

Trichostatin A causes selective loss of DNA methylation in *Neurospora*

(histone acetylation/silencing/epigenetics/5-azacytidine/deacetylase)

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ABSTRACT Both DNA methylation and hypoacetylation of core histones are frequently associated with repression of gene expression. Possible connections between these processes were investigated by taking advantage of genes controlled by methylation in *Neurospora crassa*. Trichostatin A (TSA), a potent inhibitor of histone deacetylase, derepressed a copy of *hph* that was repressed by DNA methylation which resulted from repeat-induced point mutation (RIP) acting on sequences flanking *hph*. Derepression by TSA was comparable to derepression by the inhibitor of DNA methylation, 5-azacytidine. TSA treatment also repressed an allele of *am* whose expression depends on methylation of an adjacent transposon, *Tad*. DNA methylation in the *hph* and *Tad/am* regions was greatly reduced by TSA treatment. TSA also caused hypoacetylation of other methylated alleles of *am* generated by RIP. In contrast, TSA did not affect methylation of several other methylated genomic sequences examined, including the nucleolar rDNA and the inactivated transposon *Punt*^{RIP1}. Several possible models are discussed for the observed selective demethylation induced by TSA. The implication that acetylation of chromatin proteins can directly or indirectly control DNA methylation raises the possibility that connections between protein acetylation and DNA methylation result in self-reinforcing epigenetic states.

DNA is modified by methylation of cytosines in many higher organisms, including mammals, plants, and some fungi. DNA methylation can silence genes (see refs. 1 and 2) and may serve in genome defense systems (3, 4) and in the regulation of certain endogenous genes, such as genes subjected to genomic imprinting or dosage compensation in mammals (5). Although DNA methylation does not appear to interfere directly with transcription, it can indirectly prevent transcription initiation (6) or elongation (7, 8). How methylation exerts its repressive effect remains largely undefined, but proteins that bind specifically to methylated DNA have been identified (9), and methylation appears to cause assembly of an inactive form of chromatin (6, 10). Histones H3 and H4 are hypoacetylated on the heavily methylated inactive X chromosome (11–14) and hyperacetylated in the unmethylated “CpG islands” in animal genomes (15). In a study on sequences introduced into animal cells as episomes, it was found that 5-azacytidine (5-AC) and sodium butyrate, which cause hypomethylation of DNA and hyperacetylation of histones, respectively, could both relieve repression (16). Butyrate has pleiotropic effects at the high concentrations at which it must be used (see ref. 17), but this observation raised the possibility that methylation operates through an effect on histone acetylation, or *vice versa*. Recently, a potent direct inhibitor of histone deacetylases, (R)-

trichostatin A (TSA) (18), was found to substitute for 5-AC to derepress silent, methylated rDNA genes in interspecific plant hybrids (19). Changes in methylation and/or acetylation in the rDNA, or at an undefined regulatory locus, may have caused the derepression. Effects on DNA methylation were not assessed. Two key questions are (i) Can DNA methylation affect histone acetylation? and (ii) Can acetylation affect DNA methylation? Either possibility could account for the observed correlations. If both occur, this should produce a self-reinforcing cycle that could account for stable epigenetic states.

The fungus *Neurospora crassa* offers an attractive system to investigate these possibilities. Most of the *Neurospora* genome is unmethylated and DNA methylation is nonessential (20, 21), but it is clear that DNA methylation can control some genes in this organism (8, 22, 23). Most specifically, we know that methylation of alleles of the *am* (glutamate dehydrogenase) and *mtr* (methyltryptophan-resistant) genes prevent transcription elongation (8). Inhibition of DNA methylation by the drug 5-AC or by the *dim-2* mutation, which prevents all methylation in *Neurospora*, activates methylated *am* and *mtr* alleles. Similarly, inhibition of methylation by using 5-AC or other means reactivates a methylated copy of the bacterial *hph* (hygromycin B phosphotransferase) gene (23) and causes repression of an allele of *am* whose expression depends on methylation of a transposon inserted in its upstream region (22). As a step to investigate the mechanism of methylation-dependent effects on gene expression, I investigated the effect of the histone deacetylase inhibitor TSA on expression of these genes. TSA was found to reverse the effects attributable to methylation. Analyses of DNA methylation demonstrated that TSA can cause selective loss of demethylation in *Neurospora*, implying that acetylation of histones or other proteins can somehow control DNA methylation.

