained from different protoplast preparations, each experiment included the 35S promoter construct pDH4408 as a standard control. Levels of gene expression were then expressed in arbitrary activity units relative to the pDH4408 control. Each construct was electroporated in duplicate (or triplicate) in each experiment. Values of duplicates were usually within 10% and seldom varied by more than 20% in an experiment. In our hands, this methodology yielded extremely reproducible values relative to the pDH4408 control in spite of some fluctuation in protoplast viability between experiments. The levels of enhancement with IVS2 and IVS6 over the intron-less control (approximately 12-fold and 20-fold respectively) were observed on at least ten independent occasions.

Results

Adh1 introns 2 and 6 can enhance CAT gene expression in maize

An earlier study revealed that *Adh1* intron 1 enhanced the expression of reporter genes driven by different promoters. However, the stronger the promoter, the less striking the enhancement observed. Enhancements of 170-, 70- and 8.4-fold were observed for three promoters exhibiting basal expression levels of 0.09, 0.70 and 6.7% acetylation in CAT assay respectively [2]. This study also showed that the 35S promoter of cauliflower mosaic virus (CaMV 35S) exhibited the highest basal levels of activity in maize cells, among those tested. We utilized this promoter exclusively in our CAT cassettes. Moreover, our improved CaMV 35S (*Dde* I) promoter has been shown to be 6-fold stronger (in BMS) than the version used in the above study [14]. In our hands expression of CAT cassettes containing this improved CaMV 35S promoter was only slightly (1–2-fold) enhanced by *Adh1* intron 1 (data not shown).

In contrast, introns 2 and 6 (but not 9) enhanced expression of CAT 12–20-fold when compared to the intron-less control, pDH4408 (Fig. 2). As seen for intron 1 [2], placement of these introns within the 5′ untranslated sequences was required for enhancement of CAT gene expression to occur. No enhancement was observed when these sequences were placed

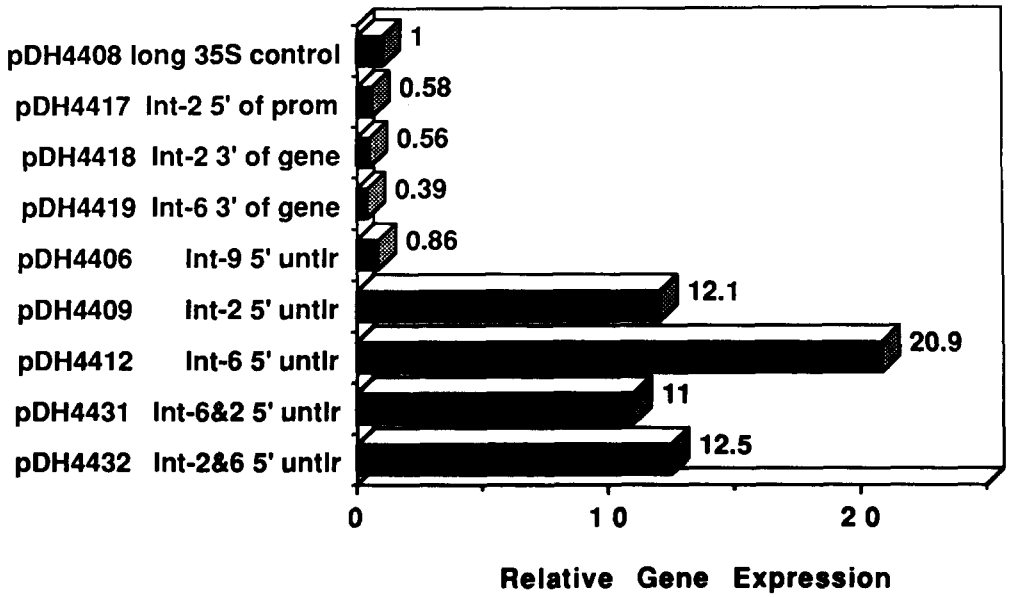

Fig. 2. Enhancement of transient gene expression in maize protoplasts is dependent on location of intron. 20 μg of plasmid DNA was electroporated into BMS protoplasts. The transient expression of each construct was measured two or more times. The results of a representative experiment are shown. All values are expressed relative to the intron-less CAT control, pDH4408. All data points represent the average of duplicate electroporations performed with each construct in one experiment. Int: intron sequences 2, 6 or 9; untlr: untranslated leader region; prom: promoter.

