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# Developmental Characterization and Chromosomal Mapping of the 5-Azacytidine-Sensitive *fluF* Locus of *Aspergillus nidulans*

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**In *Aspergillus nidulans*, a fungus that possesses negligible, if any, levels of methylation in its genome, low concentrations of 5-azacytidine (5-AC) convert a high percentage of the cell population to fluffy phenotypic variants through a heritable modification of a single nuclear gene (M. Tamame, F. Antequera, J. R. Villanueva, and T. Santos, Mol. Cell. Biol. 3:2287–2297, 1983). This new 5-AC-altered locus, designated here *fluF1*, was mapped as the closest marker to the centromere that has been identified so far on the right arm of chromosome VIII. Of all mutagens tested, only 5-AC induced the fluffy phenotype with a significant frequency. Furthermore, we determined that the wild-type, dominant allele of the *fluF* gene was primarily accessible to modification by 5-AC at the initial stages of fungal vegetative growth. These results indicated that 5-AC does not act through random mutagenic action but, rather, that *fluF* constitutes a specific target for this drug during a well-defined period of fungal development. Alteration of *fluF* by 5-AC resulted in a dramatic modification of the developmental program of *A. nidulans*. The resulting fluffy clones were characterized by massive, uncontrolled proliferation of undifferentiated hyphae, a drastic delay in the onset of asexual differentiation (conidiation), and colonies with an invasive nature. These features are reminiscent of the malignant properties of tumor cells. We propose that the locus *fluF* plays a primary role in the control of cell proliferation in *A. nidulans* and that its alteration by 5-AC produces pleiotropic modifications of the developmental program of this fungus.**

Results of a number of recent studies support the notion that DNA methylation may be involved in the epigenetic mechanisms that control eucaryotic gene expression (for reviews, see references 4, 15, 18, and 32). The cytidine analog 5-azacytidine (5-AC) is an inhibitor of DNA methyltransferases that has been widely used in such studies because it causes permanent alterations of gene expression in a number of biological systems. Acting as an inhibitor of DNA methylation, 5-AC has been shown in some cases to lead to expression of genes that are otherwise kept silent (7, 8, 20, 21). In other cases, 5-AC treatment leads to altered differentiation patterns of specific cell types that have not always been correlated with changes in the status of DNA methylation (14, 23, 26, 28). Furthermore, this compound can also produce other unrelated genetic phenomena such as mitotic recombination, chromosome decondensation, or sister chromatid exchanges in eucaryotic cells with or without detectable levels of methylated bases in their genomes (19, 33).

We have previously reported (30, 31) that 5-AC induces conversion to a new fluffy developmental phenotype in *Aspergillus* spp. with a high frequency. We have also shown (31) that this phenotype is associated with a single recessive Mendelian gene. Fluffy clones are characterized morphologically by massive, unrestricted proliferation of undifferentiated aerial hyphae that originate large, invasive, cottonlike fluffy colonies. It is of interest to note that *Aspergillus nidulans* is an organism that lacks detectable levels of genomic methylation (2). Here we present the results of chromosomal mapping studies of the responsible locus, which we designated *fluF*. We also describe the developmental alterations that were observed in fluffy clones that carried the 5-AC-altered allele *fluF1*. Furthermore, we show that the alteration of this locus by 5-AC occurs at a very specific period during development and that it cannot be induced at a significant frequency by other standard mutagenic agents or carcinogenic agents.

## MATERIALS AND METHODS

**Chemicals.** 5-AC was obtained from P-L Biochemicals, Inc. (Milwaukee, Wis.), and [4-$^{14}$C]5-AC was obtained from Moravek Biochemicals, Inc. (Brea, Calif.). All other chemicals were reagent grade.

**Organisms and growth conditions.** The strains of *A. nidulans* used in this study were obtained from the stocks of A. J. Clutterbuck (Department of Genetics, University of Glasgow, Glasgow, Scotland), their genotypes are listed below. Gene nomenclature was as described previously (12, 13).

**Strains and genotypes.** The following strains, with the indicated genotypes, were used in this study: G1059 (*adF17 pabaA1 yA2*), G94 (master strain E; *su1ad20 y ad20 W3 gal-1 pyroA4 facA303 s3 nic-8 riboB2*), G849 (*wA3 pyroA4 pdhC2*), G843 (*pabaA1 adH23 abaA14 ahrA2 chaA1*), G821 (*pabaA1 wA3 argC3 facB101 riboB2*), G852 (*pyroA4 choC3 fwA1*), G82 (*pabaA1 fwA1 facB101 riboB2 tsD15 galG7*). Fluffy clones G1059A1 and G1059A3 were obtained by treating G1059 with 5-AC as described previously (31).

**5-AC treatments and clonal analysis of the resulting fluffy variants.** 5-AC treatments and clonal analysis were carried out at 28°C for the indicated periods of time in liquid *A. nidulans* complete medium (CM) composed of *A. nidulans* minimal medium supplemented with the appropriate nutritional factors required for each strain (11, 12). After treatment, the fungal mycelium was washed twice with distilled water and transferred to fresh medium for further growth, as

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specified in each case. These mycelia were then collected by filtration through glass fiber filters, and the mycelia were incubated for 48 to 72 h under conidiation-promoting conditions as described previously (24). The conidia were then detached from the mycelia, inoculated onto selective solid CM, and grown for appropriate periods of time (~72 h) in order to perform the clonal analysis of the offspring (31). Experiments in which the effect of hydroxyurea (3) was studied were conducted in the same way. Treatments with mutagenic or carcinogenic agents were carried out as described previously (1, 17, 27).

**Genetic techniques.** Procedures to obtain heterokaryons, heterozygous diploids (for mitotic analyses), and hybrid cleistothecia (for meiotic analyses) were those described previously (11, 12, 27).