MATERIALS AND METHODS

Strains. The following strains from our laboratory collection were used in this study: N220 (*am::Tad3-2 ure-2 mat a*) (22), N644 (*am*¹³² [(*am/hph/am*)^{ec42 pJ12}]^{RIP77} *inl mat A*) (23), N669 (*am*^{RIP4} *am*^{RIPec4} *lys-1 mat a*), N672 (*am*^{RIP5} *am*^{RIPec5} *lys-1 mat A*), N617 (*am*^{RIP8MM} *mat a*), N676 (*am*^{RIP7} *am*^{ec7} *lys-1 mat A*) (24). The *am/hph/am* region of N644 was from pJ12, and consists of a direct repeat of the *am* gene separated by a 1.7-kb segment including the bacterial *hph* gene from pDH25 driven by the *Aspergillus nidulans* *trpC* promoter (25, 26). The *am*¹³² allele contains a deletion that removes all sequences homologous to the *am* probe and to *am* sequences introduced by transformation.

Cultures. Liquid cultures of *Neurospora*, inoculated with 5–10 × 10⁴ fresh conidia per ml, were grown at 32°C with shaking in sucrose (1.5–2.0%) Vogel’s medium (27) supple-

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Abbreviations: 5-AC, 5-azacytidine; TSA, trichostatin A; HAT, histone acetyltransferase; HDAC, histone deacetylase; RIP, repeat-induced point mutation; hyg, hygromycin.

mented with alanine and inositol to support growth of *am*, *inl* strains. TSA (Wako) was added to the medium immediately prior to inoculation. Plate tests were performed with solidified Vogel's medium containing sorbose (2%), fructose (0.05%), and glucose (0.05%) in place of sucrose to cause colonial growth. Glycine (20 mM) was included in some plates to tighten the selection for *am*⁺ strains. Conidia were routinely plated in 5 ml of 0.7% agar on plates with 25 ml of 1.5% agar medium. TSA (1 μ l of 10 mg/ml in dimethyl sulfoxide) was administered from 4-mm-diameter Whatman no. 1 paper discs placed in the middle of plates shortly after plating. For some experiments, an additional 5 ml of top agar with or without hygromycin B (hyg; 600 μ g/ml; Calbiochem) was added after 18 hr at 32°C. Plates were typically photographed 40 hr later.

Southern Hybridizations. DNA was isolated from 1- to 3-day liquid cultures as previously described. DNA samples (1 μ g) were digested for at least 4 hr with 10 units of restriction enzyme (NEB), fractionated on 1% agarose gels, transferred to nylon membranes, and probed sequentially as previously described (23). Probes were prepared by priming with random hexamers using the 2.6-kb *Bam*HI *am* fragment, the 650-bp *Xba*I-*Bam*HI upstream *am* fragment, the 1.0-kb *Cla*I Ψ_{63} fragment, the 9.2-kb *Kpn*I rDNA fragment, the 1.0-kb *Cla*I-*Bam*HI *hph* fragment, or a 2.5-kb *his*-3 fragment (to verify that digests were complete; not shown).

RESULTS

The possibility that TSA would activate a gene repressed by DNA methylation was first tested with the bacterial gene *hph*, which is present as a single chromosomal copy in *N. crassa* strain N644 (23). This strain was derived from a transformant in which *hph* was unmethylated and conferred resistance to hyg (26). The approximately 1-kb gene, driven by the *A. nidulans* *trpC* promoter (see *Materials and Methods*), lies between two copies of the *Neurospora am* (glutamate dehydrogenase) gene. This allowed us to render *hph*, and the *trpC* promoter, methylated by induction of RIP in the flanking repeated *am* sequences (23). RIP is a sexual-phase-specific genome defense system that results in multiple G·C to A·T transition mutations in duplicated sequences (4, 28–31). Remaining cytosines are frequently methylated after the action of RIP and the methylation can extend into adjacent unmutated sequences, such as those of the *hph* gene flanked by mutated copies of *am*. We had identified progeny of the original transformant in which RIP had indeed caused heavy methylation of the two copies of *am* plus the intervening sequences, including *hph*. The *hph* gene was silenced in most strains (e.g., N644) but expression could be restored if methylation was inhibited by treatment with 5-AC or by limiting production of the methyl-group donor, S-adenosylmethionine (AdoMet), using a conditional mutation in the AdoMet synthetase gene (23). The effect of TSA on expression of the methylated *hph* gene was tested by plating conidia (asexual spores) of strain N644 in the presence or absence of TSA (10 μ g = 33 nmol) applied to paper discs in the middle of the plates. Because hyg quickly kills strains not expressing *hph* (23), this drug was added 18 hr after plating the conidia. One day later, hyg-resistant colonies appeared near the source of TSA; no colonies were seen on the control plate without TSA (Fig. 1A). The density of colonies near the disc appeared equivalent to the density on a similar plate lacking hyg and TSA, suggesting that TSA caused derepression in virtually every colony. Other plate tests revealed that 20 nmol (5 μ g) of 5-AC induced *hph* roughly as well as 33 nmol of TSA (Fig. 1B), although 5-AC inhibited growth considerably more than did TSA (data not shown). Interestingly, TSA and 5-AC together seemed to result in greater induction than either drug alone (Fig. 1B). To test the stability of the apparent derepression by TSA, colonies from plates with TSA and hyg, and colonies from control plates lacking the drugs, were picked and

