MOLECULAR AND CELLULAR BIOLOGY, Jan. 2000, p. 61–69 Vol. 20, No. 1
0270-7306/00/$04.00+0

Histone H1 Is Dispensable for Methylation-Associated Gene Silencing in *Ascobolus immersus* and Essential for Long Life Span

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Received 27 July 1999/Returned for modification 13 September 1999/Accepted 28 September 1999

A gene encoding a protein that shows sequence similarity with the histone H1 family only was cloned in *Ascobolus immersus*. The deduced peptide sequence presents the characteristic three-domain structure of metazoan linker histones, with a central globular region, an N-terminal tail, and a long positively charged C-terminal tail. By constructing an artificial duplication of this gene, named *H1*, it was possible to methylate and silence it by the MIP (methylation induced premeiotically) process. This resulted in the complete loss of the *Ascobolus* H1 histone. Mutant strains lacking H1 displayed normal methylation-associated gene silencing, underwent MIP, and showed the same methylation-associated chromatin modifications as did wild-type strains. However, they displayed an increased accessibility of micrococcal nuclease to chromatin, whether DNA was methylated or not, and exhibited a hypermethylation of the methylated genome compartment. These features are taken to imply that *Ascobolus* H1 histone is a ubiquitous component of chromatin which plays no role in methylation-associated gene silencing. Mutant strains lacking histone H1 reproduced normally through sexual crosses and displayed normal early vegetative growth. However, between 6 and 13 days after germination, they abruptly and consistently stopped growing, indicating that *Ascobolus* H1 histone is necessary for long life span. This constitutes the first observation of a physiologically important phenotype associated with the loss of H1.

In mammals, methylation of CpG islands correlates with loss of gene expression (2). In plants, hypermethylation accompanies gene silencing (52), while silenced genes which recover expression in mutants unable to maintain silencing lose methylation (32). In the fungus *Ascobolus immersus*, repeated genes are methylated and silenced by a process named MIP (methylation induced premeiotically) that takes place during sexual reproduction (43). In *Neurospora crassa*, a related process, named RIP (repeat-induced point mutation), leads to a concomitant hypermethylation and hypermutation of the DNA repeats (47). In the latter situation, methylation may spread to an unmutated neighboring gene which becomes silenced as well, suggesting that methylation without mutation may be sufficient to initiate gene silencing (20). It has been shown that methyl-binding proteins recognizing methylated CpG's play an important role in the methylation-associated silencing in vertebrates (33). The methyl-CpG-binding protein MeCP2 can nucleate a complex containing deacetylases which remove acetyl moieties from lysine residues in the core histones H3 and H4 (41), resulting in a repressive nucleosomal array. In *Neurospora*, a connection has also been established between methylation, deacetylation, and gene silencing (48).

Methylated groups may directly prevent the binding of transcription factors to DNA (23). Methylation could also induce the formation of a silenced higher-order chromatin structure. Methylated DNA was found to be preferentially assembled in nuclease-resistant chromatin after transfection of mouse L cells (24). A ubiquitous component of chromatin might interact preferentially with methylated DNA, thereby stabilizing the higher order of chromatin structure and preventing gene expression.

Linker histones, which bind to linker DNA extending between nucleosomes, have been proposed as potential candidates for playing that role. Linker histones, such as H1, are known to seal nucleosomes, therefore stabilizing a higher order of chromatin structure (54). Histone H1 is abundant in nuclease-resistant, inactive chromatin (57), and it inhibits in vitro transcription (7, 59). Metazoan linker histones have a three-domain structure, with a central globular domain flanked by N- and C-terminal tails rich in basic residues (18). The amino acid sequence of the globular domain is the most conserved region. The basic C-terminal tail is rich in lysine, serine, proline, and alanine and is likely to be involved in the interaction with linker DNA, neutralizing its charge and facilitating chromatin condensation (1). In animals, linker histones show extensive diversification. Various subtypes display different DNA and chromatin-condensing properties in vitro (25). In addition, they exhibit highly regulated patterns of expression during development and differentiation (26).

Several studies aimed at investigating the possibility of a preferential binding of linker histones to methylated DNA have been performed, but the overall results remain inconclusive. In the mouse, 5-methylcytosine was reported to be preferentially located in nucleosomes that contain histone H1 (3). A chicken H1-like protein, MDBP-2, was reported to selectively bind methylated DNA both in vivo and in vitro (21). Further in vitro studies led to contradictory results. While H1 was reported by McArthur and Thomas (31) to bind preferentially to methylated DNA, Campoy et al. (5) and Nightingale and Wolffe (35) concluded that binding of H1 was indifferent to methylation in chromatin reconstitution experiments. It is

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difficult to make decisive conclusions from these in vitro studies, since factors playing an important role in the assembly of cellular chromatin may be missing in these assays.

