Epigenetic Control of a Transposon-Inactivated Gene in Neurospora is Dependent on DNA Methylation

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ABSTRACT

An unstable allele of the Neurospora *am* (GDH) gene resulting from integration of the retrotransposon *Tad3-2* into 5′ noncoding sequences was found in previous work. We report that reversion to Am^+ depends on DNA methylation within and upstream of *Tad*. Levels of methylation were correlated with the proportion of Am^+ conidia, whether the cultures were derived from Am^- or Am^+ isolates. Reversion to Am^+ did not occur when conidia were plated on 5-azacytidine, which reduces DNA methylation. The mutation *dim-2*, which appears to abolish DNA methylation, also prevented reversion to Am^+. The native *am* allele, in a strain that lacked *Tad* elements, was replaced with *am::Tad3-2* or with a deletion derivative that prevents transposition of *Tad*. Transformants of both classes showed instability comparable with that of the original isolates, which contain multiple *Tad* elements. Deletion of the upstream enhancer-like sequences, URS*amα* and *β*, did not prevent the instability of *am::Tad3-2*. The results suggest that *am* expression is dependent on DNA methylation but not on proliferation or transposition of the *Tad* element and that the instability does not require the upstream sequences of *am*.

THE *Tad* element of the filamentous fungus *Neurospora crassa* is a non-LTR retrotransposon similar in structure to the *L1* elements of mammals. In common with other LINE-like elements, it has two long open reading frames with homology to regions of Gag-like and reverse transcriptase-encoding genes of retroviruses and retrotransposons. It also has a 1.7-kb 3′ region of unknown function (KINSEY and HELBER 1989; CAMBARERI *et al.* 1994). Retrotransposition of *Tad* has been demonstrated by both genetic and physical criteria (KINSEY 1990, 1993). *Tad* can transpose in both the vegetative and the sexual portions of the life cycle (KINSEY and HELBER 1989; KINSEY 1990) and provides a good model system for the analysis of events that disrupt gene function. The insertion of *Tad* into the sequence just upstream of the transcriptional start sites of the *am* gene, which encodes NADP-specific glutamate dehydrogenase (GDH), resulted in the *am::Tad3-2* mutation. The original strain carrying this mutation, and most of its *am::Tad3-2* progeny, have a highly unstable $Am^{-/+}$ phenotype (KINSEY and HELBER 1989). *Tad* is typically present in multiple copies and is sensitive to repeat induced point mutation (RIP; KINSEY *et al.* 1994).

Transposon insertions into genes frequently result in the creation of null alleles and also can result in epigenetic alterations in expression of nearby genes, which in some cases correlate with the presence or absence of DNA methylation (FEDEROFF 1989; MARTIENSSEN *et al.* 1990). In this study, we show that the Am^+ phenotype in *am::Tad3-2* strains is correlated with and dependent on increased DNA methylation of sequences within and 5′ of the inserted *Tad3-2* retrotransposon. We also demonstrate that the unstable $Am^{-/+}$ phenotype does not depend on multiple copies of active *Tad* in the genome, a complete *Tad* transposon at the *am* locus or *am* enhancer-like sequences upstream of the *Tad* element.

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MATERIALS AND METHODS

Strains, culture conditions and plating assays: Standard microbiological methods for Neurospora were used (DAVIS and DESERRES 1970). Neurospora strains used in this study are listed in Table 1. To assay plating efficiency on glycine, which inhibits growth of Am^- strains, defined numbers of filtered conidia were grown, on selective (glycine) or nonselective (alanine) medium as indicated, from single conidial isolates. The conidia were suspended in water and spread on nonselective and/or selective sorbose medium to determine the proportion of Am^+ conidia. Colonies were counted after incubation at 32° for 2 days for alanine plates, 3 days for glycine plates or alanine plus 5-azacytidine (5AC) plates, and 4 days for glycine plus 5AC plates.

Southern analyses: Neurospora DNAs were isolated by one of three previously described methods (METZENBERG and BAISCH 1981; OAKLEY *et al.* 1987; CAMBARERI and KINSEY 1993). To extract DNA directly from conidia, glass beads (0.3–0.5 μm diam) were added to the extraction solution of OAKLEY

TABLE 1

Neurospora strains

Strain[a]	Relevant genotype	Other known markers	Source/reference
J1518	*am::Tad3-2*	*lys-1 ure-2 A*	KINSEY and HELBER (1989)
N216	*am::Tad3-2*	*lys-1 ure-2 A*	Vegetative derivative of J1518
N220	*am::Tad3-2*	*ure-2 a*	Vegetative derivative of J1577, a second-generation sexual derivative of J1518
J1603	*am::Tad3-2*	*ure-2 A*	Sexual progeny of J1577 and N150 (see below)
N215	*am::Tad3-2*	*ure-2 A*	Vegetative derivative of J1603
J1594	*am::Tad3-2*	*ure-2 A*	A second-generation sexual derivative of J1518
N24	am^{132}	*inl A*	SELKER *et al.* (1987)
N36	am^{132}	*inl a*	SELKER *et al.* (1987)
N150	Wild type	*A*	74-OR23-IVA; FGSC[b] # 2489
N527	*am::Tad3-2;dim-2*	*inl A*	Sexual progeny of N215 and N507 (FOSS *et al.* 1993)
N538	*dim-2*	*al-2; mus-20; pan-2 cot-1 a*	This paper
N592	*am::Tad3-2;dim-2*	*a*	This paper: sexual progeny of N215 and N536
N597	*am::Tad3-2;dim-2*		This paper: sexual progeny of N215 and N536
N614	*am::Tad3-2;dim-2*	*A*	Sexual progeny of N215 and N536 (FOSS *et al.* 1993)
N620	*am::Tad3-2;dim-2*	*A*	This paper: sexual progeny of N215 and N536
TEC36	am^{+} *hph*[c]	al^{s}	CAMBARERI and KINSEY (1994)
TEC37	*am::Tad3-2 hph*[c]	al^{s}	This paper
TEC39	am^{+} *hph5′Δ*	al^{s}	CAMBARERI and KINSEY (1994)
TEC40	*am::Tad3-2Δ hph*[c]	al^{s}	This paper
TJK10	*ursamα/β Δ am::Tad3-2 hph*[c]	al^{s}	This paper
TEC41	*amΔ hph*[d]	al^{s}	CAMBARERI and KINSEY (1994)