Haploidization, to assign the unlocated marker *fluF1* of *A. nidulans* to a particular linkage group, was achieved by inoculating diploid spores obtained from crosses between master strain G94 carrying markers for each of the eight linkage groups and our fluffy strains (G1059A1 and G1059A3) into CM containing 70 to 100 μg of *para*-fluorophenylalanine (PFFA) (25) per ml and the nutrients required by the parental strains. After 7 to 10 days of incubation at 37°C, haploid sectors that grew vigorously from the inoculum point were isolated. These segregants were assembled onto master plates of CM and onto minimal medium plates, in order to test their degree of ploidy, and were then replica plated onto CM containing appropriate combinations of growth supplements to determine their nutritional requirements. The marker with an unknown location associated with all but one of the chromosomal markers of the master strain and showed complete linkage in *trans* with it. For meiotic mapping, fluffy strain G1059A3 (carrying *fluF1* in chromosome VIII) was crossed sexually with different *A. nidulans* strains carrying markers located at different positions on the same chromosome. Ascospores from the resulting hybrid cleistothecia were then plated at a low density onto nonselective CM and replica-plated onto selective medium to classify genetically the offspring in relation to the segregating markers. The frequencies of recombination obtained between *fluF1* and each of the other markers allowed us to locate *fluF1* on the genetic map of this linkage group.

## RESULTS

**Developmental traits of the fluffy phenotype.** Treatment of *A. nidulans* with nonmutagenic concentrations of 5-AC led to the conversion of a large fraction of the cell population into abnormally developed fluffy (f) phenotypic variants showing continuous and uncontrolled vegetative growth. We have previously shown (31) that the locus responsible for the 5-AC-induced fluffy phenotype is transmitted as a single Mendelian recessive gene. Here we designate this locus *fluF1*. A comparison of the developmental patterns of f phenotypic variants and wild-type clones growing on a solid culture is shown in Fig. 1A. Several major changes were observed in the developmental pattern of fluffy clones. First, f variants exhibited a drastic delay in the onset of asexual differentiation (conidiation) when compared with wild-type $f^+$ clones. While $f^+$ colonies completely conidiated after 72 h (Fig. 1A), f colonies only developed mature conidiophores after 6 to 7 days of incubation at 37°C, but the yield of conidia was about 3 orders of magnitude lower than that in wild-type strains. In addition, f clones showed uncontrolled vegetative growth, with the continuous, unrestricted production of aerial fluffy hyphae. In sharp contrast, these aconidiogenic aerial hyphae were totally absent from $f^+$ clones after approximately 24 h of growth, when they acquired competency for conidiation (6). As a consequence of the continuous proliferation, f clones produced colonies that were double or triple the diameter of wild-type clones (Fig. 1B). Furthermore, the f clones were invasive, in that they were able to grow over and infiltrate other colonies with the wild-type phenotype (Fig. 1B). These properties (continuous proliferation, abnormal differentiation program, and a lack of contact inhibition during growth on surfaces) are reminiscent of the neoplastic behavior of mammalian transformed cells (16).

**Differential susceptibility of *fluF* to 5-AC action throughout development.** We have previously shown (31) that chronic treatment of *A. nidulans* liquid cultures with 5-AC over a 72-h period (encompassing the period from spore germination to the stationary phase) induces the fluffy phenotype in a significant percentage of conidial offspring clones. In this study we wanted to determine whether *fluF* was susceptible to 5-AC action over all phases of fungal development or just at some specific stage(s). For this purpose we studied the frequency of induction of fluffy variants after treatments with the drug during specific stages of fungal development. The results presented in Table 1 document that the locus *fluF* is mainly affected by 5-AC at the start of vegetative growth and that incorporation of this pyrimidine analog into cellular DNA is required for the induction of fluffy variants.

Treatment of the fungus with two different concentrations of 5-AC during defined time intervals of the fungal life cycle produced a maximum induction of fluffy variants when the treatment occurred between 8 and 13 h of growth, a period corresponding to the start of vegetative growth (Table 1). Much lower induction levels were obtained when treatment was performed before (germination period) or after (exponential and transition phases) that period. Treatments with 5-AC during conidiation on a solid surface also produced very low levels of fluffy variants (Table 1).

The lower frequency of fluffy variant induction observed after 13 h was not due to the depletion of nutrients or 5-AC from the growth medium. Thus, when 24-h-old mycelium was diluted in a fivefold volume of fresh medium and allowed to grow for up to 72 h in the presence of 100 μM 5-AC, a still lower percentage (5.4%) of the conidial offspring exhibited the fluffy phenotype (Table 1). Also, further addition of 5-AC to the 5-AC-pretreated cultures did not increase the level of induction (data not shown).

Simultaneous treatment of the cultures with 5-AC and the DNA synthesis inhibitor hydroxyurea (3) decreased the number of fluffy variants induced during any period of development by more than 90% (Table 1). This indicates that this induction is dependent on 5-AC incorporation into newly synthesized DNA. However, the maximal induction of fluffy variants obtained between 8 and 13 h was not merely due to a greater ability to incorporate 5-AC into DNA during that period. In fact, parallel measurements of [$^{14}$C]5-AC uptake and DNA synthesis with [6-$^{3}$H]uracil (data not shown) indicated that the incorporation of precursors (including 5-AC) into DNA was significantly higher at later periods of development, when the susceptibility to fluffy conversion was lower (Table 1).

These results indicate that there is a specific point during development of the fungus (between 8 and 13 h of vegetative growth) at which the *fluF* locus is particularly susceptible and open to modification by 5-AC. Alteration of this locus results in a new developmental program characteristic of the fluffy clones, with the features described above.