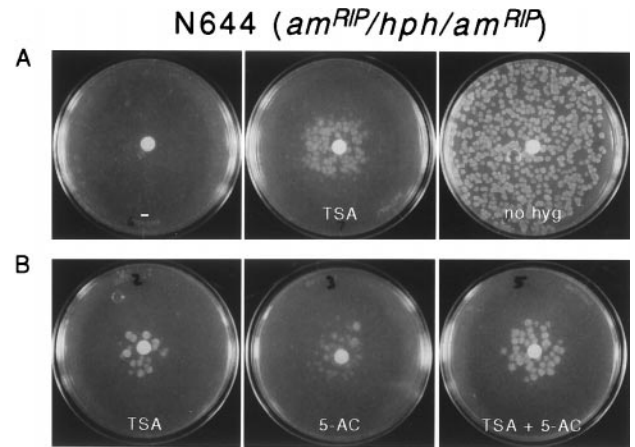


FIG. 1. Reactivation of silenced *hph* gene by TSA and 5-AC. *N. crassa* strain N644 (*am*¹³², *inl*, *am*^{RIP}/*hph*/*am*^{RIP}, *mat A*) harbors a single copy of the *Escherichia coli hph* gene that was inactivated by methylation because of the action of RIP on flanking direct repeats of the *am* gene (23, 26). Sets A and B were from separate experiments using independent solutions and cultures. In each experiment shown, \approx 1,000 conidia were plated on each of the plates, 1 μ l of TSA (33 mM in dimethyl sulfoxide), 5-AC (20 mM), both, or neither (plates marked – and no hyg) was applied to the paper discs, and, except for the right-most plate in A, hyg was added in 0.7% agar medium after 17–18 hr at 32°C. Control plates with TSA or 5-AC but lacking hyg revealed slight inhibition of growth by TSA and somewhat greater inhibition by 5-AC (not shown).

tested on plates containing hyg but no TSA. Colonies from the TSA plus hyg plate, but not the control plate, grew vigorously on hyg medium, but showed loss of resistance after conidiation (data not shown). Thus TSA caused a long-term, but not permanent, derepression of the *hph* gene.

A case in which methylation affects gene expression in the opposite direction from that normally observed provided an attractive opportunity for a second genetic test of the effect of TSA on methylated DNA. Expression of the *am* gene in strain N220 relies somehow on methylation spanning the 5' end of a LINE-like transposon, *Tad*, inserted upstream of the *am* basal promoter (22) (Fig. 2B). *am* expression is required for growth on minimal medium supplemented with glycine. Normally, \approx 2% of N220 conidia plated on restrictive (glycine) medium form colonies (ref. 22 and data not shown). When methylation is prevented, however, by using either 5-AC or a strain harboring the *dim-2* mutation, which prevents all methylation in vegetative tissue of *Neurospora* (20), the *am* gene is fully silenced. Loss of methylation somehow allows *Tad* to silence *am*. Although the cause of this methylation is not known, it is apparently not a result of RIP (22).

The effect of TSA on *am* expression in strain N220 was tested by plating \approx 400,000 conidia on permissive (alanine) or restrictive (glycine) media and then adding TSA (10 μ g) to the center of the dishes, as before. A slight inhibition of growth was detected near the source of the TSA on alanine medium, but the strain produced essentially confluent growth, as expected (Fig. 2A). TSA did not appreciably inhibit a wild-type strain on either alanine or glycine medium (not shown). In striking contrast, growth of N220 on glycine medium was limited to the region of the plate at least 2.5 cm from the source of the TSA. Thus the deacetylase inhibitor accentuated the inhibitory effect of *Tad* on *am* expression, as occurs when DNA methylation is prevented (22).

