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Characterization of the maize transposable element *Ac* by internal deletions

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We have used the ability of *Ac* to transpose in tobacco to determine which *Ac* sequences are required for transposition, using a phenotypic assay for *Ac* excision from an NPTII gene in which excisions of *Ac* result in calli resistant to the antibiotic kanamycin (Baker *et al.*, 1987). Here we show that deletion of the *Ac* DNA which encodes the untranslated leader of the *Ac* transcript does not prevent *Ac* excision. A deletion which removes 110 bp including the first 75 bp of the long open reading frame prevents *Ac* excision in tobacco cells. However, it will excise in tobacco cells previously transformed with *Ac* indicating that deletion of the region prevents the synthesis of a product required for *Ac* excision. Deletion of the *Ac* sequences between bp 44 and bp 92 or from bp 75 to bp 181 abolishes, or strongly reduces, transposition in cells which are already transgenic for an active *Ac* element. This indicates that these deleted elements have lost sequences which are required for the transposon to respond to the transposase, when the enzyme is produced *in trans*. We also describe a tobacco strain transformed with a *Ds* element stably inserted within an NPTII gene. This strain is Km^s and was retransformed with a construct containing the open reading frame (ORF) of the 3.5-kb *Ac* transcript expressed from a plant promoter. Expression of the cDNA construct promotes excision of the *Ds* element. These data suggest that the 3.5-kb transcript of *Ac* encodes the only *Ac* product required for transposition, i.e. the transposase function.

Key words: maize transposon/transgenic tobacco/transposase

Introduction

Transposable elements were first described in maize, and the *Activator* (*Ac*) element was the first to be studied in detail (McClintock, 1951). Two *Ac* elements have been isolated from different insertions within the *Waxy* (*Wx*) gene of maize (Fedoroff *et al.*, 1983; Behrens *et al.*, 1984) and were shown to be identical by sequence analysis (Pohlman *et al.*, 1984; Müller-Neumann *et al.*, 1984). Recently, *Ac* was shown to express one long transcript of 3.5 kb (Kunze *et al.*, 1987), which initiates at several positions around 330 bp from one end of *Ac*, contains an untranslated leader sequence of 600-700 nt, and, after removal of its four introns, contains one long ORF encoding 807 amino acids. The polyadenylation site is 260 bp from the other end of the element. No other *Ac* specific transcripts were detected by Northern analysis.

Ac is present at low copy number only in certain maize lines, however all maize lines contain many copies (~40) of sequences hybridizing to an *Ac* probe. Some of them are non-autonomous elements which were called *Dissociation* (*Ds*) elements by McClintock (1951). These can be transposed in the presence of an active *Ac* element. Several *Ds* elements have been isolated biochemically and their nucleotide sequence compared with that of *Ac*. This comparison has yielded information about *Ac* sequences required for transposition. For example, the *Ds9* element has suffered a deletion of 194 bp of the *Ac* sequence (Pohlman *et al.*, 1984). This deletion, which prevents autonomous transposition, removes part of the third exon of the *Ac* transcript. Similarly, a longer deletion present in the DI element removed the sequences encoding most of exon 2 and part of intron 2 of the *Ac* transcript preventing transposition in the absence of *Ac* (Dooner *et al.*, 1986). The sequences deleted from these two transposable elements must be required to produce a *trans*-acting product of the *Ac* element. This method of determining which *Ac* sequences are required for transposition is laborious as it involves the genetic isolation of *Ds* elements followed by their biochemical characterization. Moreover, the sequences deleted from *Ac* are often large portions from the middle of the element (for review see Döring and Starlinger, 1984, 1986). In order to gain more insight into the sequences required for *Ac* transposition, we have been studying *Ac* transposition in tobacco (*Nicotiana tabacum*).

Ac was shown to excise from the T-DNA of *Agrobacterium tumefaciens* after its introduction into tobacco cells, and to reinsert at new locations in the tobacco DNA in a similar way to that described in maize (Baker *et al.*, 1986). An assay for *Ac* excision was devised enabling us to follow *Ac* excision from the T-DNA phenotypically (Baker *et al.*, 1987). In this method *Ac* was inserted within the untranslated leader sequence of a neomycin phosphotransferase II (NPTII) gene preceded by the 1′ promoter of the octopine TR-DNA (Velten *et al.*, 1984) that is active in tobacco and followed by a plant polyadenylation signal. The gene was inactivated due to the presence of *Ac*. However, excision of *Ac* restored NPTII activity, and in turn allowed calli and plants to grow in the presence of kanamycin.

Here we report the insertion of a series of *Ac* deletion derivatives within the NPTII gene, and the result of their transformation into tobacco cells. In addition, we have modified the assay by constructing tobacco plants which contain *Ac* but no NPTII gene, and have used these plants to determine whether *Ac* can transactivate the *Ac* deletion derivatives within the NPTII gene. We have also studied the potential of an *Ac* cDNA clone to promote transposition.

Results

Deletions which remove most of the untranslated leader of the 3.5 kb Ac mRNA still transpose

The only *Ac* transcript so far identified contains a long untranslated leader sequence of 600–700 nucleotides. The length of the leader sequence is an unusual feature of the *Ac* transcript as most eukaryotic mRNAs contain leader sequences of no more than 100 bases in length (Lewin, 1987). Deletions were made within the untranslated leader to determine whether it is necessary for *Ac* transposition. Two of these deleted elements are shown in Table I (the construction of all plasmids is described in Materials and methods). The derivative present in pKU31 has lost 564 bases from within the leader. The deletion present in pKU33 extends from 84 bp 5′ of the major transcription start site, mapped by Kunze *et al.* (1987), to position 736 of *Ac*, which is within the mRNA. The deleted elements were inserted within the untranslated leader of an NPTII gene, and transferred to tobacco protoplasts (Baker *et al.*, 1987; see Materials and methods).