[a] At times, changes in methylation and/or plating efficiency on glycine were observed after vegetative growth of some *am::Tad3-2* strains, perhaps as a result of growth conditions and/or proliferation of *Tad* elements. To help clarify the source of the observations, some strains have more than one identification number.

[b] FGSC, Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, KS 66160.

[c] *hph* is the bacterial gene for hygromycin phosphtransferase, driven by the promoter from the *trpC* gene of *Aspergillus nidulans*. It is integrated downstream of the *am* locus and confers resistance to hygromycin (600 μg/ml).

[d] *hph* in this strain is driven by the 5′ *Tad3-2* promoter. It confers resistance to a low level of hygromycin (200 μg/ml).

et al. (1987) before vortexing for 10 min. Southern blots were performed as previously described (CAMBARERI and KINSEY 1994; MIAO *et al.* 1994). Typically, ~1 μg of genomic DNA was digested under the conditions specified by the manufacturer using a five-to 10-fold excess of enzyme to ensure complete digestion. Restriction digests were fractionated on agarose gels and transferred to Genescreen Plus (NEN) or Zetabind (Cuno) nylon membranes. Hybridization probes were made by the random primer method (FEINBERG and VOGELSTEIN 1984). All conclusions were based on DNA samples that were digested to completion, as verified by rehybridization of Southern blots with a probe of an unmethylated gene. Approximate methylation levels were determined by estimating the proportion of sites left uncleaved by a methylation-sensitive enzyme. Where indicated, the estimates were quantitated using an optical imaging system (Ambis Inc.). Assays for methylation proficiency were performed as previously described (FOSS *et al.* 1993).

Targeted gene replacements: Transformations and selection for gene replacement events at the *am* locus and construction of pEC37 were as described previously (CAMBARERI and KINSEY 1994). Plasmid pEC40, which has an internal deletion of *Tad3-2,* was constructed by religation of pEC37 digested with *Sma*I. Plasmid pJK10 was constructed by replacing the 0.5-kb *Hin*dIII-*Xba*I fragment of pEC37 with a 2.3-kb *Hin*dIII-*Sma*I fragment derived from *am* 5′ noncoding sequences between −4.4 and −6.7 kb upstream of the *am* transcriptional start site. Targeted introduction of this plasmid results in a strain with a modified *am::Tad3-2* allele that lacks the *am* 5′ noncoding sequences between −0.9 and −4.4 kb with respect to the transcriptional start site. This deletion removes both of the upstream enhancer-like elements, URS*amα* and URS*amβ* (FREDERICK and KINSEY 1990a,b). The initial *N. crassa* transformants were purified by streaking to render them homokaryotic with respect to *am:* (as determined by Southern analysis), but the purified cultures still contained a mixture of nuclei in Am^{-} and Am^{+} expression states. Cultures were enriched for Am^{+} or Am^{-} as follows. Conidia were streaked on a plate containing minimal sorbose medium plus glycine to select for am^{+} function. Well-isolated and vigorously growing hyphal colonies were picked into slants with glycine minimal medium. Similarly, non- or weakly growing conidial isolates from the same plate were picked to complete medium and allowed to conidiate in the absence of selection, resulting in predominantly Am^{-} cultures.

GDH assays: Conidia from cultures that had been enriched for either the Am^{+} or Am^{-} phenotype were inoculated (2×10^{5}/ml) into flasks of liquid minimal medium supplemented with alanine (50 μg/ml). Flasks were grown with shaking at 25° for 16–24 hr. Mycelia were harvested and lyophi-

TABLE 2

Reversion of *am::Tad3-2* strains

Strain	Reversion on glycine[a]	Reversion on glycine[a] + 5AC
J1518 *(lys-1 am::Tad302)*	33.2 ± 9.4	0.0
N527 *(am::Tad3-2;dim-2)*	0.0	0.0
N597 *(am::Tad3-2;dim-2)*	0.0	0.0
N614 *(am::Tad3-2;dim-2)*	0.0	0.0
N620 *(am::Tad3-2;dim-2)*	0.0	0.0
TEC37 *(al^s;am::Tad3-2)*	32.4 ± 12.2	0.0
TEC40 *(al^s;am::Tad3-2Δ)*	212 ± 81	0.0[b]
TJK10 *(al^s;URSamα/β Δ am::Tad3-2)*	26.4 ± 22[c]	Not done

[a] Reversion frequency per 10^4 survivors. For TEC37 and TEC40, individual Am$^-$ isolates were assayed from each of three independent transformants. Conidia from a total of four TEC37 isolates, five TEC40 isolates and two TJK10 isolates were plated in duplicate or quadruplicate, and the mean n ± 2 SD of colonies produced on glycine was tabulated. Reversion for J1518 was similarly calculated based on the mean from 38 plates from four separate experiments. Each of the *dim-2* strains was tested in quadruplicate.

[b] TEC40 exhibited weak background growth on glycine 5AC plates.

[c] Colonies formed by TJK10 were less vigorous than colonies formed by other strains.

lized before extraction. GDH extraction and assays were performed essentially as described (CODDINGTON *et al.* 1966), except that extracts were filtered through Sephadex G-50 using a spin-column. Protein concentrations were determined by a dye-binding assay using reagents purchased from Biorad.