The effects of TSA on expression of *hph* and *am* suggested that histone hypoacetylation and DNA methylation may operate in a common silencing pathway. One possibility was that the silencing resulted from hypoacetylation directed by the DNA methylation. It was also possible that hypoacetylation

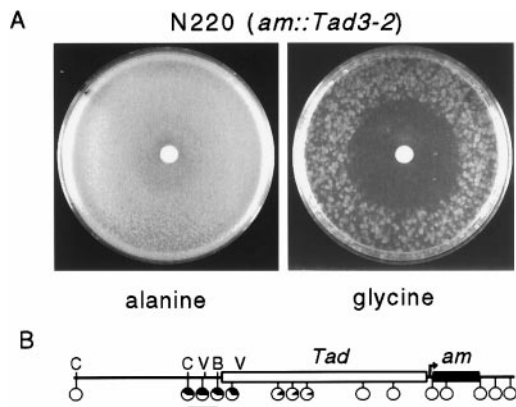


FIG. 2. TSA accentuates silencing of the *am* gene caused by the adjacent transposon *Tad*. (A) Approximately 4×10^5 conidia of *N. crassa* strain N220 (*am::Tad3-2*, *ure-2*, *mat a*) were plated on permissive (alanine) or restrictive (glycine) sorbose plates. One microliter of TSA (33 mM in dimethyl sulfoxide) was applied to the paper discs and the plates were incubated 3 days at 32°C. The difference in colony density in the portions of the plates not affected by TSA reflects the ratio of Am^+ and Am^- colonies characteristic of this strain. (B) Map of *am::Tad3-2* region of strain N220. *Tad* (open rectangle) is inserted 70 bp upstream of the transcription start sites (arrow) of *am* (black rectangle). The approximate methylation status of 15 sites in the region, as determined in a previous study (22), is depicted in black in pie charts placed close to the sites examined. Those sites for *Bam*HI (B), *Cla*I (C), and *Eco*RV (V) that are relevant to Fig. 5 are indicated. The bar beneath the map represents a 650-bp *Xba*I-*Bam*HI fragment used as a probe for the blot shown in Fig. 5.

triggered DNA methylation, which then more directly affected gene expression. I therefore investigated whether TSA affected DNA methylation. The methylation state of the *am*^{RIP}/*hph/am*^{RIP} region was examined in N644 grown in nonselective medium containing up to 1 μ g/ml (3.3 μ M) TSA, a concentration that retarded growth somewhat under the conditions of the experiment (Fig. 3A). Methylation was assessed by comparing *Sau*3AI and *Dpn*II digests by Southern hybridization. *Sau*3AI fails to cut DNA when the C in its recognition site (GATC) is methylated, whereas its isoschizomer *Dpn*II is not inhibited by cytosine methylation. A substantial reduction in methylation in the *am*^{RIP}/*hph/am*^{RIP} region was caused by growth in 3.3 μ M TSA, as shown by the fact that *Sau*3AI gave nearly complete digestion of *am* and *hph* sequences (Fig. 3B and data not shown).

Although this finding suggested that TSA may have inhibited methylation, it seemed possible that the reduced methylation was simply the result of inhibited growth, because young cultures of *Neurospora* show some reduction in overall methylation (32). Therefore DNA from N644 cultures grown for longer periods of time with and without TSA were examined. Derepression of *hph* was also tested in the same set of cultures by challenging them to grow in the liquid medium after addition of hyg. Cultures with 0.1 μ g/ml (0.33 μ M) TSA, or less, failed to grow appreciably in the presence of hyg (data not shown). In contrast, cultures supplemented with 3.3 μ M TSA grew well in hyg medium. Although all cultures with this level of TSA showed somewhat retarded growth initially, as in the previous experiment, by 46 hr they appeared to have "caught up" to the cultures without TSA. This observation was confirmed by measuring tissue weights (Fig. 4 legend and data not shown). Most interestingly, DNA prepared from mature (2- and 3-day) cultures grown nonselectively in the presence of TSA showed marked reduction in methylation in the *am*^{RIP}/*hph/am*^{RIP} region (Fig. 4A), as observed with the younger culture (Fig. 3). No difference in methylation was evident between the 2- and 3-day cultures, reinforcing the conclusion that the reduction in methylation observed here and in the