Kanamycin-resistant tobacco calli were expected only if *Ac* (from pGV3850HY :: pKU3) or the *Ac* deletion derivative (from pGV3850Hy :: pKU33 or pGV3850Hy :: pKU31) was capable of excision. As shown in Table I and Figure 1 the *Ac* deletion derivatives present in pKU33 and pKU31 excised with at least the same frequency as *Ac*.

Deletion of the 5′-end of the Ac ORF abolishes transposition

The *Ac* deletions present in plasmids pKU31 and pKU33 removed sequences upstream of the translation start site of the *Ac* open reading frame, but left the entire reading frame intact. Previous analysis of *Ds* elements isolated from maize indicated that deletions removing most of exon 2 and part of intron 2, or part of exon 3 of *Ac* prevent transposition (Pohlman *et al.*, 1984; Dooner *et al.*, 1986). In order to

Table I. Number of kanamycin-resistant calli derived from tobacco protoplasts infected with the *A.tumefaciens* strains

Plasmid[a]	Sequences deleted from *Ac*[b]	Frequency of excision[c]						Frequency of excision if *Ac* is present *in trans*[d]	
		(1)	(2)	(3)	(4)	(5)	(6)	(1)	(2)
pKU31	356–920	–[e]	142 (31)	–	135 (6)	–	(i) 50 (f) (ii) 80 (f)	–	–
pKU33	246–736	38 (37)	–	–	135 (44)	–	(i) 116 (23) (ii) 108 (26)	–	–
pKU9	950–1060	–	–	–	–	0 (21)	(i) 0 (f) (ii) 0 (18)	26 (28)	247 (80)
pKU28	75–181	–	–	1 (47)	–	0 (16)	–	0 (32)	2 (72)
pKU19	44–92	–	–	–	–	0 (8)	–	0 (21)	0 (91)
pKU3		38 (28)	40 (40)	26 (35)	34 (20)	42 (4)	39 (13)	50 (10)	177 (136)
pKU4	1783–3381	0 (34)	0 (17)	0 (55)	0 (0)	0 (30)	0 (20)	42 (47)	158 (88)
pKU2		84 (14)	–	197 (45)	160 (12)	120 (39)	166 (10)	78 (16)	400 (92)

[a]Each plasmid was used as a co-integrate with a disarmed *A.tumefaciens* Ti-plasmid pGV3850Hy, as described by Baker *et al.*, 1987.
[b]These are the *Ac* co-ordinates of the deleted sequences. The *Ac* sequence is numbered from the end nearest to the *Bam*HI restriction site.
[c]Excision is measured as the number of calli resistant to 200 mg/l kanamycin sulphate after infection of 3×10^5 *N.tabacum* SR1 protoplasts with the appropriate *A.tumefaciens* strain. After co-cultivation, half of the protoplasts (1.5×10^5) were selected with kanamycin while one quarter (0.75×10^5) were selected with 15 mg/l hygromycin and the remaining quarter (0.75×10^5) were not selected with antibiotics. The numbers of hygromycin-resistant calli are given in brackets. These dishes were designed to act as controls for the transformation frequency and for the viability of the protoplasts (see Baker *et al.*, 1987). However, the transformation frequencies often appeared much lower after hygromycin selection. Although this provided a reliable means of proving that *Agrobacterium* strains incapable of producing Km-resistant calli (e.g. pGV3850Hy :: pKU4 in experiments 1–6) were indeed able to successfully transform tobacco protoplasts, it proved an unreliable method of measuring the transformation frequency. Similar problems with hygromycin selection have been recently reported (Rogers *et al.*, 1987). As shown in Baker *et al.* (1987), a more accurate method of measuring excision of *Ac* in each experiment is by comparing the numbers of Km-resistant calli found after transformation with pGV3850Hy :: pKU2 and pGV3850Hy :: pKU3. Experiments 1–6 were performed independently with different protoplast preparations. In experiment 6 the infections with pGV3850Hy :: pKU31, pGV3850Hy :: pKU33 and pGV3850Hy :: pKU9 were performed in duplicate. Different cultures of the same *Agrobacterium tumefaciens* isolates were used for experiments 1–5, whereas independent isolates were used for experiment 6.
[d]Excision by transposase provided *in trans* was measured as described above except that protoplasts already containing *Ac* were used in the co-cultivation experiment (see Materials and methods). Experiments 1 and 2 were performed independently with different protoplast preparations. Different cultures of the same *Agrobacterium tumefaciens* isolates were used for these experiments as for the previous experiments 1–5.
[e]– denotes not tested in this experiment.
[f]Plate lost due to bacterial contamination.

determine whether the 5′-end of the first exon is required for transposition, a short deletion was made (in plasmid pKU9) between *Ac* co-ordinates 950 and 1060, removing 75 bp of the 5′-end of the first exon. No Km^r calli were detected when this element was inserted within an NPTII gene and transferred to tobacco, as shown in Table I and Figure 1. The deleted *Ac* element is therefore unable to transpose.

The *Ac* deletion derivative present in pKU9 was also transferred to tobacco protoplasts already containing *Ac*. These protoplasts were made from tobacco strain SR1 30-1 which was Km^s and was reported previously (Baker *et al.*, 1986). In this experiment Km^r calli were detected at high frequency (Table I). The deleted element present in pKU9 can therefore be complemented by an active *Ac*, indicating that it is unable to transpose as a consequence of not expressing a *trans*-acting product required for transposition.