A major contribution to understanding the structural and functional roles of linker histones in vivo came from experiments with *Xenopus laevis*. Extracts from *Xenopus* eggs depleted of histone B4, the only linker histone present in these eggs, retained the capacity to assemble chromatin from sperm nuclei, to initiate replication, and to condense their chromosomes (36). This finding indicates that linker histones facilitate the in vitro folding of nucleosomal arrays but are not required for chromatin and chromosome assembly. In somatic cells of *Xenopus*, histone H1 was shown to function as a developmentally regulated gene repressor acting specifically on the set of embryonic 5S RNA genes (4, 22, 46) and mesoderm-specific genes (53). In the case of 5S RNA genes, molecular studies indicated that the repressive effect of histone H1 is related to differential nucleosome positioning (38, 49).

In the unicellular eukaryotes *Saccharomyces cerevisiae* and *Tetrahymena thermophila*, putative linker histone genes encoding unusual products have been characterized. The *S. cerevisiae* candidate H1 histone contains two globular domains (27, 56), while that from *Tetrahymena* lacks the globular domain (61). In contrast, Ramón et al. recently characterized an *H1* gene encoding a canonic linker histone in the filamentous fungus *Aspergillus nidulans* (40). *S. cerevisiae*, *Tetrahymena*, and *A. nidulans* cells lacking linker histones are viable and display normal growth (37, 40, 61). In *A. nidulans*, the nucleosomal organization of a number of promoters was shown to be identical in a wild-type strain and in a strain harboring a complete deletion of the *H1* gene (40). Knocking out the *S. cerevisiae* linker histone gene had little effect on gene expression (37, 56). In particular, genes silenced as a consequence of their telomeric location were not activated in mutants devoid of histone H1. Deletion of the *H1* gene expressed in the macronucleus of *Tetrahymena* did not affect transcription, except for a small subset of genes that were either activated or repressed (50). This again suggests that linker histones do not play a general role in gene repression and gene silencing but can occasionally interact with some specific gene targets to modulate their expression. However, these data provide no information on a possible role of linker histone in methylation-associated gene silencing, since *S. cerevisiae*, *Tetrahymena*, and *A. nidulans* do not display cytosine methylation.

The filamentous fungus *Ascobolus immersus* represents an attractive, well-characterized experimental system with which to test in vivo by a genetic approach the possible interaction between linker histones, methylated DNA, and gene silencing. This organism displays DNA methylation, and MIP provides a convenient tool to methylate and silence at will endogenous genes (10, 13, 43). The cloning and characterization of the *H1*-like gene from *Ascobolus*, henceforth named *H1*, allowed us to inactivate the expression of this gene and to construct strains lacking *Ascobolus* histone H1. We showed that this histone is not required for methylation-associated gene silencing and protects methylated and unmethylated chromatin equally well against micrococcal nuclease (MNase) digestion. Its loss results in three clear-cut phenotypes: hypermethylation, increased accessibility of MNase to chromatin, and reduced life span.

MATERIALS AND METHODS

Transformation procedures, genetic analyses, and media. Standard genetic techniques, transformation procedures, and media were as described elsewhere (42).

Manipulation of DNA and methylation analysis. Most experimental procedures were as described previously (13). Other standard techniques were as in reference 45. PCR amplifications were performed under standard conditions (43). Cytosine methylation was analyzed by Southern hybridization, using the isoschizomers *Sau*3AI and *Nde*II, which are sensitive and insensitive, respectively, to C methylation. Methylation status was deduced from the replacement of the expected hybridizing *Sau*3AI fragments by larger fragments.

Cloning and characterization of the *Ascobolus H1* gene. *Ascobolus* genomic DNA digests were probed in Southern hybridization with the complete open reading frame (ORF) of the *A. nidulans H1* gene (40), which was kindly provided by C. Scazzocchio and colleagues. Hybridization and washings were performed at 53°C. To clone the hybridizing 1.7-kb *Hin*dIII-*Pst*I fragment, a size-fractionated (1.5- to 1.9-kb) *Hin*dIII/*Pst*I digest of the DNA from strain RN42 was subcloned into the *Hin*dIII-*Pst*I-digested pBluescript KS− vector (Stratagene). Clones were screened by colony hybridization. For reverse transcription-PCR (RT-PCR) experiments, total RNA was purified from mycelium by using the TRIzol reagent (GIBCO/BRL) and reverse transcribed, and PCR amplification of the cDNA was performed with primers H1cDNA1 and H1cDNA2, corresponding to positions 24 to 43 and 908 to 889 (Fig. 1), respectively. The PCR product obtained was sequenced with the same primers.