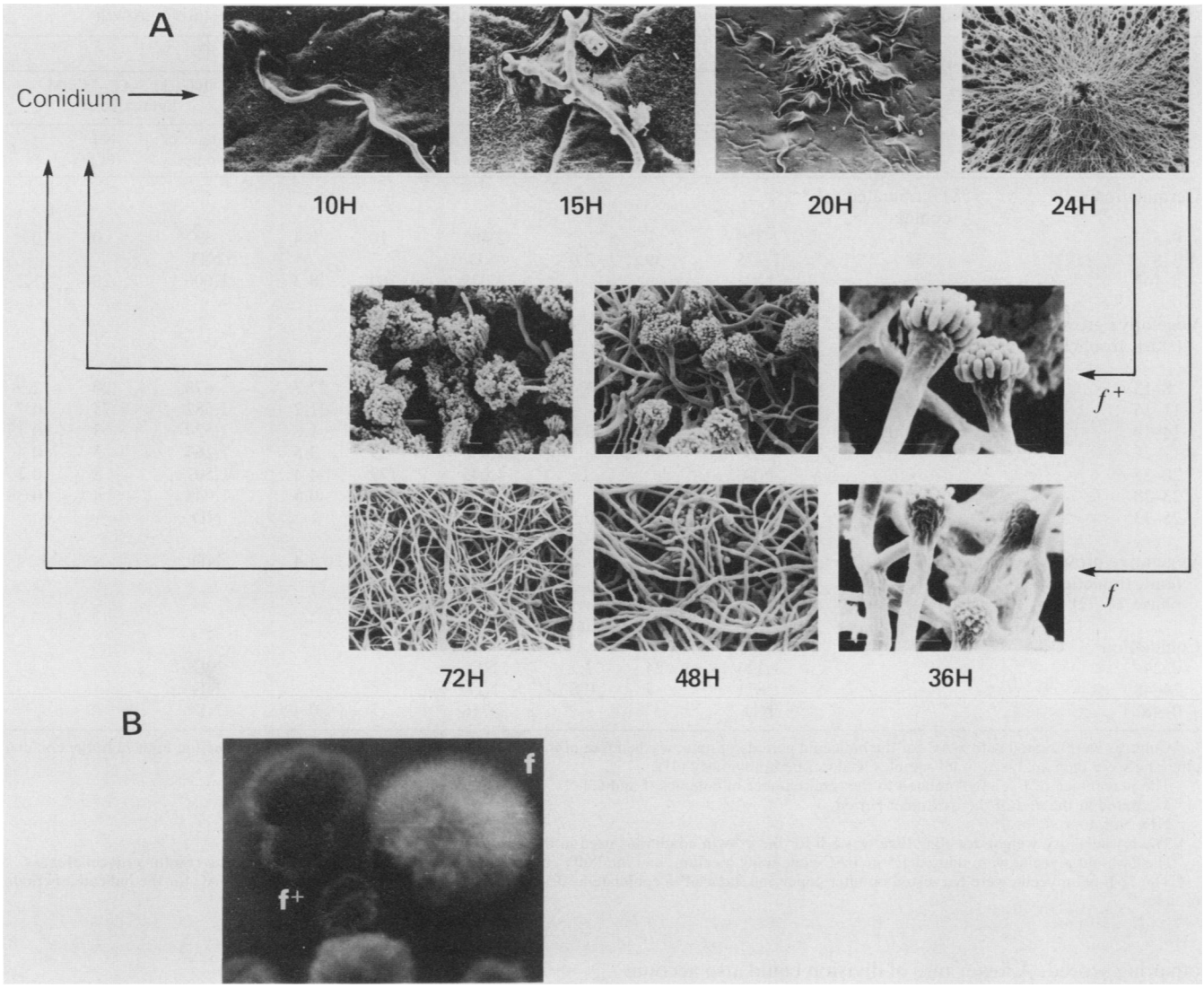


FIG. 1. Comparison of patterns of development of fluffy and wild-type strains of *A. nidulans*. (A) Scanning electron micrographs of different stages of the asexual life cycle of f and $f^+$ *A. nidulans* clones. Individual conidia from a fluffy clone (G1059A3) and its parental wild-type strain ($f^+$ G1059) were inoculated onto CM plates and incubated at 37°C, and their development was tested by examining single colonies at the indicated times. Development of $f^+$ and f colonies was similar until 20 to 24 h of growth, when $f^+$ strains stopped proliferating and started asexual differentiation (conidiation), while fluffy strains kept proliferating to produce a profuse network of aconidiogenic aerial hyphae. At 36 h $f^+$ colonies had already differentiated a very large number of conidiophores carrying differentiated conidia on top of primary and secondary sterigmata (metulae and phialidae). In contrast, f colonies very seldom possessed conidiophores at this time, and those that were rarely seen (36 h) only began to develop primary sterigmata. The increased proliferation properties and the radical delay in asexual differentiation of f clones was most prominent at 48 and 72 h, when wild-type clones had already completed the conidiation cycle. Bars, 10 μm. (B) Macroscopic aspect of 72-h-old f and $f^+$ colonies. Fluffy colonies showed invasive properties and were much larger than wild-type colonies. The average diameters of f and $f^+$ colonies at 132 h of growth at 37°C were 27 and 15 mm, respectively.