Expression of the product of the 3.5-kb transcript allows AcΔ excision

We wished to determine whether the 3.5-kb transcript encodes a product necessary for transposition, since although we had proven that deletions affecting the 5′-end of the ORF within the transcript abolished transposition, there was still the possibility that alternative splicing of the downstream sequences produced another mRNA necessary for transposition.

A cDNA clone of *Ac* containing the entire ORF and 420 bp of the untranslated leader was inserted between the plant promoter 2′, isolated from octopine TR-DNA (Velten *et al.*, 1984), and a polyadenylation signal, from gene 7 of octopine TL-DNA, in the plant expression vector pPCV720 (Figure 2). This vector also encodes hygromycin resistance (Koncz and Schell, unpublished; see Materials and methods). The cDNA was inserted both in the sense (in pKU38A) and the nonsense orientation (in pKU38B) relative to the plant promoter (Figure 2).

In parallel experiments, tobacco plants were constructed which were stably transformed with pGV3850 :: pKU4 (Baker *et al.*, 1987). The plants contained an *AcΔ* element stably inserted within an NPTII gene, and were Km^s. The element was constructed by removing the 1.6-kb internal *Hin*dIII fragment of *Ac*. The removal of this internal fragment prevents *AcΔ* acting as an autonomous element, but it retains the ability to behave as a non-autonomous element (Baker *et al.*, 1987; Table I).

We reasoned that if the cDNA encodes a function required for transposition its expression in cells containing the *AcΔ* element within the NPTII gene would result in excision of *AcΔ* and a Km^r phenotype. Indeed, after transformation of these plants with pKU38A Km^r calli were detected (Figure 2). As expected none were detected after transformation with the cDNA in the nonsense orientation, in pKU38B. This suggests that excision of the *AcΔ* element occurs only in those cells in which the cDNA is expressed.