Methylation by MIP of the endogenous *H1* gene. An ectopic duplication of the *H1* gene was created in the wild-type strain FB14 by cointegrative transformation using plasmids pH1 and pMP6. Plasmid pH1 carried the 1.7-kb *Hin*dIII-*Pst*I fragment encompassing the *Ascobolus H1* gene. Plasmid pMP6 carried the *hph* (hygromycin resistance [Hyg^r]) gene, which was used as a selectable marker (29). Two Hyg^r transformants, T21 and T32, that had integrated a single full-length copy of the *Hin*dIII-*Pst*I *H1* fragment (identified by Southern hybridization) were selected and crossed with an appropriate tester strain in order to trigger MIP and methylation of the *H1* duplication. In the progeny, strains that had segregated away the transgenic element of the duplication through meiotic segregation (Hyg^s strains) and had thus inherited only the resident *H1* gene were isolated. Methylation of *H1* was checked by Southern hybridization.

Construction of *Ascobolus* strains. To analyze the effect of *H1* silencing on preexisting methylation, strains harboring the methylated and silenced allele of the *H1* gene (*H1*^{m}) were crossed with strain FC72 in which the 5.6-kb *Hin*dIII fragment carrying the resident *met2* gene had been previously methylated and silenced by MIP (creating the *met2*^{m} gene) (43). Methylation of *H1* in the Met$^+$ and Met$^-$ strains obtained was determined by Southern hybridization, allowing identification of the four possible genotypes, *H1*$^+$,*met2*$^+$; *H1*$^+$,*met2*^{m}; *H1*^{m},*met2*$^+$; and *H1*^{m},*met2*^{m}.

To analyze the effect of *H1* silencing on MIP, we constructed strains in which the duplication of the spore color gene *b2* (required for the generation of brown ascospores) was associated either with the *H1*^{m} or *H1*$^+$ allele. *H1*^{m} strains (*H1*^{m},*b2*$^+$) were crossed with strains FD25 and FD27 (*H1*$^+$,*b2*$^+$,[*met2*^{m}-*b2*$^+$-*hph*$^+$]) harboring the *met2*^{m}-*b2*$^+$-*hph*$^+$ transgene from plasmid pLmbh (30) in order to generate duplicate-*b2* strains (*H1*^{m},*b2*$^+$,[*met2*^{m}-*b2*$^+$-*hph*$^+$] and *H1*$^+$,*b2*$^+$,[*met2*^{m}-*b2*$^+$-*hph*$^+$]). Strains derived from brown ascospores that displayed resistance to hygromycin (indicative of the presence of the transgene and thus of the *b2* duplication) were analyzed by Southern hybridization to determine the methylation state of the *H1* gene.

To analyze the effect of *H1* silencing on meiotic methylation transfer (6), *H1*$^+$,*b2*^{m} and *H1*^{m},*b2*^{m} strains were constructed by crossing *H1*^{m},*b2*$^+$ strains with strain VLM6 (*H1*$^+$,*b2*^{m}) in which the 7.5-kb *Hin*dIII fragment encompassing the resident *b2* gene had been previously methylated and silenced by MIP (*b2*^{m}) (30). The methylation state of *H1* in strains from white ascospores (*b2*^{m}) was determined by Southern hybridization.

Mycelial growth rate analysis. Ascospores from a cross between the *H1*-duplicated strain (*H1*$^+$, [*H1*$^+$-*hph*$^+$]) and wild type (*H1*$^+$) were germinated and screened for resistance or sensitivity to hygromycin. Only Hyg^s strains (that had segregated away the transgene and had thus inherited the resident *H1* gene only) were further analyzed. *H1*^{m} strains were distinguished from *H1*$^+$ strains by Southern hybridization analysis. For that purpose, a small fraction of the mycelium growing on minimal medium obtained 1 day after germination was used to inoculate a liquid culture in order to obtain mycelium for DNA extraction. In the meantime, cultures on minimal medium plates were allowed to grow, and the growth rate was measured every day. On the third day following germination, a small piece of agar cut in front of the growing mycelium was taken and transferred onto a plate containing fresh minimal medium. This procedure was repeated every 3 days.