**Do fluffy nuclei divide faster than wild-type nuclei?** Under the standard conditions used for 5-AC induction of fluffy variants in *A. nidulans* (treatment with 5-AC for appropriate times and scoring of fluffy conidial offspring in 72-h-old cultures), the percentages of induction were strikingly high (Table 1). We wondered whether the high percentages reflected the real number of primary hits by 5-AC on individual wild-type nuclei or could be secondary to some additional positive selection of the fluffy variants. In order to check this possibility, a liquid culture of wild-type *A. nidulans* was treated with 5-AC for 24 h after the moment of inoculation, and then the fluffy conidial offspring were scored at different times, while the treated culture cells were grown further in a 5-AC-free medium. A relatively low percentage of fluffy variants (1.4%) was measured immediately after treatment with 5-AC, but this percentage increased progressively with time in a dramatic way (Fig. 2). Although other explanations cannot be formally excluded, if a similar viability is assumed for wild-type and fluffy nuclei, these results would be consistent with the notion that fluffy nuclei divide much faster than wild-type nuclei in the multinucleate hyphae of the 5-AC-pretreated cultures; the faster rate of division would be responsible for the time-dependent increases in the percentages of fluffy mononucleate conidial

TABLE 1. Alteration of the *fluF* locus of *A. nidulans* by 5-AC occurs preferentially at the start of vegetative growth

| Period (h) of treatment with 5-AC | Developmental status | Clonal analysis of conidial offspring with treatments of[a]: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 25 μM 5-AC | | | 100 μM 5-AC | | | 100 μM 5-AC–75 mM hydroxyurea | | |
| | | Total no. of colonies | No. of f | % f[b] | Total no. of colonies | No. of f | % f | Total no. of colonies | No. of f | % f |
| Germination | % Germinated conidia[c] | | | | | | | | | |
| 0–5 | 1.4 | ND[d] | | | 2,481 | 10 | 0.4 | 397 | 0 | 0 |
| 3–8 | 68 | 1,375 | 39 | 2.9 | ND | | | ND | | |
| 5–10 | 92 | ND | | | 2,029 | 169 | 8.3 | 1,606 | 20 | 1.2 |
| Vegetative growth (start, trophophase) | No. of mycelial doublings[e] | | | | | | | | | |
| 8–13 | 0.6 | 879 | 127 | 14.4 | 1,208 | 335 | 27.7 | 678 | 20 | 2.9 |
| 11–16 | 0.6 | ND | | | 1,364 | 290 | 21.2 | 1,582 | 75 | 4.7 |
| 14–19 | 0.6 | 960 | 27 | 2.8 | 1,496 | 27 | 1.8 | 1,524 | 3 | 0.19 |
| 17–22 | 0.6 | ND | | | 1,095 | 39 | 3.5 | 1,167 | 5 | 0.4 |
| 20–25 | 0.6 | ND | | | 4,045 | 179 | 4.4 | 1,507 | 2 | 0.1 |
| 23–28 | 0.5 | ND | | | 2,397 | 13 | 0.5 | 1,046 | 4 | 0.38 |
| 25–33 | 0.7 | 348 | 0 | 0 | ND | | | ND | | |
| Vegetative growth (end, transition phase 24–72)[f] | 7[e] | ND | | | 1,982 | 108 | 5.4 | ND | | |
| Conidiation[g] | | | | | | | | | | |
| 0–24 | | 1,124 | 24 | 2.1 | ND | | | ND | | |
| 24–48 | | 871 | 7 | 0.8 | ND | | | ND | | |
| 0–48 | | ND | | | 716 | 3 | 0.4 | ND | | |

[a] Cultures were treated with 5-AC for the indicated periods of time; washed free of the drug and incubated further in drug-free medium until 72 h; the conidial offspring were then analyzed in all samples as described previously (31).
[b] The percentage of f colonies related to the total number of colonies (f and $f^+$).
[c] Measured at the end of the treatment period.
[d] ND; Not done.
[e] The mycelial dry weight doubling time was 8 h for the growth conditions used in this study.
[f] The 24-h-old mycelia were diluted 1:5 in 5-AC-containing medium, and the fluffy conidial offspring were scored after 72 h in the resulting mycelial mass.
[g] The 72-h-old mycelia were harvested on filter paper and induced to conidiate as described previously (31) in the presence of 5-AC for the indicated periods of time.

offspring scored. A faster rate of division could also account for the dramatic differences in size observed between fluffy and wild-type colonies of the same age growing from individual conidia on solid surfaces (Fig. 1B).

**5-AC is specific for the fluffy mutation at the *fluF* locus.** The exact alteration produced in the locus *fluF* by 5-AC for the origination of fluffy clones will remain unknown until the mutated and wild-type alleles of this locus are isolated and their sequences compared. However, the alteration of *fluF* in fluffy clones appeared to be exquisitely dependent on the action of 5-AC and not of any other carcinogen or mutagen tested. The results presented in Table 2 indicate that while micromolar concentrations of 5-AC that do not affect cell viability or growth rate produced a high percentage of fluffy clones, significantly higher concentrations of other mutagens or carcinogens tested did not generate any fluffy clones through alteration of the *fluF* locus. Of all the agents tested, only treatments with novobiocin at elevated dosages that significantly affected fungal cell growth resulted in a detectable level (0.42%) of clones with a fluffylike morphology. However, complementation analysis in diploids obtained by crossing these variants with the fluffy clone G1059A3 of *A. nidulans* yielded positive results, indicating that they do not carry the same *fluF1* mutation (data not shown). The frequency of induction of fluffylike clones with other carcinogenic or mutagenic agents, including base analogs, ICR-170,

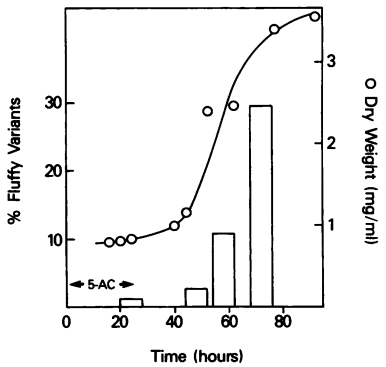


FIG. 2. Time-dependent increase of the percentage of fluffy conidial offspring in 5-AC-pretreated cultures of *A. nidulans*. A total of $10^5$ conidia per ml were inoculated into a liquid culture containing 25 μM 5-AC. After 24 h cultures were harvested and the drug was removed by washing the cultures with sterile water. The culture was then grown again in 5-AC-free medium until it reached the stationary phase. Appropriate portions were withdrawn at the indicated times and incubated under conidiation-promoting conditions in order to score the percentage of fluffy conidial offspring, as described in the text (31). The growth curve is in milligrams per milliliter. Histogram boxes represent the percentage of fluffy conidial offspring measured at the indicated times.