RESULTS

Instability of the Am$^-$ phenotype in *am::Tad3-2* strains depends on DNA methylation: The *am::Tad3-2* mutant J1518 was initially selected as a spontaneous Am$^-$ isolate of a strain derived from a cross of a laboratory strain and the *Tad* containing strain Adiopodoumé (KINSEY and HELBER 1989). Surprisingly, J1518 gave rise to Am$^+$ conidia at a high frequency (Table 2). Clonal isolates from Am$^+$ derivatives appeared to yield Am$^-$ conidia at even higher frequencies. Because *Tad* is a non-LTR retrotransposon, and because both the Am$^+$ and the Am$^-$ phenotypes are reversible, it appeared unlikely that excision of the element from the site of integration could account for the instability of the phenotype. Indeed, analysis by Southern blot of >100 derivatives isolated either on selective medium or nonselectively yielded no evidence of excision (KINSEY *et al.* 1994; data not shown).

DNA methylation is associated with epigenetic processes in many organisms. For example, inactive, but potentially transposable, elements in maize are frequently hypermethylated (see FEDOROFF 1989). We therefore explored the possibility that the instability of the *am::Tad3-2* phenotype might involve differential methylation of *am* and/or *Tad* sequences in Am$^+$ and Am$^-$ isolates. To test this possibility, we first examined the effect of 5-azacytidine (5AC) on *am* expression in *Tad3-2* strains. 5AC is a cytosine analog that prevents methylation of cytosines (SANTI *et al.* 1983). In Neurospora, this drug can result in loss of ≥90% of C methylation at a concentration of 24–48 μM (SELKER and STEVENS 1985). We found that in strain J1518, 25 μM 5AC caused ~50–60% killing, but completely abolished reversion to Am$^+$, suggesting that *am* expression in *Tad3-2* strains is dependent upon the presence of DNA methylation (Table 2). In control experiments with *am*$^+$ strains, 5AC did not interfere with *am* expression (data not shown).

The 5AC-induced loss of the high reversion frequency could be due to the loss of cytosine methylation, or to some indirect effect of 5AC such as the induction of DNA repair processes and/or a change in chromatin structure. To distinguish between these possibilities, we tested the behavior of strains with the *am::Tad3-2* allele in a *dim-2* background. The *dim-2* mutation results in the loss of detectable cytosine methylation (FOSS *et al.* 1993). The *dim-2* strains N507, N536 and N538 were crossed to an *am::Tad3-2* strain (N215, a *lys*$^+$ sexual derivative of J1518; Table 1). If normal segregation occurs and if lack of methylation confers a stable Am$^-$ phenotype on *am::Tad3-2* strains, 25% of the progeny of these crosses should be stably Am$^-$ due to the presence of both *dim-2* and *am::Tad3-2.* Additional stably Am$^-$ progeny may be expected because any cross of an *am::Tad3-2* strain (*e.g.,* Table 3, cross 4) segregates some stably Am$^-$ progeny, possibly due to extension of RIP (repeat-induced point mutation) into the unduplicated *am* sequences (FOSS *et al.* 1991; IRELAN *et al.* 1994; KINSEY *et al.* 1994). In crosses between strains with the *am::Tad3-2* allele and *dim-2*$^+$ strains, we obtained 2–10% stable Am$^-$ progeny (Table 3, cross 4). In contrast, >25% of the progeny from crosses between N215 and *dim-2* strains were stably Am$^-$ (Table 3, crosses 1–3). To determine if this stable Am$^-$ phenotype is associated with the presence of *dim-2,* we scored 12 apparently stably Am$^-$ progeny for DNA methylation at two normally methylated loci, *ζ-η* and *ψ63* (FOSS *et al.* 1993), and for the presence or absence of *Tad3-2.* All 12 progeny were *am::Tad3-2;dim-2* strains (data not shown). Twelve control progeny with the unstable Am$^{-/+}$ phe-

TABLE 3

Influence of methylation on the Am phenotypes of the *am::Tad3-2* allele

Cross	Parents	Am^+ progeny[a]	Stably Am^- progeny
1	N536 *dim-2* × N215 *am::Tad3-2*	50	18
2	N507 *dim-2* × N215 *am::Tad3-2*	37	30
3	N538 *dim-2* × N215 *am::Tad3-2*	29	11
4	N150 wildtype × J1594 *am::Tad3-2*	49	5
5	N592 *am::Tad3-2;dim-2* × N24 am^{132}	12	38
6	N620 *am::Tad3-2;dim-2* × N36 am^{132}	11	38

[a] Includes both stably Am^+ and $Am^{+/-}$ progeny.

notype were all *am::Tad3-2;dim* strains. Plating efficiencies on glycine of the unstable strains ranged from 0.1 to 17%.