Nucleus isolation and histone purification. The mycelium obtained from a 3-day culture in liquid medium was harvested by filtration, pressed dry, frozen in liquid nitrogen, and ground to a powder. Nucleus isolation was carried out at 4°C. Powdered mycelium (5 g) was introduced into a Potter homogenizer containing 20 ml of buffer A (1 M sorbitol, 7% Ficoll, 20% glycerol, 5 mM EGTA, 5 mM EDTA, 50 mM Tris-HCl [pH 7.5]). Homogenization was repeated seven times. The material obtained was transferred into a beaker, and 40 ml of buffer B (10% glycerol, 5 mM EGTA, 25 mM Tris-HCl [pH 7.5]) was slowly added and mixed. The mixed material was distributed into four 30-ml tubes, each containing 10 ml of a mix of buffers A and B (1/1.7 vol/vol) by pouring without disturbing the interface. After centrifugation at 4,300 rpm in a Sorvall HB-4 rotor for 7 min, the upper 15 ml of each tube was transferred to a new 30-ml tube containing 3.5 ml of buffer C (1 M sucrose, 10% glycerol, 25 mM Tris-HCl [pH 7.5]) without

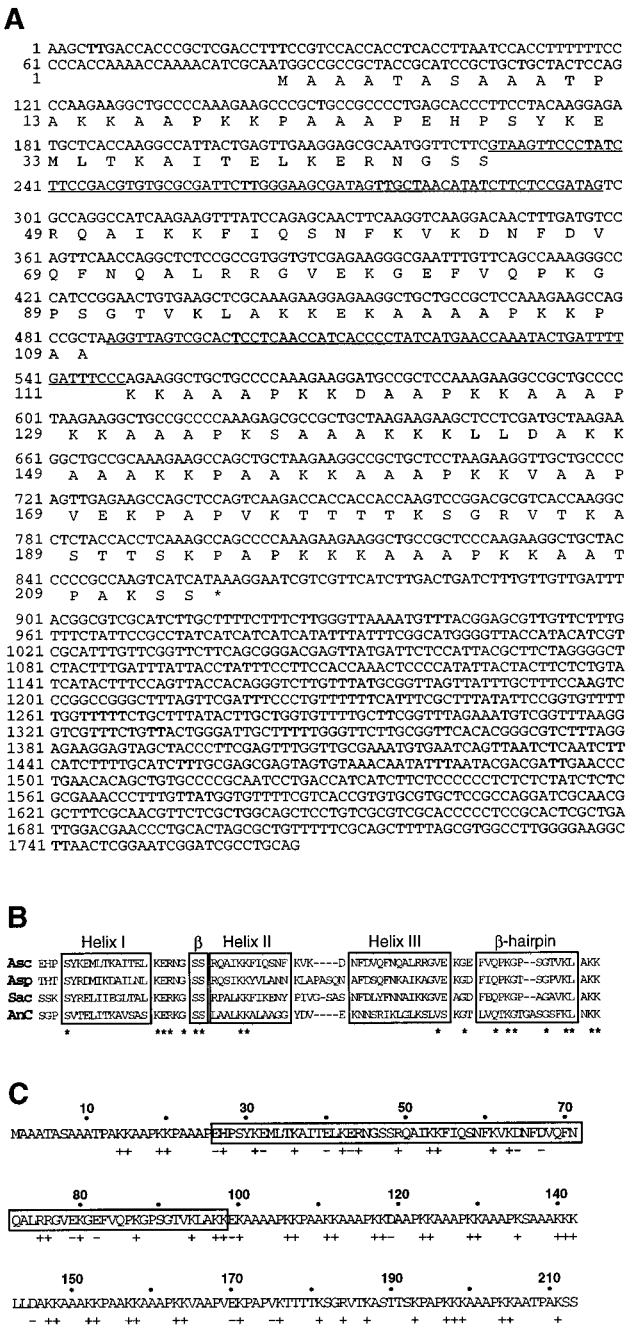

FIG. 1. Primary structure of histone H1 from *Ascobolus*. (A) Nucleotide and derived amino acid sequences of the *H1* gene. The entire 1.7-kb *Hin*dIII-*Pst*I fragment is shown. The two introns are underlined. (B) Comparison of the amino acid sequence of the globular domain of *Ascobolus* H1 (Asc) with the globular domains of H1 from *A. nidulans* (Asp) and *S. cerevisae* (Sac; the first globular domain is shown) and of the H1 consensus sequence resulting from the comparison of 30 animal H1 sequences (58). Boxes indicate the regions identified as α helix and β sheet in the H5 crystal structure (39). Asterisks indicate the conserved amino acids. (C) Tripartite organization of the *Ascobolus* H1 protein. The box indicates the globular domain flanked by the N- and C-terminal tails. Positions of basic (+) and acidic (−) residues are indicated.

disturbing the interface. After centrifugation at 9,000 rpm in a Sorvall HB-4 rotor for 20 min, the supernatant composed of two aqueous phases was discarded, and the pelleted nuclei were resuspended in 1 ml of buffer D (0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl [pH 7.5]) and immediately used or frozen at −80°C.