upstream of the CaMV 35S promoter or downstream of the CAT coding sequence (Fig. 2). These results suggest that the 'enhancing' effects of these intron sequences are distinct from those of classical enhancers of transcription.

When introns 2 and 6 were placed together (in either order) at the 5′ end of the CAT transcript, the levels of enhancement were similar to those observed when intron 2 was used alone (Fig. 2).

To examine whether the levels of intron-mediated enhancement relative to control were affected by gene dosage, the experiment shown in Fig. 3 was performed. The results showed that, within the range of concentrations used in this study, there was an approximately linear relationship between the concentration of input DNA and CAT enzyme activity observed 40 hours after electroporation for constructs with or without introns.

The possibility that enhanced expression was due to increased stability of mRNA was investigated indirectly by following the time-course of appearance of enzyme activity after electroporation. The results (Fig. 4) did not reveal any major differences in the transient activity profile of control and intron-containing constructs.

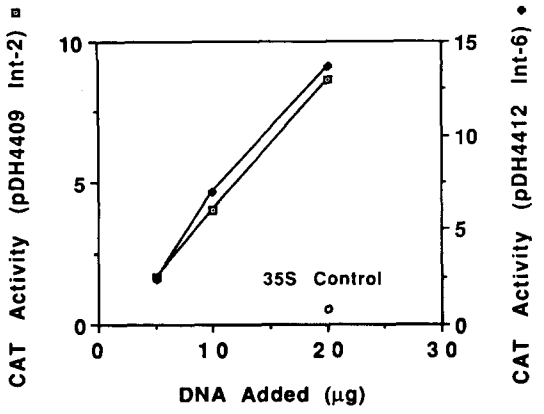

Fig. 3. DNA concentration and transient gene expression in BMS protoplasts. 5 to 20 μg of plasmid DNA was electroporated into BMS protoplasts. Data represent activity relative to 20 μg of intron-less control plasmid, pDH4408. For details, see legend to Fig. 2.

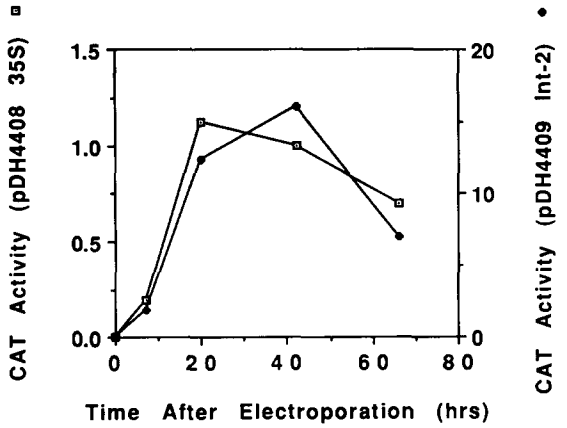

Fig. 4. Time course of transient gene expression of control and intron containing plasmids. 20 μg of the indicated plasmids was electroporated into BMS protoplasts. At various times, samples were removed and assayed for CAT activity. Results calculated relative to activity of pDH4408 control at 42 h. For details, see legend to Fig. 2.

Enhancement is influenced by intron-flanking sequences

The influence of adjacent exons on the efficiency with which an intron is spliced *in vitro* (and *in vivo*) has been documented in mammalian systems [15]. It is also believed that failure to splice a functional intron sequence from a transcript *in vivo* does not lead to accumulation of the unspliced product – presumably because this species is rapidly degraded [12]. We reasoned that if the primary transcript was modified by deletion such that the structural context of the intron was compromised, then splicing – and, possibly, intron-mediated enhancement – might be adversely affected.