If the *dim-2* mutation were responsible for preventing *am* expression in the stably Am^- *am::Tad3-2* strains, the unstable $Am^{-/+}$ phenotype should reappear among the progeny when these strains are crossed with $dim\text{-}2^+$ strains. To test this prediction, two of the 12 *am::Tad3-2;dim-2* strains isolated above, N592 and N620, were crossed with am^{132};$dim\text{-}2^+$ strains N24 and N36, which carry a deletion that removes the entire *am* gene (KINSEY and HUNG 1981). Approximately 25% of the progeny from a cross between strains N592 and N24 showed some growth on glycine (Table 3, cross 5). A cross between strains N620 and N36 gave similar results (Table 3, cross 6). Crosses between two *dim-2* parents, one carrying the *am::Tad3-2* allele and the other carrying the *am* deletion am^{132}, performed to control for possible effects resulting simply from passage through a sexual cycle, failed to produce mature perithecia (H. FOSS and E. SELKER, unpublished data). Because *am::Tad3-2*-bearing strains may also segregate progeny that are stably Am^+, perhaps due to RIP of *Tad* sequences (KINSEY *et al.* 1994), we also tested the stability of the Am^+ phenotype in five progeny strains from cross 5. Assays for growth on nonselective (alanine) and selective (glycine) media indicated that all five strains had produced Am^- conidia. Verification that the $Am^{-/+}$ strains were dim^+ and contained the *am::Tad3-2* allele came from analysis of the *am* locus in DNA samples from 11 progeny from cross 5. As expected, all 11 contained the *am::Tad3-2* allele and showed methylation at the *am* locus (data not shown). These observations indicate that the *am::Tad3-2* allele retained the potential for epigenetic switching while in the *dim-2* background. The failure of the *am::Tad3-2* allele to yield Am^+ revertants when grown on 5AC or in the *dim-2* genetic background indicates that reversion to Am^+ requires DNA methylation.

DNA methylation within and 5′ of *Tad3-2*: The effects of 5AC and the *dim-2* mutation show that DNA methylation is an essential factor that allows the expression of *am* in *am::Tad3-2* strains. The implication is that differential DNA methylation controls the state of Am expression. We wanted to know if the DNA sequences involved in this differential methylation were located within *am* or *Tad* sequences. For most of our studies, we used J1518 (or its vegetative reisolate N216) or J1577 (or its vegetative reisolate N220), which was derived from J1518 by two crosses with a wild-type strain. All of these strains showed the unstable $Am^{-/+}$ phenotype, although they sometimes displayed differences in the efficiency of producing Am^+ isolates. Normally, both wild-type and defective alleles of *am* are unmethylated (MIAO *et al.* 1994). Examination of the unstable $Am^{-/+}$ *am::Tad3-2* cultures, however, revealed evidence of methylation in upstream *am* sequences and in the adjacent *Tad* DNA (summarized in Figure 1A). Data obtained using the methylation-sensitive enzymes *Bam*HI, *Bsp*106I, *Bgl*II, and *Xba*I, for example, show evidence of methylation: unexpected higher molecular weight bands and a reduction in the intensity of expected lower molecular weight bands (Figure 1, B and C). Data are shown for *am::Tad3-2* strains N216 and N220, and for Am^+ isolates from each of these strains (N216-G1 and G2, N220-G1 and G2). The unstable Am^+/Am^- strains both showed methylation, although N216 showed less than N220. Furthermore, methylation at sites within the 5′ region of *Tad3-2* and *am* sequences just upstream of *Tad* was increased in the Am^+ revertants grown on selective medium. Sites in the 3′ region of *Tad3-2* were lightly methylated in the Am^+ revertant N220-G1, but not in N220, N216, or N216-G1. Reprobing of the blots from Figure 1B (data not shown) and Figure 1C with part of the coding region of *am* demonstrated that the sites 3′ of Tad were not methylated (Figure 1D). Similar analyses were conducted for *Eco*RI and *Pvu*II sites (data not shown). The higher level of methylation in the 5′ region of *Tad3-2* coincided with higher plating efficiency on glycine (~0.35% in N220 as compared with ~0.07% in N216). These data suggest that methylation is associated with *am* expression and that such methylation occurs in the 5′ region of *Tad 3-2* and *am* sequences upstream of *Tad*.

Stability of *am::Tad3-2* methylation: The increase in methylation associated with *am* expression occurred only during the first round of selection. Further growth on selective medium through up to four more cycles