Total histones or histone H1 alone was purified from nuclei by the following protocol, performed at 4°C. A 100-μl aliquot of the nuclear suspension was centrifuged at 15,000 × *g* for 30 s. The pelleted nuclei were mixed with 50 μl of 5% $HClO_4$ (for histone H1 isolation) or 50 μl of 0.6 N H_2SO_4 (for total histone isolation) and incubated 45 min with occasional vigorous shaking. Centrifugation was then carried out at 15,000 × *g* for 30 min. The supernatant was transferred into a new tube, trichloroacetic acid was added to a final concentration of 20% (vol/vol), and the mixture was incubated overnight. Centrifugation was then carried out at 15,000 × *g* for 30 min. The histone pellet was washed once with acidic acetone (acetone–0.3 N HCl) and once with acetone. Finally, the histone pellet was dried and resuspended in Laemmli sample buffer (Bio-Rad) for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), performed with the PhastSystem (Pharmacia) and 20% polyacrylamide gels.

Nucleosomal repetition and chromatin analysis. Protoplasts from the different strains were prepared as described elsewhere (9). For the nucleosomal repetition analysis, 2×10^7 protoplasts were resuspended in 250 μl of permeabilization buffer (300 mM sucrose, 0.2% NP-40, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 15 mM Tris-HCl [pH 7.5]), and increasing amounts (0.75, 1.5, 3.0, 6.0, and 12 U for the $H1^m$ strain; 2.25, 4.5, 9.0, 18, and 36 U for the wild-type strain) of freshly added MNase (Boehringer Mannheim) were added. Samples were incubated 3 min at 25°C, and reactions were stopped by the addition of 250 μl of stop buffer (50 mM Tris-HCl pH 7.5, 20 mM EDTA, 1% SDS). After extraction, the DNA was size separated on a 1.5% agarose gel further stained with ethidium bromide.

For chromatin analysis, the same protocol was used except that 1.5, 4.5, 15, and 45 U of MNase were added, and DNA was digested overnight with 15 U of *Eco*RV before being size separated on a 1.5% agarose gel. Southern blots were probed with a 253-bp random-primed ^{32}P-labeled fragment of *met2* located just upstream from the *Eco*RV site corresponding to the 3′ end of the coding sequence, obtained by *Eco*RV digestion of the PCR product amplified by using primers corresponding to the sequences located at positions 2174 to 2191 and 2828 to 2811 of the published *met2* sequence (14).

Nucleotide sequence accession number. The GenBank accession number for the sequence reported in this paper is AF190622.

RESULTS

Isolation of the histone H1 gene from *Ascobolus*. Several restriction enzyme digests of *Ascobolus* DNA were probed in low-stringency conditions with the coding sequence of the *H1* gene from *A. nidulans* (40). A faint hybridization band was found with almost every DNA digest (data not shown). The hybridizing 1.7-kb *Hin*dIII-*Pst*I fragment was cloned. Its sequencing revealed the presence of a discontinuous ORF, split in three putative exons separated by two introns of 72 bp (positions 227 to 298) and 62 bp (positions 487 to 548), respectively (Fig. 1A). The presence and location of the two introns was confirmed by RT-PCR amplification followed by sequencing of the PCR product. The putative protein is 213 amino acids long, with a calculated molecular mass of 21.88 kDa.

Comparison of the amino acid sequence of the putative protein with protein databases showed similarity to H1 protein sequences only, suggesting that we had cloned the *H1* gene from *Ascobolus*. By using the *Hin*dIII-*Pst*I fragment as a probe in Southern hybridization, we could not detect any extra hybridizing fragments (data not shown).

Sequence alignment of the *Ascobolus* H1 protein with the H1 histones from other organisms revealed that residues 26 to 98 could be aligned with the globular domain, which is the most conserved region of the H1 family (Fig. 1B), showing that the *Ascobolus* H1 protein presents the characteristic three-domain structure of metazoan H1 histones: an N-terminal region of about 25 amino acids; a globular region of about 73 amino acids; and a positively charged C-terminal region of about 115 amino acids (Fig. 1C). Moreover, the nuclear location of this protein was shown by constructing strains expressing the green fluorescent protein fused to the carboxyl terminus of the H1 protein (data not shown).