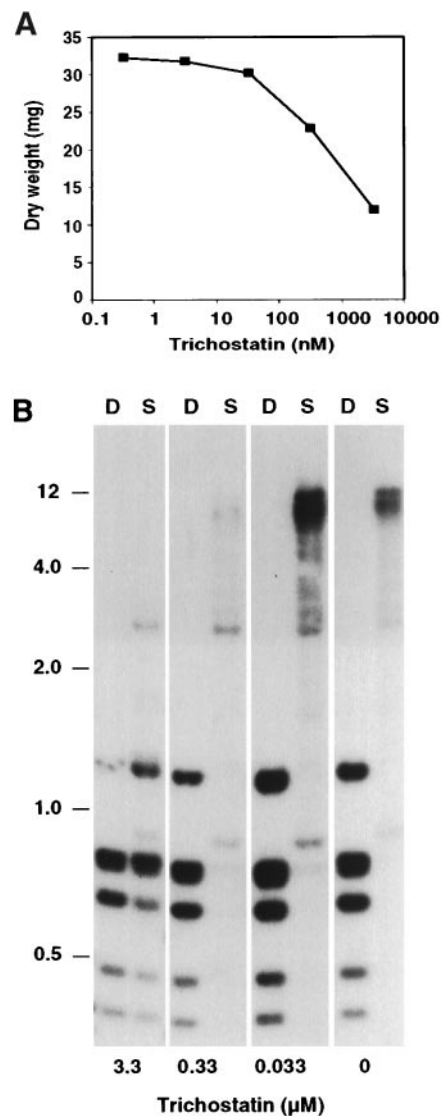


FIG. 3. Effect of TSA on growth and DNA methylation. (A) Liquid cultures inoculated with *N. crassa* strain N644 (7×10^4 conidia per ml) were supplemented with up to 1 μ g/ml (3.3 μ M) TSA and grown 27 hr. The untreated culture yielded 32.8 mg of dry tissue. (B) Southern hybridization of selected samples from the cultures. Samples of DNA were prepared and digested with *Dpn*II (D) or *Sau*3AI (S) and probed for *am* sequences. Stronger signals in the 0.33 μ M lanes is partially due to 2 \times heavier loadings of DNA in these lanes. The positions of selected size standards (kb) are indicated.

previous experiment was not somehow due to differences in growth. DNA from a culture grown in TSA and hyg showed the greatest reduction in methylation (Fig. 4A), presumably because selection for hyg favored cells with reduced methylation of *hph* (23).

The marked reduction in methylation caused by TSA in the *am*^{RIP}/*hph/am*^{RIP} region was particularly surprising because no change in overall methylation was apparent from inspection of the ethidium bromide-stained genomic DNA samples (Fig. 4B). We had previously noted that comparisons of *Sau*3AI and *Dpn*II (or *Mbo*I) digests of *N. crassa* DNA provide a simple indicator of overall methylation (33). Thus it seemed possible that TSA caused a highly selective loss of methylation in the genome, in contrast to the rather uniform reduction in methylation achieved by 5-AC treatment, by use of any of several mutants defective in methylation (refs. 20, 32, and 34; H. Foss, C. Roberts, and E.S., unpublished results; and A. Hagemann, M. Freitag, and E.S., unpublished results) or by limiting