Figures 5 and 6 summarize the results obtained when portions of introns 2 and 6 and adjacent exons were deleted from CAT constructs. Enhancement of gene expression mediated by intron 2 was virtually abolished by a deletion leaving only 6 bp of the adjacent 5′ exon intact (pDH4411). However, a similar deletion of the 3′ exon did not have this effect (pDH4421). In the case of intron 6, truncation of the 5′ exon also reduced gene expression (pDH4430). Deletion of a 104 bp *Dde*I fragment internal to intron 6 (pDZ4521) did not appear to affect enhancement. This deletion removed exactly one iteration of the

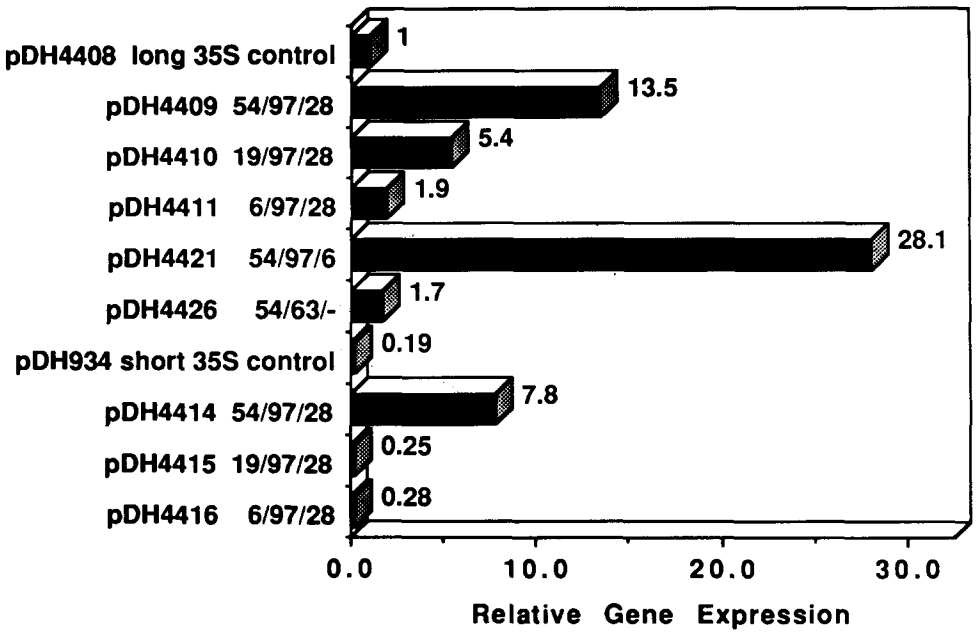

Fig. 5. Influence of exon sequences on enhancement of gene expression by *Adh1* intron 2. 20 μg of the indicated plasmids were electroporated into BMS protoplasts. The level of gene expression obtained is indicated relative to activity of the pDH4408 control plasmid (see legend to Fig. 2 for details). The numbers refer to the size of the 5′ exon/intron/3′ exon fragment.