TABLE 2. Induction of fluffy clones by treatment of *A. nidulans* with different mutagens[a]

| Mutagenic agent | Dose | % Induction of fluffylike clones |
|---|---|---|
| Novobiocin | 3 mM | 0.42[b] |
| 5-bromodeoxyuridine | 10 mM | <0.02 |
| 5-bromodeoxycytidine | 10 mM | <0.01 |
| ICR-170 | 0.6 mM | <0.1 |
| UV light (254 nm) | 25 J/m$^2$ | <0.1 |
| L-Ethionine | 0.5 mM | <0.01 |
| Hydroxylamine | 0.1 mM | <0.02 |
| 5-AC | 25 μM | 14.4 |

[a] Treatments with each of the agents, scoring of the conidial offspring, and complementation analysis were performed as described in the text and elsewhere (1, 17, 27, 31).

[b] Complementation analysis indicated that fluffylike clones induced by novobiocin were not carrying the *fluF1* mutation.

UV light, L-ethionine, and hydroxylamine, was negligible and about 3 orders of magnitude lower than that obtained with 25 μM 5-AC (Table 2). These results suggest that *fluF* is particularly susceptible to molecular alteration by 5-AC but not by other agents. Whatever the molecular lesion is, it should involve a permanent modification of cellular genomic sequences since the *fluF1* mutation is hereditary and mitotically and meiotically stable (31).

**Chromosomal mapping of the *fluF1* locus. (i) Mitotic analysis.** Mitotic haploidization analysis of pertinent diploid strains was used initially to assign the *fluF1* marker to one of the eight linkage groups of *A. nidulans*.

Parasexual crosses of two independent fluffy clones (G1059A1 and G1059A3) with the master strain G94 (carrying markers for every chromosome) were used to originate heterozygous diploids that were then treated with PFFA (see above) to induce mitotic haploidization. The resulting haploid sectors were randomly selected and classified genetically (Table 3). These results indicate that the new marker *fluF1* is located in linkage group VIII. In fluffy haploid sectors from either of the two crosses studied, the mutant *fluF1* allele associated freely with both alleles of markers for all chromosomes, with the exception of *riboB2*, the marker for chromosome VIII (Table 3). Regarding the *riboB2* marker, all f sectors were prototrophic for riboflavin (*ribo*$^+$), while all f$^+$ sectors were auxotrophic (*ribo*$^-$). This absolute linkage in *trans* between *fluF1* and *riboB2* identified chromosome VIII as the bearer of the new marker.

**(ii) Meiotic analysis.** The *fluF1* locus was further mapped within chromosome VIII by analysis of the cleistothecia produced by meiosis in sexual crosses between fluffy and other strains of *A. nidulans* carrying markers at different positions in this chromosome. Analysis of the offspring of the sexual crosses of strains G1059A3 and G849 (Table 4) indicated that the new *fluF1* marker is located on the right arm of chromosome VIII, since the map distance (percentage of recombination) relative to marker *pdhC2*, which is located on the left arm of chromosome VIII (13), was nearly 50%. Furthermore, results from the cross G1059A3 × G843 indicated that *fluF1* maps to the proximal half of the right arm of chromosome VIII, since the recombination frequency with the marker *chaA1*, which is located on the distal end of this arm, was 52%. Finally, the frequencies of recombination of *fluF1* with the markers *riboB2* and *facB101*, which are located on the central zone of the right arm, and with *choC3* and *fwA1*, which are located to the left one third of this arm, were used to draw the genetic map shown in Fig. 3, in which *fluF1* is shown to be the closest marker to the centromere that has been mapped so far on the right arm of chromosome VIII. The frequency of recombination measured between *fluF1* and *tsD* in the cross G1059A3 × G82 was not consistent with those measured in all other crosses studied (Table 4) and was not considered in the genetic map shown in Fig. 3. In this case, the recombination frequencies obtained were not additive (*fluF1*:*tsD*, 26.5%; *tsD*:*fwA1*, 28.6%; *fluF1*:*fwA1*, 35.6%) and would locate *fluF1* even closer to the centromere than described in Fig. 3. The markers *choC3* and *tsD* are known to map very closely in chromosome VIII, but their distance to the centromere is unknown (13).

## DISCUSSION

In *A. nidulans*, a fungus that possesses negligible, if any, levels of methylated bases in its genome (2), 5-AC induces a high frequency of fluffy phenotypic variants that are characterized by massive, uncontrolled proliferation of aerial hyphae and a drastic delay of the start of asexual differentiation (conidiation). We have previously shown (31) that this new phenotype depends on a single Mendelian recessive factor that presumably results from modification of its wild-type allele by 5-AC.