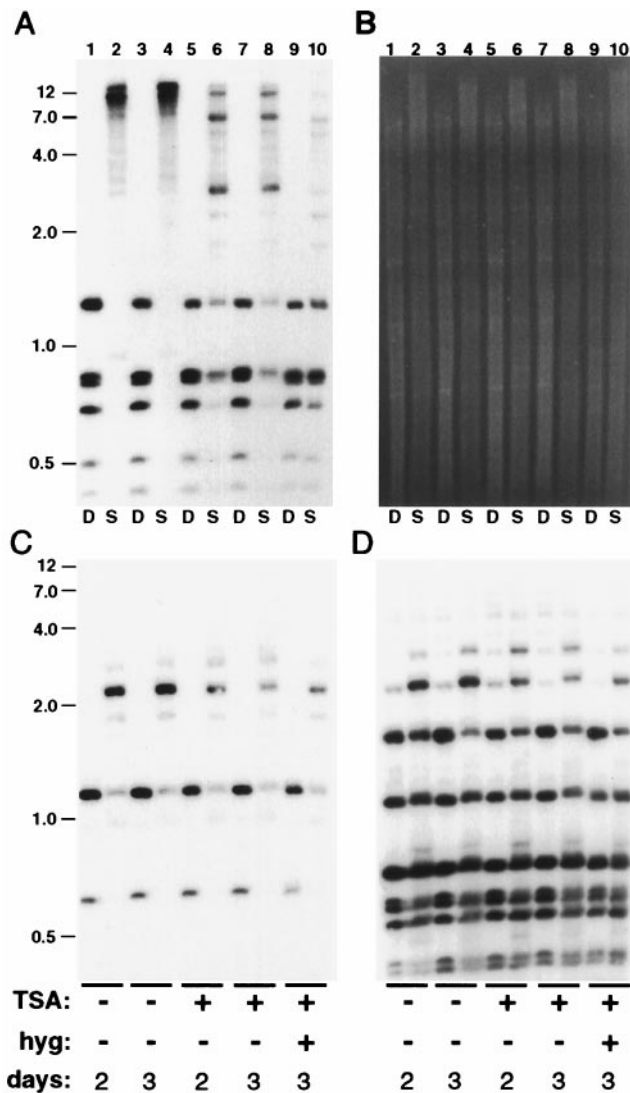


FIG. 4. TSA causes selective hypomethylation of DNA. Strain N644 was grown for 2 or 3 days, as indicated, with or without TSA (1 μ g/ml; lanes 5–10) and hyg (added to a concentration of 0.1 mg/ml 17 hr after inoculation; lanes 9 and 10). The dry weights of the cultures illustrated were, from left to right, 31.1 mg, 38.5 mg, 37.5 mg, 37.3 mg, and 32.8 mg. DNA was isolated and analyzed by digestion with *DpnII* (D) or *Sau3AI* (S) and by probing for *am* (A), Ψ_{63} (C), rDNA (D), or *hph* (not shown). (B) Total genomic DNA visualized by staining with ethidium bromide. The positions of selected size standards (kb) are indicated.

methylation by using strains harboring mutations in genes required for *S*-adenosylmethionine biosynthesis (20, 34). To test directly whether TSA caused differential hypomethylation, the blot shown was reprobed for two other regions known to be methylated in the *N. crassa* genome. These were Ψ_{63} , a 5S rRNA pseudogene interrupted by a transposon that has been inactivated by RIP (20, 35), and the tandemly repeated rRNA genes (36). Hybridization results revealed that neither Ψ_{63} (Fig. 4C) nor rDNA (Fig. 4D) sequences were hypomethylated. Thus the histone deacetylase inhibitor caused striking hypomethylation in the *am*^{RIP}/*hph*/*am*^{RIP} region but caused no apparent change in methylation of the other regions.

The effect of TSA on methylation was also examined in the upstream *am/Tad* boundary in strain N220 and in several additional alleles of *am* that were methylated as a result of RIP. Digestion of N220 DNA with *Bam*HI, *Bsp*106I, or *Sau*3AI revealed TSA-induced hypomethylation at the upstream *am/Tad* boundary (Fig. 5A), as found in the *am*^{RIP}/*hph*/*am*^{RIP}

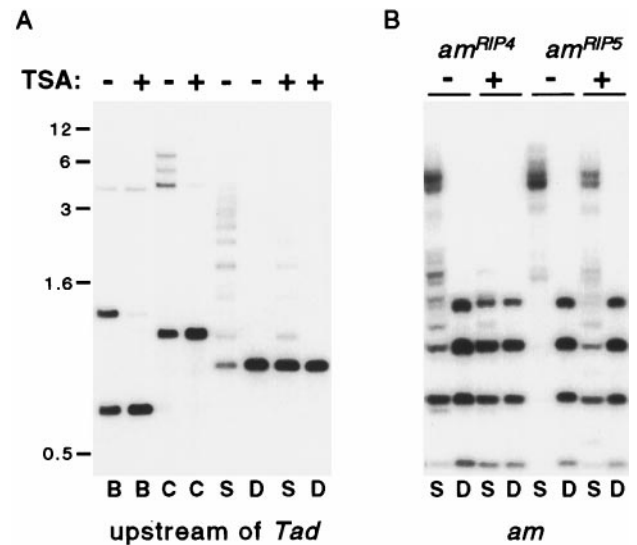


FIG. 5. TSA-induced hypomethylation of *am::Tad3-2* and *am*^{RIP} alleles. Strains containing *am::Tad3-2* (N220), *am*^{RIP4} and *am*^{RIPec4} [N669 (24)] or *am*^{RIP5} and *am*^{RIPec5} [N672 (24)] were grown from conidia for 2 days in the presence or absence of 1 μ g/ml TSA, as indicated. (A) DNA samples of N220 were digested with *EcoRV* (which is not inhibited by cytosine methylation) plus *Bam*HI (B), *Bsp*106I (an isoschizomer of *Cla*I; C), *Sau*3AI (S), or *Dpn*II (D) and probed for sequences upstream of *Tad* (see Fig. 2B). The positions of selected size standards (kb) are indicated. (B) DNA of N669 or N672 was digested with *Sau*3AI or *Dpn*II and probed sequentially for *am*, Ψ_{63} (not shown), and rDNA (not shown).

region of N644. The *am*^{RIP}/*hph*/*am*^{RIP} construct of N644 was built using only sequences downstream of the *Bam*HI site (Fig. 2B); thus the methylated upstream *am/Tad* sequences include less than 180 bp found in strain N644.