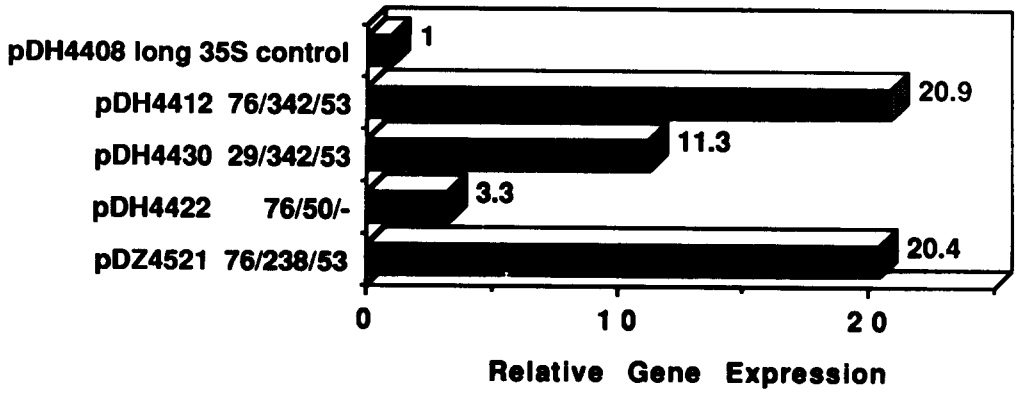

Fig. 6. Influence of exon sequences on enhancement of gene expression by *Adh1* intron 6. 20 μg of the indicated plasmids were electroporated into BMS protoplasts. The level of gene expression obtained is indicated relative to activity of the pDH4408 control plasmid (see legend to Fig. 2 for details). The numbers refer to the size of the 5′ exon/intron/3′ exon fragment.

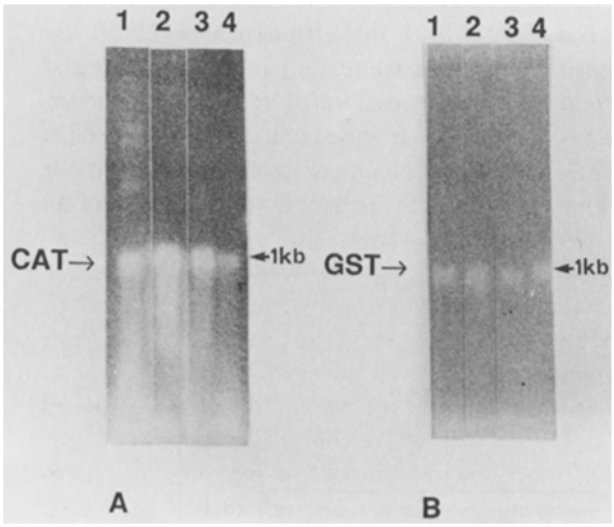

Fig. 7. CAT mRNA levels in BMS protoplasts electroporated with various intron constructs. BMS protoplasts were electroporated with 20 μg plasmid DNA. 42 hours after electroporation, an aliquot was removed for CAT enzyme assay (Table 1). Total RNA was extracted from remaining protoplasts and northern analysis performed as described [14] except that a double (sandwich) blot was performed on the same gel. One blot (A) was probed with a CAT probe and the other (B) with a maize glutathione-S transferase (GST) cDNA probe as a control. CAT and GST mRNAs each co-migrate with the 1 kb size marker in formaldehyde gels, as expected. Owing to the photographic method used, the shades in the autoradiogram are reversed. Lane 1: pDH4408, intron-less control; 2: pDH4409, *Adh1*-IVS2 (54/97/28); 3: pDH4410, *Adh1*-IVS2 (19/97/28); and 4: pDH4411, *Adh1*-IVS2 (6/97/28).

three 104 bp near-perfect tandem repeats spanning this intron.

The modest levels of enhancement observed with pDH4426 and pDH4422 (1.7- and 3.3-fold respectively), which lack 3′ splice sites, may result from aberrant splicing at cryptic sites elsewhere in the transcript (upstream of the CAT translational initiation codon).

We next considered the possibility that the mechanism by which the CaMV 35S leader elevates the expression of downstream genes [14] overlaps the mechanism of intron-mediated enhancement. Deletion of most (128 bp) of the 131 bp corresponding to the 5′ untranslated region of CaMV 35S reduced basal expression 6- to 7-fold [14] and magnified the effect of truncating the 5′ exon of intron 2. However, when 54 bp of this exon were left intact (pDH4414) CAT gene expression was only 2-fold lower than with the comparable construct (pDH4408) containing the long 35S leader (Fig. 5).