Here we have characterized further the 5-AC-affected locus in relation to (i) its genetic identity and chromosomal location, (ii) its specificity as a target for 5-AC, and (iii) its

TABLE 3. Assignment of *fluF1* to chromosome VIII by mitotic haploidization analysis[a]

| Chromosome | Markers (f$^+$/f) | Assortment (no.) of chromosomal markers in: | | |
|---|---|---|---|---|
| | | f haploid sectors of f$^+$/f diploids in the following crosses: | | f$^+$ haploid sectors of f$^+$/f diploids of cross G94 × G1059 A1 |
| | | G94 × G1059 A1 | G94 × G1059 A3 | |
| I | *su1ad20* +/+ *pabaA1* | 11 *suad20* +, 16 + *paba* | 17 *su1ad20* +, 8 + *pabaA1* | 4 *suad20* +, 6 + *paba* |
| II | *w3*/+ | 16 *w*, 11 *w*$^+$ | 16 *w*, 9 *w*$^+$ | 4 *w*, 6 *w*$^+$ |
| III | *gal-1*/+ | 20 *gal*, 7 *gal*$^+$ | 6 *gal*, 19 *gal*$^+$ | 7 *gal*, 3 *gal*$^+$ |
| IV | *pyro4*/+ | 18 *pyro*, 9 *pyro*$^+$ | 10 *pyro*, 15 *pyro*$^+$ | 8 *pyro*, 2 *pyro*$^+$ |
| V | *facA303*/+ | 12 *fac*, 15 *fac*$^+$ | 7 *fac*, 18 *fac*$^+$ | 7 *fac*, 3 *fac*$^+$ |
| VI | *S3*/+ | 8 *S3*, 19 *S3*$^+$ | 20 *S3*, 5 *S3*$^+$ | 5 *S3*, 5 *S3*$^+$ |
| VII | *nic-8*/+ | 8 *nic*, 19 *nic*$^+$ | 11 *nic*, 14 *nic*$^+$ | 8 *nic*, 2 *nic*$^+$ |
| VIII | *riboB2*/+ | 0 *ribo*, 27 *ribo*$^+$ | 0 *ribo*, 25 *ribo*$^+$ | 10 *ribo*, 0 *ribo*$^+$ |

[a] Heterozygous diploids resulting from crosses between two fluffy strains and the master strain G94, which carried markers for every chromosome, were submitted to mitotic haploidization by treatment with PFFA, as described in the text and elsewhere (25). The resulting haploid sectors were randomly selected and classified phenotypically, according to the fluffy character and the associated chromosomal markers.

TABLE 4. Meiotic mapping of the *fluF1* gene of *A. nidulans* (cleistothecium analysis)[a]

| Cross | Parental genotype | Genotype of ascospore offspring[b] | | | |
|---|---|---|---|---|---|
| | | Total no. of ascospores | Parental types | Recombinant types | Recombination frequency (%) |
| G849 × G1059A3 | + *pdhC2* / *fluF1* + | 174 | *pdhC2* +,+ *fluF1* (90) | *pdhC2 fluF1*,+ + (84) | *pdhC2*:*fluF1* (48.2) |
| G843 × G1059A3 | + *chaA* / *fluF1* + | 100 | + *chaA*,*fluF1* + (48) | + +,*fluF1 chaA* (52) | *fluF1*:*chaA* (52) |
| G821 × G1058A3 | + *riboB2* / *fluF1* + | 171 | + *riboB2*,*fluF1* + (105) | + +,*fluF1 riboB2* (66) | *fluF1*:*riboB2* (38.5) |
| | + *facB101* / *fluF1* + | 162 | + *facB101*,*fluF1* + (104) | + +,*fluF1 facB101* (58) | *fluF1*:*facB101* (35.8) |
| G852 × G1059A3 | + *fwA1* / *fluF1* + | 248 | + *fwA1*,*fluF1* + (226) | + +,*fluF1 fwA1* (22) | *fluF1*:*fwA1* (8.9) |
| | + *choC3* / *fluF1* + | 248 | + *choC3*,*fluF1* + (238) | + +,*fluF1 choC3* (10) | *fluF1*:*choC3* (4.03) |
| | *choC3 fwA1* / + + | 248 | *choC3 fwA1*,+ + (232) | *choC3* +,+ *fwA1* (16) | *choC3*:*fwA1* (6.45) |

[a] Hybrid cleistothecia that arose from the indicated sexual crosses were isolated (12), and their ascospores were released (11) and classified genetically by monitoring the segregation of the indicated markers.

[b] Values in parentheses represent frequencies of the indicated genotypes.

participation and possible role in processes of fungal development.

Genetic analysis confirmed the uniqueness of this locus. The recessive, 5-AC-mutated allele *fluF1* was mitotically and meiotically stable, with a reversion rate lower than $10^{-6}$. Mitotic and meiotic analyses allowed us to map this gene as the closest marker to the centromere that has been located so far on the right arm of chromosome VIII (Fig. 3). We determined that this genetic locus had not been described previously and did not coincide with any of the other *flu* loci previously mapped in this linkage group (13). We designated this newly characterized gene *fluF*.

Several specific features of the action of 5-AC in this particular system were particularly noticeable. In particular, the extraordinary target specificity shown by this compound and the very high percentage of induced fluffy variants were striking. Under our experimental conditions, the low concentrations of 5-AC used always modified the *fluF* locus,

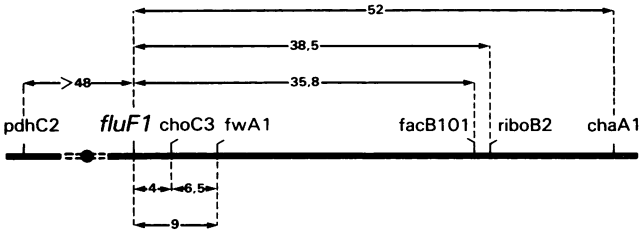


FIG. 3. Location of *fluF1* in chromosome VIII of *A. nidulans*. The map was constructed based on the frequencies of recombination measured between *fluF1* and other markers located at different positions in chromosome VIII. Symbols and abbreviations: ●, centromere; *pdhC2*, acetate requirement; *fluF1*, fluffy morphology; *choC3*, choline requirement; *fwA1*, fawn conidia; *facB101*, fluoroacetate resistant, acetate nonutilization; *riboB2*, riboflavine requirement; *chaA1*, chartreuse conidia; double dashed line, unknown distance.

while they did not affect either cell growth or viability (31). The action of the drug was so specific that even the percentage of the resulting fluffy variants could be predicted from the concentrations of 5-AC used. All these characteristics indicate that 5-AC does not act randomly as a conventional mutagen on the genome of *A. nidulans* but, rather, that *fluF* is singled out as a specific target for the drug under the experimental conditions used.