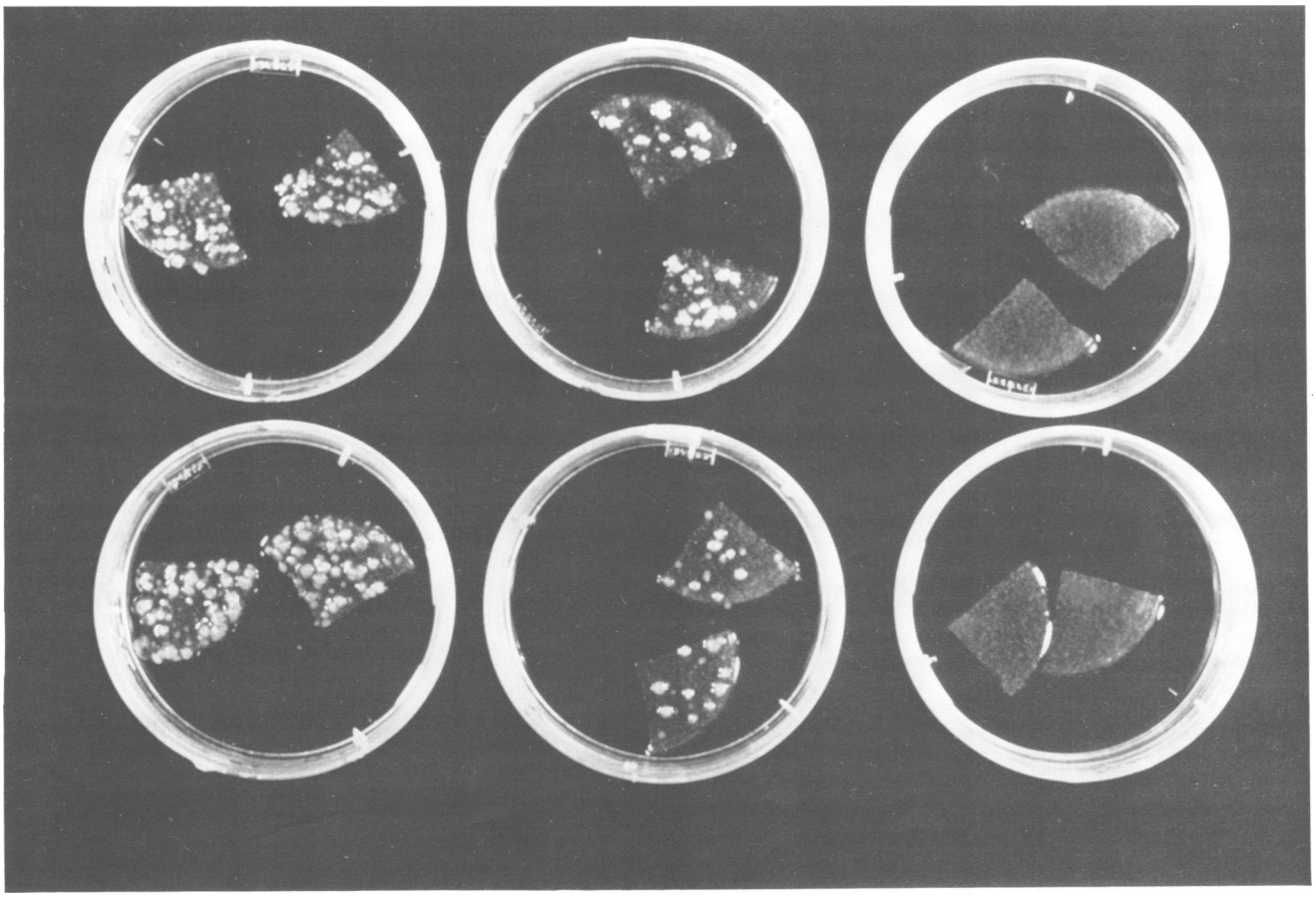

Fig. 1. *N.tabacum* SR1 protoplasts co-cultivated with different *A.tumefaciens* strains and selected in 200 mg/l kanamycin sulphate. These dishes are taken from experiment 6 in Table I. The *A.tumefaciens* strains used carried the following plasmids: **Top row**, left to right pGV3850Hy :: pKU33, pGV3850Hy :: pKU31, pGV3850Hy :: pKU9. **Bottom row**, left to right pGV3850Hy :: pKU2, pGV3850Hy :: pKU3, pGV3850Hy :: pKU4.

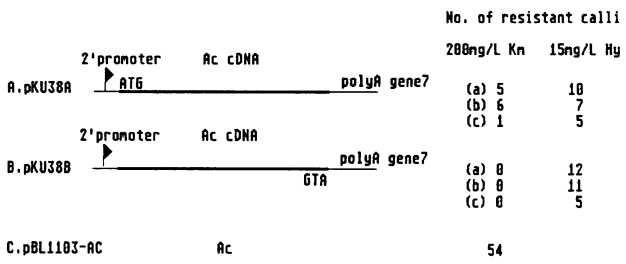

Fig. 2. Schematic representation of the fusion of the *Ac* cDNA to the 2′-promoter. The calli resistant to 200 mg/l kanamycin sulphate were detected after transfer of these constructions to protoplasts previously transformed with the T-DNA of pGV3850 :: pKU4. The construction of pKU38A and pKU38B are described in Materials and methods, and pBL1103-*Ac*, which carries an intact *Ac* element, was described by Baker *et al.*, 1986. This experiment was performed with one batch of protoplasts. The same *A.tumefaciens* isolate containing pKU38A was used to infect the protoplasts in dishes (a) and (b), whereas a second isolate was used in dish (c). Similarly, in the case of pKU38B the same *A.tumefaciens* isolate was used to infect dishes (a) and (b), whereas a second one was used to infect dish (c).

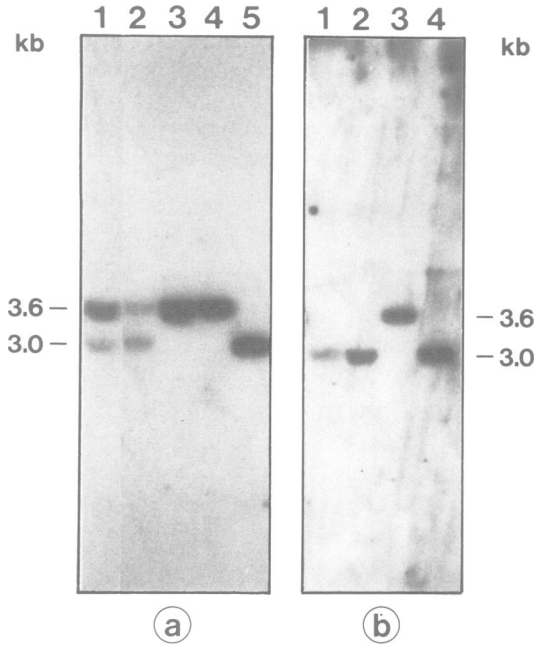

Fig. 3. Panel A. A Southern blot of DNA extracted from hygromycin selected plants transformed with both the T-DNAs of pKU38A and pGV3850 :: pKU4 (**tracks 1** and **2**) or with the T-DNA of pGV3850 :: pKU4 alone (**track 3**). The DNA was cleaved with *Eco*RI and *Hin*dIII. The radioactive probe contained NPTII gene sequences which hybridize only to the pGV3850 :: pKU4 T-DNA. The NPTII containing *Eco*RI–*Hin*dIII fragment in pKU4 is 3.6 kb long and if *Ac*Δ excises the fragment should be 3.0 kb long, almost identical to that present in pKU2 (Baker *et al.*, 1987). **Tracks 1** and **2**: DNA from hygromycin-resistant plants transformed with T-DNAs of pKU38A and pGV3850 :: pKU4. **Track 3**: DNA from a plant transformed only with the T-DNA of pGV3850 :: pKU4. **Track 4:** plasmid DNA of pKU4. **Track 5:** plasmid DNA of pKU2. **Panel B.** A Southern blot of DNA extracted from plants selected for kanamycin resistance and transformed with the T-DNAs of pKU38A and pGV3850 :: pKU4. The DNA was cleaved with *Eco*RI and *Hin*dIII. The radioactive probe contained NPTII gene sequences which hybridize only to the pGV3850 :: pKU4 T-DNA. **Tracks 1** and **2**: DNA from two kanamycin-resistant plants transformed with pKU38A and pGV3850 :: pKU4. **Track 3**: plasmid DNA of pKU4. **Track 4**: plasmid DNA of pKU2.

Similarly, NPTII tests of six calli selected with hygromycin, and therefore containing the T-DNA from pKU38A, revealed expression of NPTII in four calli, whilst in six calli transformed with pKU38B no NPTII signal was detected.