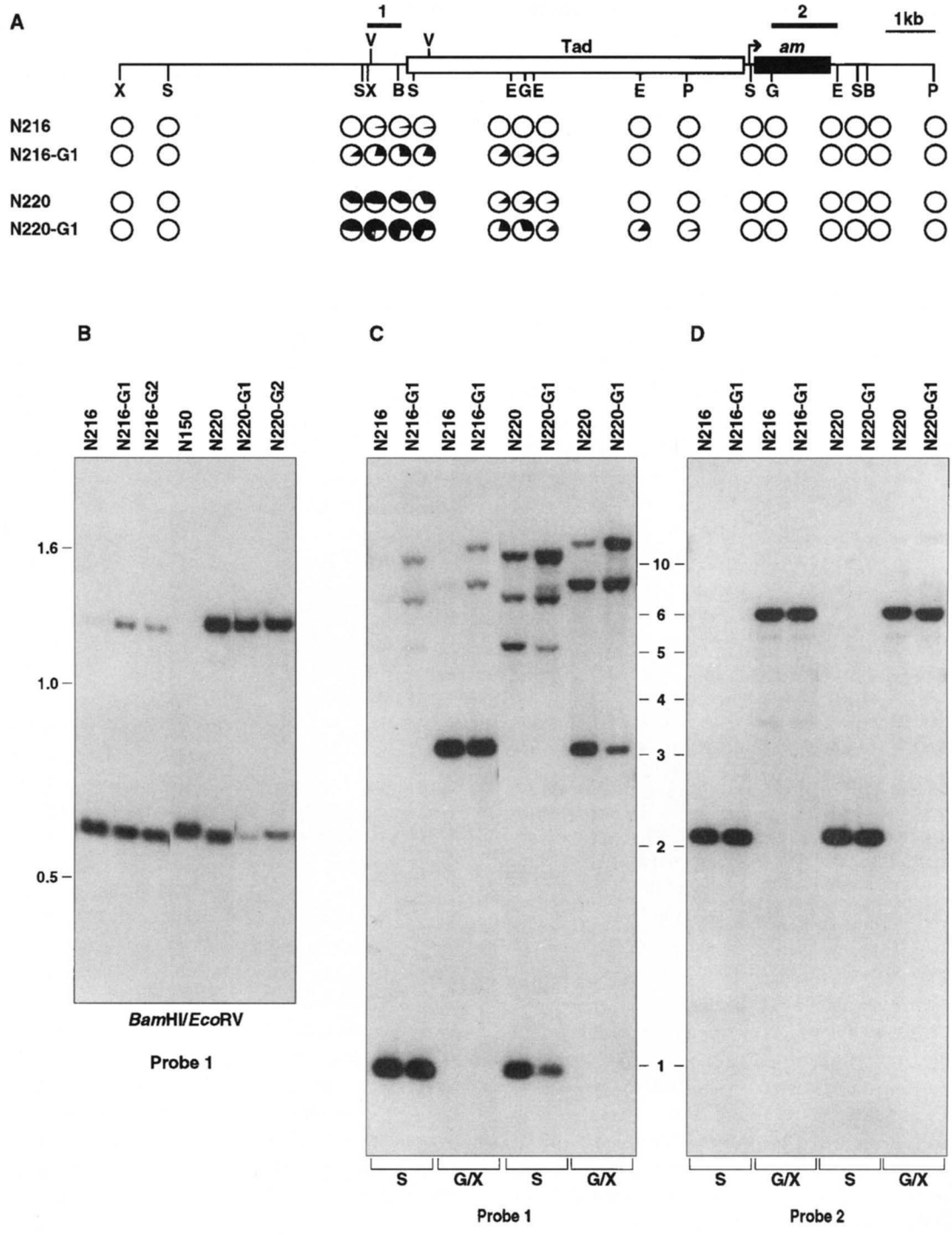

FIGURE 1.—Methylation associated with the Am^+ phenotype of the *am::Tad3-2* allele. (A) Map of the *am::Tad3-2* allele. The *Tad* element (open box) is inserted upstream of the transcription start sites (arrow) of *am* (black box). The recognition sites for the methylation-sensitive restriction enzymes *Bam*HI (B), *Bgl*II (G), *Bsp*106-I (S), *Eco*RI (E), *Pvu*II (P), and *Xba*I (X) are indicated below the horizontal line; the recognition sites for the methylation-insensitive restriction enzyme *Eco*RV (V) are indicated above it. Only the relevant restriction sites are shown. The location and sizes of probes 1 and 2 are indicated above the map. The approximate degree of methylation at each restriction site (except *Eco*RV) is depicted in black in a pie chart below each site for strains N216 and N220, and one Am^+ derivative from each (N216-G1 and N220-G1, respectively). (B) Southern blot of genomic DNAs from strains N216 and N220, and two Am^+ derivatives from each (N216-G1 and N216-G2; N220-G1 and N220-G2, respectively) digested with *Bam*HI and *Eco*RV and hybridized with probe 1. Wild-type strain N150 served as a control. (C and D) Southern blots depicting *Bsp*106-I and *Bgl*II/*Xba*I digests of strains N216, N216-G1, N220, and N220-G1 hybridized to probe 1 (C) or probe 2 (D). The *Bgl*II site 3′ of *am* (not shown) is 6 kb from the *Bgl*II site in *am*. Size standards are indicated in kilobases to the left of B and between C and D.

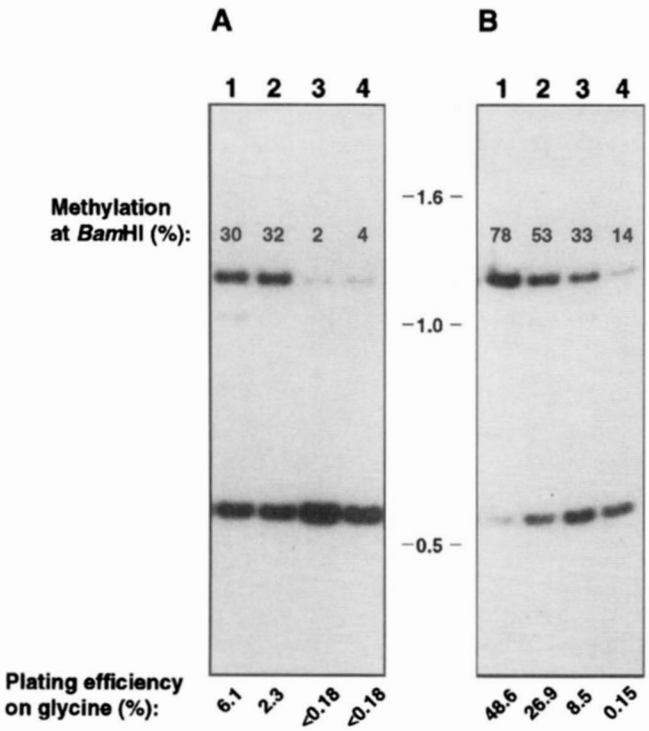

FIGURE 2.—Stability of methylation and associated Am^+ phenotype of the *am::Tad3-2* allele. Southern blots of genomic DNA extracted from conidia, digested with *Bam*HI and *Eco*RV, and hybridized to probe 1 (see Figure 1A). DNA digests were derived from an Am^+ revertant of strain N216 (A) or strain N220 (B) after growth on glycine (lane 1) or alanine (lane 2). From each culture grown on nonselective medium, two conidia were isolated and, again, grown on nonselective medium for further analysis (lanes 3 and 4). The approximate methylation level at the *Bam*HI site (percent) is shown above the 1.2-kb band for each isolate. The plating efficiency on glycine (percent) is displayed below the blots for each isolate. Size standards are indicated in kilobases between the panels.