In fact, we determined (Table 2) that treatment of *A. nidulans* with a variety of chemical or physical mutagens that were selected for their ability to induce demethylation or epigenetic changes in eucaryotes did not produce any measurable induction of fluffy clones by modification of the *fluF* locus. Only novobiocin, which has been described as an inhibitor of the DNA topoisomerases in some eucaryotic systems (10), induced fluffylike phenotypic variants with a detectable frequency. However, complementation analysis demonstrated that those variants were mutated in a locus different than *fluF1*.

A similar case of hypermutability of a specific locus at a specific period of development has been described for the gene *regA* of *Volvox carteri* (22). However, unlike *fluF* (which is only open to modification by 5-AC), *regA* was susceptible to the action of a variety of chemical and physical mutagens (22).

At present we can only speculate about the biochemical nature of the alteration produced by 5-AC in the genomic sequences of *fluF*. 5-AC has been shown to be a mutagenic agent in yeast and mammalian cells, producing point mutations, mitotic recombination, endoreduplications, and sister chromatid exchanges (19, 33). Although we have detected some measurable generalized mutagenic potential of 5-AC in *A. nidulans* (31), it seems clear that, because of the peculiarities of the system, the 5-AC-mediated induction of fluffy variants is not the result of standard mutagenic mechanisms. The best-known effect of 5-AC is inhibition of DNA methylation (7, 8, 20, 21). Although DNA methylation levels fall

below detectable levels in global genomic analysis of this fungus (2), we cannot rule out the possibility that a few methylated genes could have escaped detection in the global analysis and still be the target for hypomethylation by 5-AC. However, it is also possible that 5-AC may be acting in this fungal system through other epigenetic mechanisms that are unrelated to DNA demethylation. For example, it has been shown that 5-AC-containing DNA can form stable complexes with DNA methyltransferases (29) and other nonhistone nuclear proteins (9). It has been suggested that such complexes can mediate heritable changes in gene activity (5, 9).

Regarding fungal development, results of this study indicate that the *fluF* locus is particularly susceptible to modification during a well-defined period of development and that its alteration by 5-AC leads to significant changes in the overall developmental program of the fungus. There was a precise interval, between 8 and 13 h of vegetative growth in liquid medium, at which the maximum frequency of induction of fluffy phenotypes was reached (Table 1). Although incorporation of 5-AC into the DNA was necessary for the induction of f variants (as shown by experiments carried out in the presence of hydroxyurea), the levels of incorporation of [$^{14}$C]5-AC between 8 and 13 h were not greater than those reached during other periods. Therefore, the maximum levels of induction obtained during this period can only be attributed to the fact that the *fluF* gene is more susceptible and open to modification by 5-AC in the initial stages of vegetative growth.

The main developmental features of the fluffy phenotype of *A. nidulans* included massive, uncontrolled proliferation of fluffy hyphae, a drastic delay of the asexual differentiation program, and invasivity of their colonies. Some of these properties are reminiscent of the malignant properties of mammalian tumor cells. It seems apparent that f clones do not respond to the stimuli which determine the cessation of vegetative growth and the start of asexual differentiation in $f^+$ wild-type clones. The developmental alterations observed suggest that *fluF* is involved in processes that occur at the onset of the asexual differentiation pathway in *A. nidulans*, and it can be speculated that its protein product may be needed either to control vegetative growth or to turn on the sporulation process. The observation that *fluF* is particularly susceptible to 5-AC at the start of the proliferative phase (Table 1) and the indications that fluffy nuclei divide faster than wild-type nuclei (Fig. 2) under conditions in which differentiation signals are not yet a factor suggest that the first possibility is more likely.

However, a definitive answer on the role of *fluF* in fungal development, as well as the lesion produced by 5-AC in this locus, awaits the molecular cloning of this gene and its mutated allele. Current efforts are directed to the isolation of the wild-type *fluF* gene by complementation of the recessive fluffy character in fluffy strains transformed with genomic libraries constructed with DNA from wild-type *A. nidulans*.