Only a low number of Km^r calli were detected after transformation with pKU38A, and therefore Southern analyses were performed on DNA isolated from calli containing both the T-DNA of pGV3850 :: pKU4 and pKU38A to prove that expression of the NPTII gene was a consequence of *Ac*Δ excision and not of an unexplained DNA rearrangement. In these experiments an NPTII gene probe was used as this hybridizes only to DNA fragments from the pGV3850 :: pKU4 T-DNA, which also contains the *Ac*Δ element. A 3.0-kb fragment hybridizes to the probe after digestion of genomic DNA with *Eco*RI and *Hin*dIII, if the *Ac*Δ element has excised, but a 3.6-kb fragment is detected if *Ac*Δ remains at its original location. In two hygromycin-resistant calli tested which expressed the NPTII gene, a 3.0-kb fragment was indeed detected as well as the 3.6-kb fragment. In one Km-resistant callus transformed with the pKU38A T-DNA the 3.0-kb fragment, but no 3.6-kb fragment was detected. The DNA from plants regenerated from calli transformed with pKU38A was also examined. Four plants regenerated from three hygromycin-resistant calli all possessed the 3.0 kb fragment, indicating *Ac*Δ excision, as well as the 3.6-kb fragment, showing that in some cells *Ac*Δ was still at its original location (Figure 3A). However, two Kanamycin-resistant plants possessed only the 3.0-kb *Eco*RI–*Hin*dIII fragment indicating that *Ac*Δ had excised in all cells (Figure 3B). We conclude that NPTII expression is a consequence of *Ac*Δ excision, and that expression of the ORF present in the cDNA is sufficient for excision of the *Ac*Δ element in tobacco.

Sequences inside the terminal inverted repeats of Ac are required for the response to the Ac transposase

In order to determine whether sequences inside the terminal inverted repeats are required *in cis* for transposition deletions were made near one terminus of *Ac*. The deleted elements were then tested for their ability to excise in the presence of *Ac*. One of the deletion derivatives had lost the *Ac* sequences between bp 44 and bp 92 (pKU19) while the other had lost the sequences between bp 75 and bp 181 (pKU28). These deleted elements were inserted within the NPTII gene and transferred to tobacco protoplasts which either did or did not contain *Ac* (Table I). With pKU19 no Km-resistant calli were detected in either experiment, while with pKU28 a very much reduced number of Km-resistant calli were found (Table I, Figure 4). The sequences inside the inverted repeat must, therefore, be required for passive transposition in the presence of *Ac* transposase.

Discussion

We have constructed and tested a series of deletion derivatives of the maize transposon *Ac* for their ability to excise in transgenic tobacco plants. In addition we have designed assay systems which allowed us to show that the 3.5-kb *Ac* cDNA encodes a product required to activate a deleted *Ac* element, and to determine which sequences are required for transposability in the presence of an active *Ac*.

Our first observation was that the sequences between bp 356 and bp 920 of *Ac* which are located within the

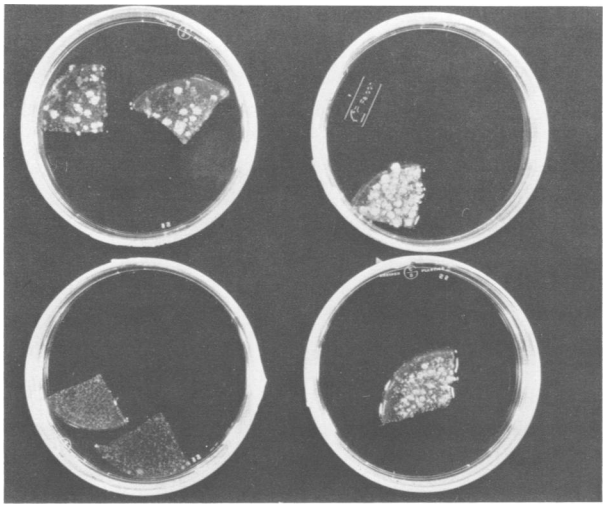

Fig. 4. *N.tabacum* SR1 (*Ac*) protoplasts transformed by *A.tumefaciens* strains carrying pGV3850Hy :: pKU28 or pGV3850Hy :: pKU4, and selected with 200 mg/l kanamycin sulphate or 15 mg/l hygromycin. The dishes in the top row were transformed with pGV3850Hy :: pKU4, the dish on the left was selected with kanamycin, that on the right with hygromycin. The dishes in the bottom row were transformed with pGV3850Hy :: pKU28, the dish on the left was selected with kanamycin, that on the right with hygromycin.

untranslated leader sequence of the 3.5-kb *Ac* mRNA (Kunze *et al.*, 1987) are unnecessary for transposition in tobacco. Indeed, the excision frequencies of the leader deletion pKU31 are consistently higher than those observed for the wild-type *Ac* element in pKU3. While we are aware that the test employed shows experimental variations and that small factors are to be treated cautiously, we also realize that a factor of three would mean excision of *Ac* in 100% of the transformants and therefore cannot be exceeded. It is even possible that the approach to 100% excision does not follow zero order kinetics. We thus do not dismiss the possibility that the leader sequence lowers the transposition activity of *Ac* and are performing experiments to test this directly.

The deleted *Ac* element present in pKU33 has lost the leader sequence and sequences extending 84 bp upstream of the major transcription start site used in maize and tobacco (Kunze *et al.*, 1987; and unpublished results). The plasmid pKU33 is clearly able to produce *Ac* transposase; however, it is not yet clear whether pKU33 produces a transcript initiating from a new position within *Ac*. Another possibility is that the 1′-promoter, which is located upstream of the deleted *Ac* element and expresses the NPTII gene after excision (Baker *et al.*, 1987), allows initiation of a read-through transcript extending into *Ac* and permitting expression of the transposase. Transcripts starting at the 1′-promoter and extending into the *Ac* sequence have been detected in regenerated plants (Feldmar, Kunze and Starlinger, unpublished data), but it is unclear whether these are translated.