Histone H1 is dispensable for gene silencing. If the *Ascobolus* H1 protein were required for methylation-associated gene silencing, it would be impossible to silence the *H1* gene via MIP. To test this prediction, an ectopic duplication of the *Hin*dIII-*Pst*I fragment containing the *H1* gene was created via

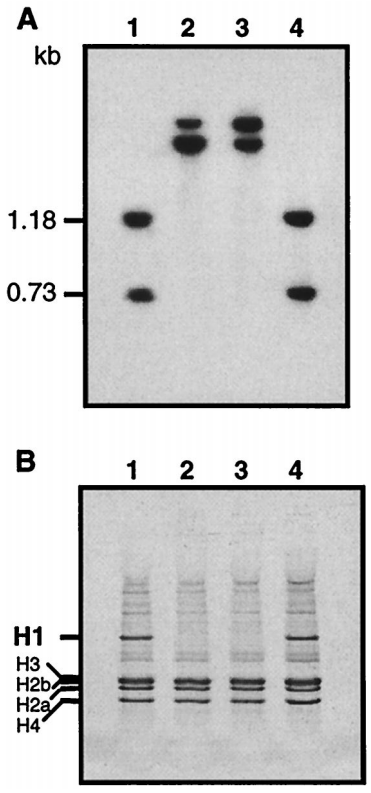

FIG. 2. Methylation and silencing of the *H1* gene. (A) Southern analysis of methylation in four strains derived from the *H1*-duplicated strain and harboring only the resident *H1* copy. *Sau*3AI DNA digests were probed with the cloned 1.7-kb *Hin*dIII-*Pst*I fragment carrying the *H1* gene (this work). Two strains (lanes 1 and 4) harbored the unmethylated allele, as revealed by the presence of the 1,184- and 728-bp expected fragments. The two other strains (lanes 2 and 3) harbored the methylated allele, as revealed by the presence of larger fragments. (B) SDS-PAGE of H_2SO_4-soluble proteins extracted from nuclei of the same strains as in panel A. The bands corresponding to H1 and to the core histones are indicated.

integrative transformation of wild-type strain FB14. Two independent transformants harboring the duplication were used in crosses in order to trigger MIP and target methylation at the native *H1* gene. Progeny strains that had segregated away the transgene through meiotic segregation were used for further analyses. The methylation status of the native *H1* gene was analyzed by Southern hybridization using restriction enzymes sensitive to cytosine methylation. Methylation was found in 20% of the strains (Fig. 2A). We then determined whether the methylated *H1* gene was silenced, as expected for genes that have undergone MIP. Total histone proteins were extracted from nuclei of two strains either with or without the native *H1* gene methylated. The complete and specific disappearance of histone H1 in *H1*-methylated strains indicated that the *H1* gene was completely silenced (Fig. 2B). Accordingly, when the protocol for extracting only histone H1 from the *H1*-methylated strains was used, no protein was detected by SDS-PAGE (data not shown).

The above results indicate that H1 is dispensable for gene silencing resulting from MIP, since its own expression can be stably silenced by MIP. We further showed that in the absence of H1, maintenance of silencing also occurred for endogenous single-copy genes that had been previously methylated by MIP. By appropriate crosses, we constructed $H1^m$,$met2^m$ strains in which the silenced *H1* gene was associated with the silenced *met2* gene. These strains exhibited the characteristic Met^- phenotype expected from the silencing of *met2*. We previously showed (43) that Met^- strains, in which *met2* was silenced by MIP, were always able to revert spontaneously to prototrophy after growth on nonselective medium (supplemented with methionine) and transfer onto selective medium (without methionine). Reversion is observed after a period of time following transfer ranging from a few days to more than a month. $H1^m$,$met2^m$ strains were also able to revert spontaneously to prototrophy, which occurred within a period of time similar to that needed for reversion of the control ($H1^+$,$met2^m$) strains. This indicated that the lack of H1 had no effect on the stability of silencing. We also constructed $H1^m$,$b2^m$ strains in which the silenced *H1* gene was associated with the silenced ascospore color gene *b2*. Crosses between two $H1^m$,$b2^m$ strains gave pure noncolored ascospore progeny only (the fertility of crosses involving two $H1^m$ strains is described below) indicating that the silencing of *b2* was faithfully maintained (data not shown).