Strains bearing *am* alleles inactivated by RIP showed similar results. Several strains examined each contain two *am*^{RIP} alleles, one at the native *am* locus and another at the unlinked site where the 2.6-kb *Bam*HI *am* fragment that triggered RIP had integrated (24, 29, 37). Strain N669 was chosen because the methylation of both *am* sequences (*am*^{RIP4} and *am*^{RIPec4}) in this strain appears to be dependent on maintenance methylation. That is, if the methylation of these alleles is removed by treatment with 5-AC, or if one of these alleles is isolated and reintroduced into *Neurospora* in an unmethylated state, the methylation is not reestablished (24). In contrast, in strain N672, the *am*^{RIP5} and *am*^{RIPec5} alleles become methylated *de novo*, as is most common for sequences bearing moderate or heavy damage by RIP (4). Interestingly, both of the *am*^{RIP} alleles in both strains N669 and N672 showed striking reduction of methylation in response to TSA treatment (Fig. 5B). The alleles that do not trigger *de novo* methylation and therefore must depend on maintenance methylation (*am*^{RIP4} and *am*^{RIPec4}) were most affected. Curiously, in strain N672, the allele at the native *am* locus appeared more sensitive to TSA than the ectopic allele, as indicated by the relatively weak signal of the *Sau*3AI fragment diagnostic of the ectopic copy [1.4-kb fragment matching the largest *Dpn*II fragment (24)]. A strain (N617) containing only a single *am* allele (*am*^{RIP8}), at its native locus, also showed dramatic reduction in methylation (data not shown). Reprobings of the blot with Ψ_{63} and rDNA (data not shown) revealed no apparent loss of methylation from these regions, as with strain N644 (Fig. 4).

DISCUSSION

The exposed N-terminal tails of each of the four core histones are subject to a variety of posttranslational modifications that may affect chromatin function. Early evidence that acetylation

of lysines in the tails is associated with gene expression has been consistently supported and extended (see refs. 38–40). The general picture that has emerged is that hyperacetylation is a prerequisite for transcription, whereas hypoacetylation can result in transcriptional repression (see refs. 41–43). Several transcription factors and transcriptional coactivators have been demonstrated to possess histone acetyltransferase (HAT) activity (44, 45), and a number of transcriptional repressors have been shown to recruit histone deacetylases (HDACs) (46–51). Studies with antibodies specific to histones acetylated at particular sites (52) or with probes for sequences in hyper- or hypoacetylated chromatin (53) revealed gross variation in histone acetylation in different chromosomal regions, presumably reflecting differential availability of HATs and HDACs. DNA sequences associated with epigenetic silencing, such as on the inactive X chromosome of mammals (11–14), in heterochromatin of insects (54), in the silent mating type genes in yeasts (55, 56), and in the centromere regions of fission yeast (57), are typically associated with hypoacetylated histones. Methylated sequences in animals have also been found associated with hypoacetylated histones (15).

Direct evidence that histone acetylation can affect gene expression came from studies in which genes for HDACs were mutated (58) or inhibited with drugs (16, 17, 19, 49, 57, 59, 60). Curiously, two cases were found in which genes could be activated by using either an inhibitor of DNA methylation (5-AC) or an inhibitor of HDACs (butyrate or TSA). In the first, Hsieh (16) showed that butyrate could enhance expression of a methylated episome transfected into human cells. No effect on methylation was detected. In the second study, both butyrate and a specific inhibitor of HDACs, TSA, were found to substitute for 5-AC to derepress silent, methylated rDNA genes in interspecific plant hybrids (19). Effects on DNA methylation were not assessed. Thus, changes in methylation and/or acetylation in the rDNA, or at an undefined regulatory locus, may have caused the derepression. Nevertheless, these findings raised two possibilities: the repressive effect typical of DNA methylation may be mediated by deacetylation of histones, or deacetylation may lead to DNA methylation. These possibilities were investigated by using genes in *Neurospora* whose expression is known to be negatively or positively controlled by DNA methylation: a bacterial transgene (*hph*) repressed by methylation (23), an allele of the *Neurospora am* gene that is controlled indirectly by methylation of a transposon (*Tad*) inserted upstream of the *am* basal promoter (22), and several methylated alleles of *am* generated by RIP (24). TSA treatment induced expression of *hph* and silenced expression of the *am* allele downstream of *Tad*. Surprisingly, DNA methylation was dramatically reduced in the *hph* and *Tad/am* regions, as well as in all *am*^{RIP} alleles examined. Thus, the changes in gene expression may have resulted directly from histone hyperacetylation, as has been observed in other systems, or may have resulted indirectly from an effect of hyperacetylation on methylation. Although it is conceivable that TSA affected a process other than histone acetylation, TSA is a direct, noncompetitive inhibitor of HDACs and no other effects of the drug have yet been found (18). As far as I know, this is the first indication that DNA methylation may depend, directly or indirectly, on the acetylation state of histones. Interestingly, TSA did not affect methylation of other genomic sequences examined, including the nucleolar rDNA and a transposon inactivated by RIP.