Previously, transposition of an unmutated *Ac* was shown to be independent of its orientation relative to the upstream 1′-promoter (Baker *et al.*, 1987), indicating that readthrough transcription from the upstream promoter is not necessary for the transposition of *Ac* when the complete *Ac* promoter is present. Whether the 1′-promoter leads to the synthesis of antisense RNA is not known, but since transposition of *Ac* in those constructs is orientation-independent, an antisense RNA cannot be inhibitory. In any case it is clear that the deleted portion of the untranslated leader does not encode sequences indispensable for transposase expression.

The *Ac* deletion derivatives present in plasmids pKU31 and pKU33 were derived from a plasmid containing an insertion of seven tandem *BglII* linkers at *Ac* position 480 bp, that is within the untranslated leader of the *Ac* mRNA (see Materials and methods). Later the number of linkers was reduced to one. Both elements showed severely reduced excision frequencies (data not shown). While it is possible that this reduction is a consequence of the linker insertion interfering with translation or stability of the mRNA, we cannot presently rule out the possibility that an unknown sequence alteration was introduced in the *Ac* sequence during the ligation steps.

We have also reported that an *Ac* derivative with a 110-bp deletion including the sequences encoding the first 25 codons of the long ORF present in the 3.5-kb *Ac* mRNA was unable to transpose. Though we cannot exclude the possibility that the deletion renders the mRNA unstable, we prefer the interpretation that the first ATG of the ORF is necessary for correct initiation of translation and/or the encoding of amino acids necessary for the function of the protein, supporting the *in vitro* translation data of Kunze *et al.* (1987). Larger deletions in this ORF were detected in *Ds* elements isolated from maize. These deletions remove 2 kb from the interior of *Ac* (Döring *et al.*, 1984), 200 bp of the third exon of the transcript (Pohlman *et al.*, 1984), or most of exon 2 and part of intron 2 (Dooner *et al.*, 1986). However, no *Ds* element isolated from maize contained short deletions in the 5′-region of the ORF (see Döring and Starlinger, 1986). Preliminary experiments (Li, Coupland and Starlinger) suggest that mutations located 3′ to the starting AUG and located within the region encoding the first 110 amino acids do not abolish transposition altogether. It is thus conceivable that the first ATG is necessary for correct initiation and that the unaltered structure of the first protein domain is not a prerequisite for transposition. That an N-terminal domain of a protein may be of only minor importance for its biological function was demonstrated for gene A5 of bovine papilloma virus (Green and Loewenstein, 1987). It is also possible that the deletion has an effect on the stability of the mRNA and thus prevents transposase formation post-transcriptionally.

Further evidence that the ORF present in the long transcript encodes a product necessary for transposition was shown by a cDNA construct in a plant expression vector which was introduced into protoplasts made from plants already containing an internally deleted *Ac* element inserted in the leader of the NPTII gene. These plants were stably transformed with the intention that they should act as an indicator for the transposase activity of *in vitro* constructed *Ac* derivatives. Indeed, the deleted *Ac* element was able to transpose in the presence of the expressed cDNA. This strongly suggests that the 807 aa protein encoded by the mRNA is essential for transposition, although it is formally possible that after expression the mRNA, encoded by the cDNA, is further processed to produce different mRNA species. The (as yet undetected) formation of an additional spliced mRNA molecule from the cDNA could formally be

described as the retention of an intron within the known mRNA (and the cDNA derived thereof). mRNA molecules with 'retained introns' have been described elsewhere (Breitbart *et al.*, 1987). Our experiments suggest that the mRNA encodes the only *Ac* product(s) necessary for transposition, although we cannot rule out that the deleted *Ac* element present in the transformed plants encodes a second function. As the deletion removes the central third of the element (the 1.6-kb *Hin*dIII fragment), and we know that the 564 bp between bp 356 and bp 920 of *Ac* are not required to encode an essential product (see above), it seems likely that the deleted element encodes no active product. However, it would be possible for the deleted *Ac* element to produce a protein of a length of 102 amino acids encoded by a different reading frame located completely within the 5′-end of the long ORF (Kunze *et al.*, 1987). Experiments are in progress to test this possibility by mutating the overlapping ORF, but leaving the longer ORF intact, and also testing whether the cDNA can activate larger internal deletions of *Ac*. With these reservations the most probable explanation of our data is that the 807 aa protein is the only *Ac*-encoded protein required for transposition, although it remains unclear whether host-encoded proteins are required.

During the course of this work it became evident that the cDNA used in these experiments had suffered a deletion at the carboxy terminus (see Materials and methods). Four amino acids have been removed and replaced by six amino acids prior to the new translation stop. Until we have determined the activity of an intact cDNA clone we cannot be certain that the deletion has not caused a qualitative change in the excision frequency of the deleted *Ac*. However, the alteration does not detract from our major observation that the expression of the cDNA results in excision of a deleted *Ac* element, and indeed it reveals that the four amino acids at the carboxy terminus are not essential for transposase activity.