We also tested whether the loss of histone H1 could affect the occurrence and frequency of MIP in premeiotic cells (43) as well as methylation transfer in meiotic cells (6), two processes which lead to gene silencing. To analyze the effect of *H1* silencing on MIP, the two types of strains harboring a duplication of the *b2* gene, containing the $H1^m$ or the $H1^+$ allele, were crossed with $H1^m$ and H^+ strains, respectively. Both types of crosses gave the same frequencies of MIP (data not shown), indicating that histone H1 is dispensable for MIP of *b2*. To analyze the effect of *H1* silencing on meiotic methylation transfer, $H1^+$,$b2^m$ and $H1^m$,$b2^m$ strains were crossed with $H1^+$,$b2^+$ and $H1^m$,$b2^+$ strains. All four types of crosses gave rise to similar progeny, consisting of ~90% of asci exhibiting the expected 4 brown:4 white segregation and ~10% of asci with an excess of white spores, reflecting methylation transfer. This indicated that histone H1 is dispensable for this process as well (data not shown).

The lack of histone H1 results in hypermethylation. DNA from *H1*-silenced strains was more resistant to digestion with methylation-sensitive enzymes than the wild type, as revealed by the presence of a large amount of uncut DNA in ethidium bromide-stained agarose gels (Fig. 3A). We checked that this was not the result of an incomplete digestion by probing the digests with the unmethylated *met2* gene from *Ascobolus* (Fig. 3D, lanes 1 to 4).

Global hypermethylation was confirmed by the finding that the 5-methylcytosine content of *H1*-silenced strains (kindly determined for us by J. Desgrès and A. Costa) was 13.35% of total C's on average (12.66, 13.35, and 14.05%), while it was 8.4% in wild-type strains (7.39, 8.94, and 9.03%).

We found that in *H1*-silenced strains, hypermethylation affected the native DNA repeats that exhibit, in wild-type strains, methylation patterns characteristic of MIP (16). For instance, methylation exhibited by the ~60 copies of *Mars3*, a copia-like retroelement, was much more homogeneous and dense in *H1*-silenced strains than in wild-type strains, as revealed by the presence of a major strong high-molecular-weight hybridizing band released by the methylation-sensitive enzyme (Fig. 3C). Hypermethylation also affected the single-copy gene *met2* that had previously undergone MIP (Fig. 3D, lanes 5 and 7). Importantly, the size of the largest methylated *met2* fragment (~6 kb) was the same in *H1*-silenced and wild-type strains (Fig. 3D, lanes 4 to 8), indicating that hypermethylation did not spread outside the region initially methylated. This was also confirmed by probing the flanking regions and showing that they remained totally unmethylated at the sites tested in *H1*-silenced strains (data not shown). Moreover, the unmethylated *met2* gene did not become methylated in *H1*-silenced strains (Fig.

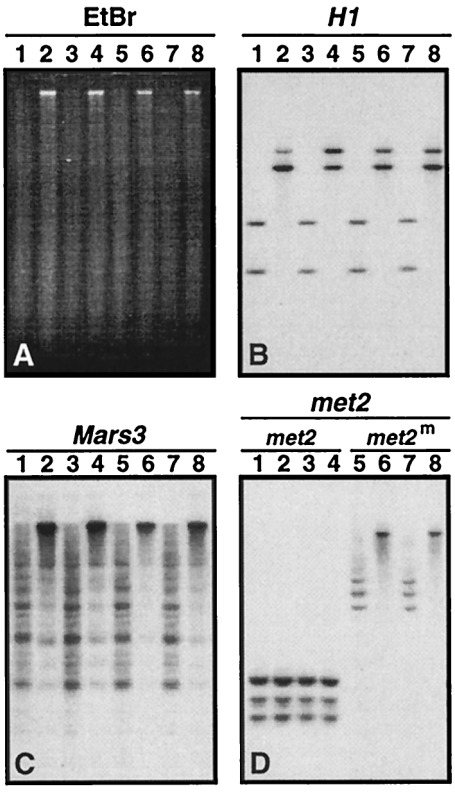

FIG. 3. Hypermethylation in strains lacking histone H1. (A) Ethidium bromide staining of the gel used for Southern analyses of the *Sau*3AI DNA digests shown in panels B to D. As determined from panel B, strains analyzed in lanes 1, 3, 5, and 7 harbored the native unmethylated *H1* allele, and strains analyzed in lanes 2, 4, 6, and 8 harbored the methylated allele. The gel was probed with *H1* (B), *Mars3* (C), and *met2* (D). The *H1* probe was as in Fig. 2. The *Mars3* probe corresponded to the *Eco*RI fragment carrying *Mars3* in plasmid pCG57 (16). The *met2* probe was a PCR product corresponding to the 2.9-kb *Hin*cII-*Bgl*II fragment carrying the *met2* gene (14). Strains 5 to 8 had inherited a silenced allele of *met2*, while strains 1 to 4 expressed this gene. The largest *met2 Sau*3AI fragments from strains 5 and 7 are hardly visible on the autoradiograph presented in panel D.