Enhancement is accompanied by increased levels of CAT mRNA

A modest elevation of CAT mRNA levels was seen in cells electroporated with pDH4409 when compared to the 35S control (Fig. 7). Probing an equivalent blot for glutathione-S-transferase message indicates that the RNA extractions were

Table 1. Relative CAT mRNA and activity levels in protoplasts.
The amount of CAT mRNA in the bands shown in Fig. 7 was determined as follows. After autoradiography the regions on the blot corresponding to the CAT mRNA band were excised from each lane and subjected to scintillation counting (^{32}P). Background (determined by counting similar regions from blank lanes) was subtracted and the corrected values were taken as representative of the amounts of CAT mRNA present in each sample. The amount present in the control (pDH4408) sample was assigned an arbitrary value of 1.0 and other values were expressed relative to control. CAT enzyme activity was measured on a separate aliquot of the same electroporated protoplasts from which the RNA was extracted. Enzyme activity is expressed relative to activity obtained with the intron-less CAT control (pDH4408).

Band	Plasmid	CAT mRNA	CAT activity
1	pDH4408	1.0	1.0
2	pDH4409	3.9	12.1
3	pDH4410	2.1	4.4
4	pDH4411	0.6	1.9

consistent for the various treatments. The 4-fold elevation seen in CAT mRNA is not, however, sufficient to explain the 12-fold increase in CAT enzyme activity observed in the same experiment (Table 1). Thus a separate effect on translation efficiency cannot be ruled out.

In these experiments we were unable to observe unspliced transcripts – as judged by mobility in gels. Also, the presence of unspliced intron 2 or intron 6 sequences prevented *in vitro*-generated CAT mRNA transcripts from directing the synthesis of CAT enzyme when added directly to maize protoplasts by electroporation (data not shown).

Discussion

Intron-mediated enhancement of CAT gene expression requires appropriate placement of intron sequences within the chimeric transcript. Sequences immediately adjacent to the intron can exert a profound influence on the enhancement effect, both at the mRNA level and the level of enzyme activity. Cells electroporated with a construct containing intron 2 exhibited a 4-fold increase in CAT mRNA (compared to control). In the same cells, CAT enzyme activity was elevated 12-fold. A similar discrepancy was observed when the 'enhancing' segment of the CaMV 35S leader was investigated [14]. This segment is also present in most of our constructs. Its deletion reduced expression 6- to 7-fold in constructs without introns, but only 2-fold when intron 2 was present. These observations raise the possibility of overlapping mechanisms. Further, they suggest that both the quantity and translational competence of CAT mRNA may be increased by these sequences.

Although our data do not rule out the formal possibility of an increased rate of message initiation, the intron sequences tested here do not exhibit the spatial versatility exhibited by classical enhancers of eukaryotic transcription.

Northern analysis with a CAT probe revealed a single mRNA species in protoplasts electroporated with intron-containing CAT constructs. The mobility of this species is consistent with the predicted size of the spliced product, although we have no direct evidence that it is indeed spliced. A previous study [2] using a similar experimental system concluded that intron-containing CAT transcripts were spliced in electroporated maize protoplasts. Unspliced transcripts were not observed – within the limits of detection imposed by RNAse protection studies. These observations are in keeping with results obtained in mammalian systems which suggested that unspliced transcripts were degraded *in vivo* [16].

The effect of adjacent exons on the efficiency of splicing *in vitro* has been documented for mammalian systems [15]. A recent report indicates that exon sequences may also be important for intron recognition and processing in plants [8]. Our deletion analysis indicated that intron 2 mediated enhancement of CAT gene expression was sensitive to 5′ (but not 3′) exon sequences. The results could be explained if these sequences affected the efficiency of splicing and if, as discussed above, unspliced products are subject to degradation. In a construct missing the 3′ splice site to intron 2 (and thus, presumably unable to

undergo splicing) but containing the 5′ exon, enhancement is largely abolished – thus ruling out a direct enhancer function for these exon sequences.