Transformation of the internally deleted *Ac* element into protoplasts made from plants containing *Ac* allowed excision of the mutated *Ac* element, although after transformation to non-transformed tobacco protoplasts the mutated *Ac* element was completely stable. This experiment confirmed that the *Ac* element(s) present in the protoplasts was producing the transposase and allowed us to determine which sequences are required for excision in the presence of transposase produced *in trans*. The *Ac*-containing protoplasts were made from plantlets which were the progeny of a plant regenerated from callus transformed with *Ac* (see Materials and methods). This suggests that *Ac* remains active in tobacco plants in subsequent generations. This observation is supported by the detection of the 3.5-kb *Ac* transcript in the progeny of a selfed tobacco plant transformed with *Ac* (Kunze *et al.*, 1987). We therefore believe that *Ac* can remain active in transgenic tobacco plants through at least one generation.

The introduction of mutated *Ac* elements into the *Ac*-containing plants revealed that sequences inside the inverted repeats are required for transposition even in the presence of the transposase. Most *Ds* elements which transpose in the presence of the *Ac* transposase contain at least 300 bp of *Ac* at each end (see Döring and Starlinger, 1986). However, the *Ds1* element which has been isolated from several mutable alleles in maize (Sutton *et al.*, 1984; Wessler *et al.*, 1986) transposes in the presence of *Ac*, although it only contains short sequences (13 and 19 bp) at the ends that are homologous to *Ac*. They do not have homology to the region of *Ac* deleted in pKU19. Why *Ac* should require sequences for transposition which are not required by *Ds1* is unclear, although it is possible that the short length (405 bp) and high AT base composition (~75%; Sutton *et al.*, 1984; Wessler *et al.*, 1986) of *Ds1* may allow transposition in the absence of the specific sequences present in *Ac*. We are attempting to understand this problem by further defining the sequences required at the ends of *Ac* required for transposition.

Materials and methods

Bacterial strains

Most plasmids to be transferred to tobacco were derived from plasmid pKU3, and were transferred to tobacco via the co-integrate vector Ti plasmid pGV3850Hy (Kreuzaler, unpublished; Baker *et al.*, 1987). They were first conjugated into *Escherichia coli* strain GJ23 (van Haute *et al.*, 1983), and then transferred to *Agrobacterium tumefaciens* strain C58C rif harbouring plasmid pGV3850Hy (Kreuzaler, unpublished results; Zambryski *et al.*, 1983; Baker *et al.*, 1987). The resulting co-integrate plasmids were checked by Southern analysis prior to their transfer to tobacco.

Plasmids pKU38A and pKU38B were based on the binary vector pPCV720 (Koncz and Schell, 1986 and unpublished results). These were transferred to *E.coli* strain SM10 (Simon *et al.*, 1983) and then conjugated with *Agrobacterium tumefaciens* strain GV3101 harbouring plasmid pMP90RK (van Larebeke *et al.*, 1974; Koncz and Schell, 1986). To ensure that the resulting *Agrobacterium* strains indeed contained pKU38A and pKU38B these were transferred back to *E.coli* (strain HB101), plasmid DNA was extracted and the structure confirmed by restriction enzyme analysis.

Construction of E.coli plasmids

In order to make deletions within the untranslated leader of the *Ac* transcript, the smaller *Eco*RI fragment of plasmid pKU3 (Baker *et al.*, 1987) was inserted within the polylinker of pUC9 (Viera and Messing, 1982). The resulting plasmid (pKU8) contained the 1′−2′ promoter and 2485 bp of *Ac* initiating at the end containing the *Bam*HI restriction site and ending at the *Eco*RI restriction site within *Ac*. pKU8 contains three *Eag*I restriction sites, within 40 bp of each other, within the untranslated leader sequence of the *Ac* transcript. pKU8 was linearized by partial digestion with *Eag*I, the linear fragment extracted from an agarose gel, the *Eag*I ends blunt ended with the Klenow fragment of DNA polymerase I and then *Bgl*II linkers (CAGATCTG) were added and the plasmid re-ligated as described by Maniatis *et al.* (1982). The resulting plasmid was called pKU18. Sequence anlaysis revealed that there were seven copies of the linker inserted at the *Eag*I site at position 480 bp in *Ac*. To construct the deletions (finally present in plasmids pKU31 and pKU33) pKU18 was digested with *Bgl*II and then for appropriate times with *Bal*31. After *Bal*31 treatment the ends were blunt ended with the Klenow fragment of DNA polymerase I, and *Bgl*II linkers inserted. This resulted in a series of deletions containing *Bgl*II linkers inserted at the deletion end points. These deletions were analysed by restriction enzyme analysis and then by DNA sequencing. This revealed the end-points of the deletions and that pKU31 contained one copy of the *Bgl*II linker, while pKU33 contained two copies.

To determine whether the alterations to the *Ac* element affect the activity of the element the appropriate *Eco*RI fragments from pKU18 or the deletion derivatives were used to replace the corresponding fragment in pKU3. Plasmid pKU30 contained the larger *Eco*RI fragment from pKU18 while pKU31 and pKU33 contained the *Eco*RI fragments which had been digested with *Bal*31 from two of the deletion plasmids.

pKU8 (described above) was also used to construct plasmid pKU19. Firstly pKU8 was cleaved with *Cla*I which only cuts once 75 bp within the *Ac* sequence, the linearized pKU8 was then digested for varying times with *Bal*31 and *Bgl*II linkers inserted at the deletion end points. The resulting plasmids were screened for small deletions which ought not to have lost the terminal inverted repeat of *Ac*. The deletion end points of one such plasmid were determined by DNA sequencing and was shown to have lost the *Ac* DNA between co-ordinates 44 bp and 92 bp. The appropriate *Eco*RI fragment from this plasmid was used to replace the wild-type fragment in pKU3 to construct plasmid pKU19.