of conidiation did not change the overall methylation observed with either N216 or N220 (data not shown). Similarly, the lower levels of methylation observed in the absence of selection did not change as a result of passaging (data not shown). We wanted to determine whether the increase in methylation in the Am^+ revertants would be maintained on nonselective medium. Thus, we tested for maintenance of methylation at the *Bam*HI site 5′ of *Tad* in strains N216 and N220. Colonies derived from Am^+ revertants that had been passaged on selective (glycine) medium five times were grown on glycine and divided in half. Half of each colony was allowed to grow and conidiate on selective medium; the other half was propagated on nonselective medium. The resulting conidia were then assayed for methylation at the *Bam*HI site 5′ of *Tad3-2* (Figure 2, A and B, lanes 1 and 2). Two colonies derived from the conidia that had been grown nonselectively were picked off alanine plates and put through a second cycle of nonselective growth. Again, the resulting conidia were tested for methylation (Figure 2, A and B, lanes 3 and 4). Methylation associated with *am::Tad3-2* in the Am^+ derivative of N216 was maintained for one round of nonselective growth (Figure 2A, lane 2) but dropped during a second round of nonselective growth (Figure 2A, lanes 3 and 4) to levels approximating those seen in N216 Am^- isolates (Figure 1). Methylation in the Am^+ derivative of N220 dropped after the first round of nonselective growth (Figure 2B, lane 2) and continued to drop during the second round of nonselective growth (Figure 2B, lanes 3 and 4) to levels below those observed for Am^- isolates of N220 (Figure 1). Tests for plating efficiency on glycine of the conidia used for each DNA analysis showed a direct correlation between methylation and the Am^+ phenotype (Figure 2). Thus, in the absence of selection, the Am^+ nuclei gave rise to predominantly Am^- cultures with decreased levels of methylation. Nevertheless, the residual elevation in methylation after one round of growth on nonselective medium indicates that methylation, once initiated, remained somewhat stable through an estimated 25 nuclear divisions.

Targeted replacement of *am*$^+$ by the *am::Tad3-2* allele in a strain devoid of *Tad*: We wished to know whether the presence of other *Tad* elements in the strains analyzed above played a role in the observed epigenetic effect. Numerous cases have been reported in which the copy number of transposable elements or transgenes had affected gene expression. In maize, for example, the expression of genes that have an insertion of a defective *Spm* transposon can be controlled by the presence of other, active *Spm* elements in the genome (McCLINTOCK 1961, 1971). It is also known that in Neurospora, and in some plants, extra copies of a gene added to the genome via DNA-mediated transformation can result in the loss of expression of all copies of that gene during vegetative growth (PANDIT and RUSSO 1992; ROMANO and MACINO 1992). This effect is in some cases associated with DNA methylation (for review, see MATZKE and MATZKE 1993).

To determine if multiple copies of a *Tad* sequence are required for the unstable phenotype of *am::Tad3-2* strains, we made use of a system that allows simple targeted gene replacement of sequences at the *am* locus without requiring expression of *am* (CAMBARERI and KINSEY 1994). In this way, we could directly test if a single copy of the transposon upstream of the *am* coding sequences is sufficient to produce the unstable phenotype. Figure 3A shows the strategy used to replace the chromosomal *am* gene with a cloned copy of the *am::Tad3-2* allele in a strain that has no sequences that hybridize with *Tad* at high stringency. Three transformants, TEC37-12, -13, and -15, were analyzed. We also used the plasmid pEC40, which contains a deletion within *Tad*, to construct a strain (TEC40-1) with a single *Tad* element that is incapable of transposition. This deletion (between *Sma*I sites 2130 bp from the 5′ end and 620 bp from the 3′ end of *Tad*) removes both the internal *Bgl*II site and the reverse transcriptase coding