Although we have been able to show that introns 2 and 6 enhance expression, we did not observe similar effects when intron 9 was used. One possible explanation is that not all introns 'enhance'. Alternatively, the presence of two 'ATG' sequences in the 5′ exon may have been utilized to produce an inactive translation product.

Although other explanations of our data exist, we favor the interpretation that the splicing of introns *per se* elevates the level of CAT gene expression by affecting both the quantity and possibly the quality of CAT mRNA. This might occur, for instance, via the association of spliced mRNAs with RNA-binding proteins or other factors in the nuclear environment playing a role in the transport, longevity, and translational competence of mRNA molecules.

The use of introns to augment heterologous gene expression could be intrumental in achieving the requisite levels of genetic activity for the successful introduction of new agronomic traits into maize.

References

1. Barker RF, Idler KB, Thompson DV, Kemp JD: Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTI15955. Plant Mol Biol 2: 335–350 (1983).
2. Callis J, Fromm M, Walbot V: Introns increase gene expression in cultured maize cells. Genes and Development 1: 1183–1200 (1987).
3. Chee PP, Klassy C, Slightom J: Expression of a bean storage protein 'phaseolin minigene' in foreign plant tissues. Gene 41: 47–57 (1986).
4. DeGreve H, Dhaese P, Seurinck J, Lemmers M, Van Montagu M, Schell J: Nucleotide sequence and transcript map of the *Agrobacterium tumefaciens* Ti plasmid-encoded octopine synthase gene. J Mol Appl Genet 1: 499–511 (1983).
5. Freeling M, Bennett DC: Maize *Adh1*. Ann Rev Genet 19: 297–323 (1985).
6. Fromm M, Taylor LP, Walbot V: Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc Natl Acad Sci USA 82: 5824–5828 (1985).
7. Gilbert W: Why genes in pieces? Nature 271: 501–502 (1978).
8. Goodall GJ, Filipowicz W: The AU-rich sequences present in the introns of plant nuclear pre-mRNAs are required for splicing. Cell 58: 473–483 (1989).
9. Gorman C, Moffat L, Howard B: Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. Mol Cell Biol 2: 1044–1051 (1982).
10. Gruss P, Lai CJ, Dhar R, Khoury G: Splicing as a requirement for biogenesis of functional 16S mRNA of simian virus 40. Proc Natl Acad Sci USA 76: 4317–4321 (1979).
11. Hamer DH, Leder P: Splicing and the formation of stable mRNA. Cell 18: 1299–1302 (1979).
12. Hamer DH, Smith KD, Boyer SH, Leder P: SV40 recombinants carrying rabbit β-globin coding sequence. Cell 17: 725–735 (1979).
13. Hohn T, Richards K, Lebeurier G: Cauliflower Mosaic Virus on its way to becoming a useful plant vector. Curr Top Microbiol Immunol 96: 193–236 (1982).
14. Pierce DA, Mettler IJ, Lachmansingh AR, Pomeroy LM, Weck EA, Mascarenhas D: Effect of 35S leader modifications on promoter activity. In: UCLA Symp Mol Cell Biol, New Series vol. 62 pp. 301–310. Alan R. Liss, New York (1987).
15. Reed R, Maniatis V: A role for exon sequences and splice-site proximity in splice-site selection. Cell 46: 681–690 (1986).
16. Sharp P: Splicing of messenger RNA precursors. Science 235: 766–771 (1987).
17. Triesman R, Novak U, Favaloro J, Kamen R: Transformation of rat cells by an altered polyoma virus genome expressing only middle-T protein. Nature 292: 595–600 (1981).
18. Young SL, Jackson AE, Puett D, Melner MH: Detection of chloramphenicol acetyl transferase activity in transfected cells: A rapid and sensitive HPLC-based method. DNA 4: 469–475 (1985).