The deletions at the 5′-end of the *Ac* ORF were constructed by first cleaving a pBR322 derivative containing *Ac* with *Mst*II. This enzyme cleaves only once in the plasmid 30 bp within the *Ac* ORF, at bp 1015 within *Ac*. After cleavage the plasmid DNA was digested with *Bal*31, filled in with

the Klenow fragment of DNA polymerase I, ligated and transferred into *E.coli*. The plasmid DNA present in the resulting colonies was screened for loss of the *Acc*I restriction site which is present 64 bp within the ORF. The end points of the deletion present in one of the resulting plasmids were determined by DNA sequencing. The plasmid containing the *Ac* deletion derivative was then cleaved with *Bss*HII, restriction sites for which flank *Ac* (Baker *et al.*, 1987), filled in with the Klenow fragment of DNA polymerase I, and the *Ac*-containing fragment inserted into the blunt ended *Bam*HI site of pKU2 (Baker *et al.*, 1987). The resulting plasmid contained the deleted *Ac* element inserted within the untranslated leader of the NPTII gene in the same orientation as *Ac* in pKU3, and was called pKU9.

pKU28 was also constructed by first making a deletion in an *Ac* element inserted in a pBR322 derivative. The *Cla*I and *Bam*HI restriction sites within *Ac* are unique in this plasmid. In order to construct the deletion, the plasmid was cleaved with *Cla*I and *Bam*HI, blunt ended with the Klenow fragment of DNA polymerase I and religated. The *Ac* deletion derivative was cleaved out of the resulting plasmid with *Bss*HII, the ends of the DNA fragment blunt ended before inserting it into the *Bam*HI restriction site of pKU2. This plasmid, pKU28, contains *Ac* inserted in the same orientation as pKU3.

pKU38A and pKU38B were constructed by inserting a *Bam*HI fragment containing a cDNA clone derived from the 3.5-kb mRNA of *Ac* into the binary vector pPCV720. The cDNA clone was assembled from the partial clones of Kunze *et al.* (1987) and ought to have contained 419 bp of the untranslated leader sequence, the entire ORF and 339 bp after the translational stop. However, subsequent sequence analysis of the fragment revealed that recombination between two direct repeats 7 bp in length resulted in a deletion of 96 bp. This deletion removed the four terminal codons of the ORF, the nonsense codon and 81 bp of the untranslated 3′-tail. pPCV720 is a binary vector which contains within the T-DNA a hygromycin resistance gene active in plants as well as the 1′−2′ promoters of octopine T-DNA followed by polyadenylation signals. The insertion of an ORF between the 1′ or 2′ promoter and the following polyadenylation signal results in a chimeric gene suitable for expression in plants (Koncz and Schell, 1986 and unpublished results). In pKU38A and pKU38B the *Bam*HI fragment containing the cDNA was inserted in both orientations at a unique *Bam*HI restriction site between the 2′-promoter and the polyadenylation signal of gene 7 of octopine T-DNA.

Transformation of tobacco by co-cultivation

The preparation of tobacco protoplasts, their co-cultivation with the *Agrobacterium tumefaciens* strains, and the selection of antibiotic resistant calli were performed as described by Baker *et al.* (1987). Tobacco plantlets were regenerated from callus by growth on MS medium (Murashige and Skoog, 1962) containing 0.5 mg/l kinetin, 0.1 mg/l NAA. Roots were regenerated on 50% MS medium.

Preparation and analysis of DNA from tobacco tissues

Plant DNA was isolated by the method of Taylor and Powell (1983). Tobacco DNA (5−10 μg) was used in each lane. DNA probes were labelled according to the multiprime method of Feinberg and Vogelstein (1983) using the multiprime DNA labelling system marketed by Amersham. The hybridization solution was essentially that of Langer *et al.* (1981): 5 × SSC, 50 mM sodium hydrogen phosphate, 5 × Denhardt's, 250 mg/ml calf thymus DNA, 10% dextran sulphate and 50% formamide The same solution was used for hybridization and pre-hybridization. For hybridization 25 μl hybridization solution/cm^2 was used, and incubated shaking at 42°C overnight.

Plant material

Tobacco protoplasts were isolated from ~6-week-old plantlets of *Nicotiana tabacum* cv petit Havanna SR1.

The *Ac*-containing tobacco plantlets were made in the following way. *Agrobacterium tumefaciens* harbouring the *Ac*-carrying plasmid pGV3850 :: pBL1103-Ac was used to transform SR1 protoplasts and transformed calli were identified as those containing nopaline (Baker *et al.*, 1986). Plants were regenerated from transformed callus line number 30 (Baker *et al.*, 1986). One of the regenerated plants was shown to contain several copies of *Ac* by Southern analysis and this plant was then selfed. The progeny of this plant were assumed to contain *Ac* and were used to make *Ac*-containing protoplasts. In each experiment the presence of *Ac* in the protoplasts was confirmed by its ability to promote excision of the *Ac*Δ element present in pKU4 in an independent transformation assay.

The tobacco plantlets stably transformed with pKU4 were constructed in a similar way. SR1 protoplasts were co-cultivated with *Agrobacterium tumefaciens* containing pGV3850 :: pKU4 (Baker *et al.*, 1987). Transformed calli were identified as those containing nopaline. Plants were regenerated from the calli and re-checked for nopaline. The integrity of the T-DNA in the plants was confirmed by Southern analysis, and then they were selfed. The seeds were germinated, and those seedlings containing nopaline were used to make protoplasts.

DNA sequencing

This was performed by the chain termination method of Sanger *et al.* (1977).

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