What is the most straightforward interpretation of these results? In every system examined, TSA has been found to cause hyperacetylation of the core histones, apparently because their state of acetylation reflects the balance of HATs and HDACs working in opposition (18, 19, 57, 59, 61). Variation in the distribution of HATs and HDACs in different chromosomal regions should result in regional differences in

the degree of hyperacetylation in response to TSA, but this possibility has not been carefully examined. In principle, the observed loss of methylation caused by TSA in our system could have resulted either from hyperacetylation of the methylated regions or from hyperacetylation elsewhere in the genome. For example, if a hypothetical negative regulator of a DNA methyltransferase were activated by increased acetylation in the vicinity of its gene, this might lead to decreased DNA methylation. This is not the most economical model, however, especially considering that TSA strongly affected methylation in some chromosomal regions but left other areas unaffected. There are several possible explanations for the differential effect of TSA on DNA methylation. TSA may not have inhibited the action of HDACs in all regions. Alternatively, deacetylation may have been inhibited globally but only some regions had access to HATs and were therefore re-acetylated. Consistent with this line of reasoning, it has recently been shown that TSA activates the WAF1/Cip1 promoter in a human cell line through Sp1 sites (59). This is particularly interesting in light of evidence that deletion or mutations in Sp1 sites of the mouse and hamster *aprt* genes lead to methylation of their CpG islands (62–64). Thus, it does not seem unreasonable to suppose that in our system the *am* enhancer region upstream of *Tad3-2* would recruit a HAT and thereby increase acetylation in the region after TSA treatment, whereas the Ψ_{63} pseudogene, with its heavily mutated transposon (35), would lack sites to which HATs are recruited and thus remain hypoacetylated. It is worth noting in this context that the upstream region of the *Neurospora am* gene includes a CCAAT site that binds a protein (AAB) equivalent to HAP5 of yeast, which is a member of an activation complex dependent on the HAT encoded by GCN5 (65, 66). The distribution of regulatory sites and DNA methylation in rDNA is not yet well defined in *Neurospora*, but it is interesting that butyrate was not found to influence the gross acetylation level of histones in rDNA of human chromosomes (53).

The observed loss of methylation could be a direct or indirect result of TSA-induced hyperacetylation. It is possible that hyperacetylation released transcription in some chromosomal regions and the transcription, rather than hyperacetylation, *per se*, inhibited DNA methylation. We know that absence of transcription is not sufficient to trigger methylation and that methylation of *am* prevents transcription elongation (8), but it is conceivable that activation by hyperacetylation could overcome this effect. A second possibility consistent with the observations is that acetylation directly controls DNA methylation. Hypoacetylation could trigger DNA methylation or hyperacetylation could inhibit methylation (or both). Either DNA methylation or hypoacetylation could be responsible for repression of transcription in this model. The observation of interchangeable effects of 5-AC and TSA implies, however, that hypoacetylation cannot be solely responsible for repression unless the state of methylation feeds back on the state of acetylation. Recent suggestions that methyl-DNA binding proteins may recruit HDACs are consistent with this possibility (67, 68). If methylated sequences recruit HDACs, which then cause deacetylation, and deacetylation promotes methylation, this should produce a rather stable repressed state. This self-reinforcing epigenetic state could account for observed maintenance of methylation, including maintenance of heterogeneous methylation at nonsymmetrical sites (69). To distinguish between these models it will be necessary to determine whether transcription is required for TSA-induced changes in methylation and whether DNA methylation can directly affect histone acetylation. A variety of studies will be required to discover all the connections between modifications of DNA and chromatin and between these modifications and gene expression.

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