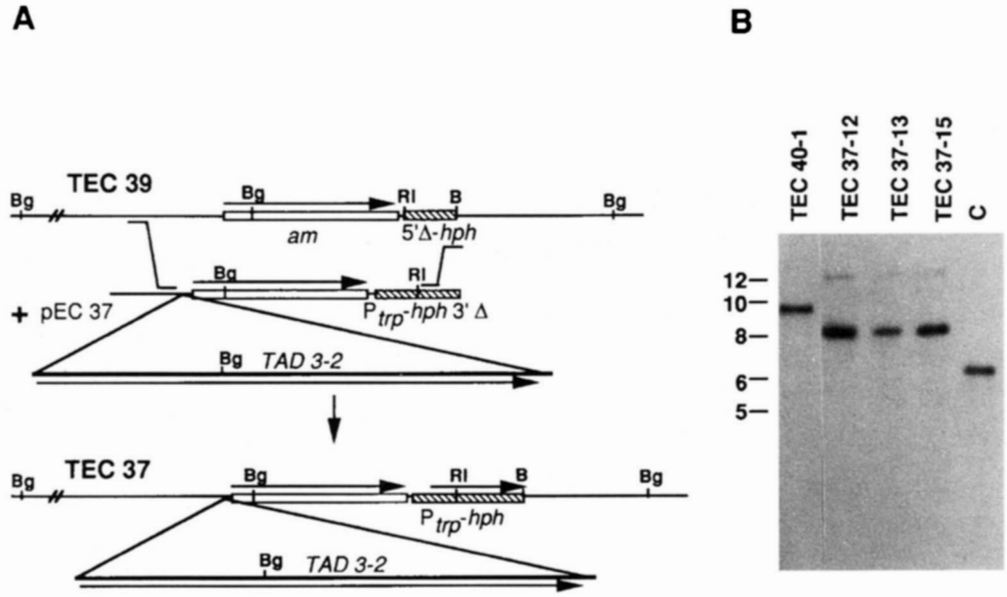

FIGURE 3.—Gene replacement at the *am* locus of TEC39 using plasmid pEC37 or pEC40. (A) Selection for hygromycin resistance depends on joining two truncated halves of the *hph* gene by a homologous recombination event (CAMBARERI and KINSEY 1994), as shown for pEC37. A second recombination event upstream of *am* will result in gene replacement. Transformants with the plasmid pEC40, which contains a *Tad* internal deletion that removes the *Bgl*II site, were made in the same way. P_{trp} is a fragment of DNA containing the promoter region of the *Aspergillus nidulans trpC* gene. *Hph* refers to the bacterial hygromycin phosphotransferase gene. Restriction sites for *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (RI) are indicated. (B) *Bgl*II digests of genomic DNA from transformants TEC40-1, TEC37-12, TEC37-13, TEC37-15, and from the transformation host TEC39 (C) were fractionated, blotted and hybridized with a *Hin*dIII fragment immediately adjacent to the *am* upstream sequences present in the targeting vector. The increase in the size of the upstream *Bgl*II fragment is as expected for insertion of the pEC37 or pEC40 fragments.

portion of *Tad*. Southern blots of genomic DNA from the transformants confirmed that the expected single copy gene-replacement events at *am* had occurred (Figure 3B). The higher molecular weight bands, of lighter intensity, seen in the TEC37 lanes are presumably due to blockage of digestion of the internal *Tad Bgl*II site by DNA methylation. This interpretation was supported by additional probings of the blot with labeled fragments from nearby regions (data not shown). Hybridization with *Tad* DNA revealed up to three new bands of subgenomic intensity in the TEC37 strains presumably due to new transpositions during passages for purification and for DNA isolation. As expected, no evidence of transposition was seen in the TEC40 strain (data not shown).

Instability of Am in strains transformed with *am::Tad3-2* or its derivatives: We wished to test whether the strains containing the *am::Tad3-2* gene replacements would show Am instability equivalent to that of the original *Tad3-2* insertion mutant. We had previously shown that the presence of the *trpC/hph* construct in the 3′ flanking sequences of the *am* gene had no effect on *am* expression (CAMBARERI and KINSEY 1994). To test for instability, we plated the transformants on selective medium (glycine) with or without 5AC. The transformants, like the original *am::Tad3-2* strain, showed high reversion frequencies. As with the original strain, 5AC prevented reversion in the transformants tested (Table 2).

We then asked if activity of GDH in the transformants was similar to that of the original mutants. Table 4 shows typical results of GDH enzyme assays from Am^+ and Am^- isolates of one TEC40 transformant, two TEC37 transformants, and control strains. Am^- isolates had extremely low levels of GDH, while Am^+ isolates showed levels comparable with Am^+ revertants of the original mutant strain J1518 (15–24% of wild type). The instability of the $Am^{-/+}$ phenotype of the TEC transformants indicates that multiple active copies of *Tad* are not necessary for reversion to occur. They also show that the protein products of the ORF2 region of *Tad* are not necessary for the reversion to occur, since this region is almost completely deleted in the TEC40 strains. As with the original *am::Tad3-2* strains, Am^+ isolates of all of these transformants showed methylation of sequences at the 5′ end of *Tad* (data not shown).

The *am* gene has two enhancer-like elements, *UR-Samα* and *β*, that are 1.3 and 2 kb upstream, respectively, of the transcriptional start sites (FREDERICK and KINSEY 1990a,b). Deletion of either element results in a 50% decrease in *am* transcription and GDH activity. Deletion of both results in an 85–90% decrease in GDH levels. These elements seem to act as simple drivers of *am* expression with little or no effect on the regulation of *am* expression (CHEN and KINSEY 1994). Since the Tad element in the *am::Tad3-2* allele is inserted between the upstream enhancer elements and the basal promoter of *am*, we wanted to determine if the en-

TABLE 4

Glutamate dehydrogenase activity of targeted transformant strains

Strain	Glutamate dehydrogenase activity (% of TEC36)[a]	
	(+)[b]	(−)[b]
TEC36	100	100
TEC37-13	29	02
TEC37-15	24	03
TEC40-1	38	04
TJK10-1	09	01
TJK10-2	09	02

[a] Each GDH value is the mean of duplicate or triplicate repeats of specific activity determinations from a given strain compared to the specific activity for the TEC36 strain. All cultures used for GDH assays were grown on minimal medium supplemented with alanine (nonselective conditions). TEC36 is a transformant strain with the wild-type *am* gene precisely reconstructed. It has the same genetic background as the other transformant strains.

[b] The designations (+) and (−) refer to cultures enriched for Am^+ or Am^- nuclei as described in the MATERIALS AND METHODS. TEC36 strains do not undergo epigenetic switching, so the values used for controls in both sets were the same.

hancer-like sequences are functional in this allele and if they influence its instability. We therefore tested if deletion of the upstream sequences would prevent the instability. The plasmid pJK10, which carries a modified *am::Tad3-2* allele that lacks URS*amα* and URS*amβ*, was targeted to *am* to create strains TJK10-1 and TJK10-2. Reversion analysis of these strains demonstrated instability of the Am phenotype similar to that seen in strains in which the enhancers-like elements were intact (Table 2). Two significant differences were evident, however. First, the reversion frequency for TJK10 strains was more variable. Second, the Am^+ colonies derived from TJK10 strains were less vigorous than Am^+ colonies derived from TEC37 strains. Consistent with this observation, the levels of GDH expression in Am^+ isolates derived from TJK10 strains were less than half those of TEC37 strains and only ~9% of wild-type strains (Table 4), suggesting that insertion of *Tad3-2* did not prevent the enhancers from functioning.