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## LITERATURE CITED

1. **Alderson, T., and M. J. Hartley.** 1969. Specificity of spontaneous and induced forward mutation at several loci in *Aspergillus nidulans*. Mutat. Res. **8**:255–264.
2. **Antequera, F., M. Tamame, J. R. Villanueva, and T. Santos.** 1984. DNA methylation in the fungi. J. Biol. Chem. **259**:8033–8036.
3. **Bergen, L. G., and N. R. Morris.** 1983. Kinetics of nuclear division cycle of *Aspergillus nidulans*. J. Bacteriol. **156**:155–160.
4. **Bird, A. P.** 1986. CpG-rich islands and the function of DNA-methylation. Nature (London) **321**:209–213.
5. **Brown, D. D.** 1984. The role of stable complexes that repress and activate eucaryotic genes. Cell **37**:359–365.
6. **Champe, S. P., M. B. Kurtz, L. N. Yager, N. J. Butnick, and D. E. Axelrod.** 1981. Spore formation in Aspergillus nidulans: competence and other developmental processes, p. 253–276. *In* G. Turian and H. R. Hohol (ed.), The fungal spore: morphogenetic controls. Academic Press, Inc., London.
7. **Charache, S., G. Dover, K. Smith, C. Conover, J. R. Talbot, M. Moyer, and S. Boyer.** 1983. Treatment of sickle cell anemia with 5-azacytidine results in increased fetal hemoglobin production and is associated with non-random hypomethylation of DNA around the α-δ-β-globin gene complex. Proc. Natl. Acad. Sci. USA **80**:4842–4846.
8. **Chen, E., R. W. Karr, J. P. Frost, T. A. Gonwa, and G. D. Ginder.** 1986. Gamma interferon and 5-azacytidine cause transcriptional elevation of class I major histocompatibility complex gene expression in K-562 leukemia cells in the absence of differentiation. Mol. Cell. Biol. **6**:1698–1705.
9. **Christman, J. K., N. Schneiderman, and G. Acs.** 1985. Formation of highly stable complexes between 5-azacytidine-substituted DNA and specific non histone nuclear proteins. J. Biol. Chem. **260**:4059–4068.
10. **Clarkson, J. M., and D. L. Mitchell.** 1983. The effect of various inhibitors of DNA synthesis on the repair of DNA photoproducts. Biochim. Biophys. Acta **740**:355–361.
11. **Clowes, R. C., and W. Hayes.** 1968. *In* Experiments in microbial genetics, p. 159–190. Blackwell Scientific Publications, Ltd., Oxford.
12. **Clutterbuck, A. J.** 1974. Aspergillus nidulans, p. 447–510. *In* R. C. King (ed.), Handbook of genetics, vol. 1. Bacteria, bacteriophages and fungi. Plenum Publishing Corp., New York.
13. **Clutterbuck, A. J.** 1987. Loci and linkage map of the filamentous fungus Aspergillus nidulans, p. 325–335. *In* S. J. O'Brien (ed.), Genetic maps. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Darmon, M., J. F. Nicolas, and D. Lamblin.** 1984. 5-Azacytidine is able to induce the conversion of teratocarcinoma derived mesenchymal cells into epithelial cells. EMBO J. **3**:961–967.
15. **Doerfler, W.** 1983. DNA-methylation and gene activity. Annu. Rev. Biochem. **52**:93–124.
16. **Dorn, G. L.** 1970. Genetic and morphological properties of undifferentiated and invasive variants of *Aspergillus nidulans*. Genetics **66**:267–279.
17. **Fincham, J. R. S., P. R. Day, and A. Radford (ed.).** 1979. Fungal genetics, p. 254–281. Blackwell Scientific Publications, Ltd., Oxford.
18. **Holliday, R.** 1987. The inheritance of epigenetic defects. Science **238**:163–170.
19. **Hori, T. A.** 1983. Induction of chromosome decondensation, sister-chromatid exchange and endoreduplications by 5-azacytidine, an inhibitor of DNA methylation. Mutat. Res. **121**:47–52.
20. **Jeggo, P. A., and R. Holliday.** 1986. Azacytidine induced reactivation of a DNA repair gene in Chinese hamster ovary cells. Mol. Cell. Biol. **6**:2944–2949.
21. **Jones, P. A.** 1985. Altering gene expression with 5-azacytidine. Cell **40**:485–486.
22. **Kirk, D. L., G. J. Baran, J. F. Harper, R. J. Huskey, K. S. Huson, and N. Zagris.** 1987. Stage-specific hypermutability of the regA locus of Volvox, a gene regulating the germ-soma dichotomy. Cell **48**:11–24.
23. **Konieczny, S. F., and C. P. Emerson, Jr.** 1984. 5-Azacytidine induction of stable mesodermal stem cell lineages from $10T^{1/2}$ cells: evidence for regulatory genes controlling differentiation. Cell **38**:791–800.
24. **Law, D. J., and W. E. Timberlake.** 1980. Developmental regu-

lation of laccase levels in *Aspergillus nidulans*. J. Bacteriol. **144:** 509–517.

25. **McCully, K. S., and E. Forbes.** 1965. The use of p-fluorophenylalanine with master strains of *Aspergillus nidulans* for assigning genes to linkage groups. Genet. Res. **6:**352–359.
26. **Olsson, L., and J. Forchammer.** 1984. Induction of the metastatic phenotype in a mouse tumor model by 5-azacytidine, and characterization of an antigen associated with metastatic activity. Proc. Natl. Acad. Sci. USA **81:**3389–3393.
27. **Pontecorvo, G., J. A. Roper, L. M. Hemmons, K. D. MacDonald, and A. W. J. Bufton.** 1953. The genetics of Aspergillus nidulans. Adv. Genet. **5:**141–238.
28. **Samid, D., D. M. Flessate, and R. M. Friedman.** 1987. Interferon-induced revertants of *ras*-transformed cells: resistance to transformation by specific oncogenes and retransformation by 5-azacytidine. Mol. Cell. Biol. **7:**2196–2200.
29. **Santi, D. V., A. Norment, and C. E. Garret.** 1984. Covalent bond formation between a DNA-cytosine methyltransferase and DNA containing 5-azacytosine. Proc. Natl. Acad. Sci. USA **81:** 6993–6997.
30. **Tamame, M., F. Antequera, J. R. Villanueva, and T. Santos.** 1983. 5-Azacytidine induces heritable biochemical and developmental changes in the fungus *Aspergillus niger*. J. Gen. Microbiol. **129:**2585–2594.
31. **Tamame, M., F. Antequera, J. R. Villanueva, and T. Santos.** 1983. High frequency conversion to a "fluffy" developmental phenotype in *Aspergillus* spp. by 5-azacytidine treatment: evidence for involvement of a single nuclear gene. Mol. Cell. Biol. **3:**2287–2297.
32. **Yisraeli, J., and M. Seyf.** 1984. *In* A. Razin, H. Cedar, and A. D. Riggs (ed.), DNA methylation: biochemistry and biological significance, p. 353–378. Springer-Verlag, New York.
33. **Zimmerman, F. K., and I. Scheel.** 1984. Genetic effects of 5-azacytidine in *Saccharomyces cerevisiae*. Mutat. Res. **139:**21–24.