DISCUSSION

We have documented that the Am^- phenotype of the *am::Tad3-2* allele is unstable, reverting to Am^+ at high frequencies. Mutant cultures were enriched for Am^+ revertants by growth on selective medium. The increase in the proportion of Am^+ conidia in a culture was correlated with increased DNA methylation near the 5′ end of the transposon and with an increase in GDH activity. Methylation was light or absent at the 3′ end of *Tad3-2* and completely absent in the coding region of *am*. In the absence of selection, the levels of methylation associated with the Am^+ phenotype remained high through one round of growth (~25 nuclear divisions) but eventually decreased.

Factors other than growth conditions also had an influence on the level of methylation at *am::tad3-2*. For example, strains N216 and N220 typically differed from each other with respect to reversion frequencies and methylation levels at the *am* locus even when they were grown under similar conditions. The differences could be due to RIP mutations in *Tad* (KINSEY *et al.* 1994), although sequence analysis of a ~200-bp segment at the 5′ end of *Tad3-2* in N220 showed no evidence of RIP (M. ROUNTREE and E. SELKER, unpublished results).

Cultures in which DNA methylation was prevented, either through addition of the drug 5AC to the growth medium or by means of a mutation, *dim-2*, were unable to generate Am^+ revertants. Such cultures, or their dim^+ *am::Tad3-2* sexual progeny, regained the ability to yield unstable Am^+ revertants when methylation proficiency was restored by growth on medium lacking 5AC, or by introduction of the dim^+ allele through a cross, respectively. These results demonstrate that the observed sporadic alleviation of the mutant phenotype of *am::Tad3-2* strains is dependent on DNA methylation.

A possible source of the unstable methylation of the *am::Tad3-2* allele lies in the *Tad* element itself. It has been suggested that methylation serves to restrict the movement of mobile elements by inactivating them (CHANDLER and WALBOT 1986; BESTOR 1990). In humans, LINE elements are subject to DNA methylation (THAYER *et al.* 1993). In plants, inactivation of transposons has been shown to be associated with methylation of the element (CHANDLER and WALBOT 1986; CHOMET *et al.* 1987; FEDEROFF *et al.* 1988; FEDEROFF 1989; MARTIENSSEN *et al.* 1990), although the causal relationship between DNA methylation and transposon inactivation is unclear. Inactivation as a consequence of methylation could result from the methyl adduct in the major groove interfering with the binding of proteins directly, or indirectly through the presence of methyl-DNA binding proteins (IGOUCHI-ARIGA and SCHAFFNER 1989; MEEHAN *et al.* 1989; BOYES and BIRD 1991), causing heritable gene repression (HOLLIDAY and PUGH 1975). Conversely, it is possible that DNA methylation is a consequence of gene inactivation.

In attempts to identify a sequence or function of *Tad* that could influence methylation of the *am::Tad3-2* locus, we have shown that limiting the number of *Tad* elements in the genome to a single copy of *Tad3-2* does not eliminate methylation-dependent reversion from Am^- to Am^+. Moreover, deletion of the *Tad* sequences that encode the reverse transcriptase did not abolish the instability of the Am^- phenotype. These results suggest that any methylation-related inactivation of *Tad* could not simply be regulated by the number of elements in the nucleus or by their transposition activity. They also argue against repeat-dependent gene silenc-

ing mechanisms (MATZKE and MATZKE 1993). Finally, deletion of the upstream enhancer-like elements, URS*amα* and *β*, did not eliminate the instability of the Am$^-$ phenotype, supporting the idea that these enhancer-like regions are not involved in triggering methylation of the *am::Tad3-2* allele.

It is possible that the origin and instability of methylation at the *am::Tad3-2* locus is unrelated to *Tad* function. A signal triggering methylation could, for example, be inherent in a DNA sequence created by insertion of the *Tad* element. Such signals are common in sequences altered by RIP (SELKER *et al.* 1987, 1993), but the methylation associated with RIP is generally stable. If methylation at the *am::Tad3-2* locus were unrelated to *Tad* function, its sporadic occurrence coupled with its persistence through ~25 rounds of replication could be accounted for by a weak methylation signal combined with an imperfect system of "maintenance methylation" such as that implicated for Neurospora by the work of SINGER *et al.* (1995).

There are several possible mechanisms for the reversible silencing of *am* expression caused by the *am::Tad3-2* mutation. One possibility is that, when the allele is unmethylated, *Tad3-2* sequences are transcribed causing interference with *am* transcription. Methylation would shut off *Tad* transcription and thus allow transcription of *am*. Another possibility is that a sequence within *Tad* acts as a "silencer" that prevents transcriptional activity of *am*, perhaps by directly perturbing its transcription complex. That the expression of the downstream *hph* gene driven by the *trpC* promoter appeared unaffected by *Tad3-2* suggests either that the silencing affects selected sequences or positions only, or that its effects are limited by distance. Silencing action over limited distances could take place by altering chromatin structure as is hypothesized for the action of suppressor of hairy-wing/mod(mdg4) proteins in Drosophila, which can bind to a gypsy element to form a chromatin insulator that prevents enhancer elements from interacting with the promoter (GERASIMOVA *et al.* 1995).

Although chromatin structure might be involved in methylation-dependent instability, the silencing associated with the unmethylated *am::Tad3-2* allele could not be a matter of simply insulating the promoter from its upstream enhancer-like elements. If it were, Am$^-$ derivatives of *am::Tad3-2* strains would be expected to exhibit GDH levels characteristic of strains carrying a deletion of these elements (10–15% of wild type), instead of the extremely low levels (1–4%) observed. Analysis of GDH activity in *am::Tad3-2* strains, enriched for Am$^+$ nuclei, that either have the upstream enhancer-like elements deleted (TJK10) or intact (TEC37) demonstrated that the upstream enhancer-like elements were still contributing to *am* expression even when separated from the basal promoter by an additional 7 kb. These results indicate that *Tad3-2* can silence *am* whether the upstream enhancer-like elements are present or absent.

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