3D, lanes 2 and 4), suggesting that hypermethylation caused by the loss of H1 affects only previously methylated genes.

We have previously shown (15) that while all C's can be methylated as a result of MIP, not all DNA molecules derived by replication from the molecule that had undergone MIP are methylated with the same intensity. While C's belonging to CpG sites are nearly always methylated, other C's display lower levels of methylation, ranging from 75 to 83%, which causes the heterogeneity of the methylation patterns obtained by Southern hybridization. Therefore, the hypermethylation observed following the loss of H1 corresponds to an increased methylation at non CpG sites, which accounts for the lack of cleavage of the GATC sites by *Sau*3AI. This was further confirmed by showing that the AGCT sites remained uncut by the C-methylation-sensitive enzyme *Alu*I in the hypermethylated genes analyzed (data not shown).

We then asked whether hypermethylation was maintained in strains restored for histone H1. We analyzed the progeny of crosses between *H1*-silenced and wild-type strains. Thirty asci were dissected, and methylation of the *H1* gene was analyzed by Southern hybridization in individual meiotic products (data not shown). *H1* methylation showed the expected 2:2 segregation. The overall genomic hypermethylation always cosegregated with the methylated *H1* allele and loss of the H1 protein, hypermethylation never being found in products that had inherited the unmethylated *H1* allele and had a normal content of histone H1. These results show that hypermethylation per se is not heritable and therefore that it is strictly correlated with the lack of histone H1.

The methylation-associated chromatin modification does not require histone H1. The above results show that H1 is dispensable for both gene silencing and methylation and that its loss results in DNA hypermethylation. This led us to ask whether this loss would affect the chromatin changes associated with methylation. In another study (J. L. Barra, G. Grégoire, G. Almouzni, J.-L. Rossignol, and G. Faugeron, unpublished data), we had shown, by analyzing MNase-controlled digests of chromatin, that methylation in the *Ascobolus met2* gene was associated with a change in chromatin that was confined to the methylated portions of the gene. Here we compared, in the same way, the chromatin configuration of the unmethylated and methylated *met2* gene in *H1*-silenced and wild-type strains (Fig. 4). The loss of H1 did not lead to any change in the MNase banding patterns. Notably, the chromatin changes associated with methylation were similar in the two types of strains, which indicates that the chromatin changes that accompany methylation do not depend on H1. As expected, an analysis of the overall genomic nucleosomal pattern showed that the chromatin was more accessible to MNase in strains lacking H1. Indeed, three to four times less enzyme was sufficient to produce the same nucleosomal patterns as in the wild type (Fig. 5). Although the nucleosomal patterns were similar, oligonucleosome bands were much less diffuse in the absence of H1 than in its presence (Fig. 5). The size heterogeneity exhibited by wild-type oligonucleosomes may result in part from the fact that some of the nucleosomes contain H1 and others do not. In the *met2* region also, the lack of H1 made the chromatin more accessible to MNase. Indeed, equal amounts of MNase gave higher levels of digestion in strains lacking H1 than in the wild-type control (Fig. 4A). Interestingly, this happened independently of the methylation status of *met2*, which indicates that in *Ascobolus* histone H1 interacts similarly with methylated and unmethylated DNA. These results indicate that there is no preferential binding of histone H1 to methylated DNA in *Ascobolus*. Furthermore, the hypermethylation phenotype and the increased accessibility of MNase to chromatin in strains devoid of histone H1 indicate that this protein is a chromatin constituent, as expected for linker histones.

Vegetative and sexual phenotypes of strains lacking histone H1: essential role of H1 for normal life span. The loss of H1 did not confer any noticeable phenotype to the mutant strains either during early vegetative life or during sexual reproduction. Spore germination was not affected, and mycelial growth occurred at the normal rate. In contrast to *Tetrahymena*, where histone H1 knockout cells display enlarged 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei (51), we did not detect any difference in the sizes of the nuclei from either protoplasts (Fig. 6) or mycelium in strains devoid of H1 or wild-type strains after DAPI staining.

The sexual reproduction cycle of *Ascobolus* consists of an ordered series of differentiation steps consisting in fertilization, formation of the fruiting bodies, differentiation of the dikaryotic cells, karyogamy leading to diploid cells, meiosis, and ascospore formation. No differences in any of these steps could be detected between crosses involving two parental *H1*-silenced strains, which were normally fertile, and wild-type crosses.

However, a short-life-span phenotype consistently appeared when *H1*-silenced strains were grown for more than 6 days

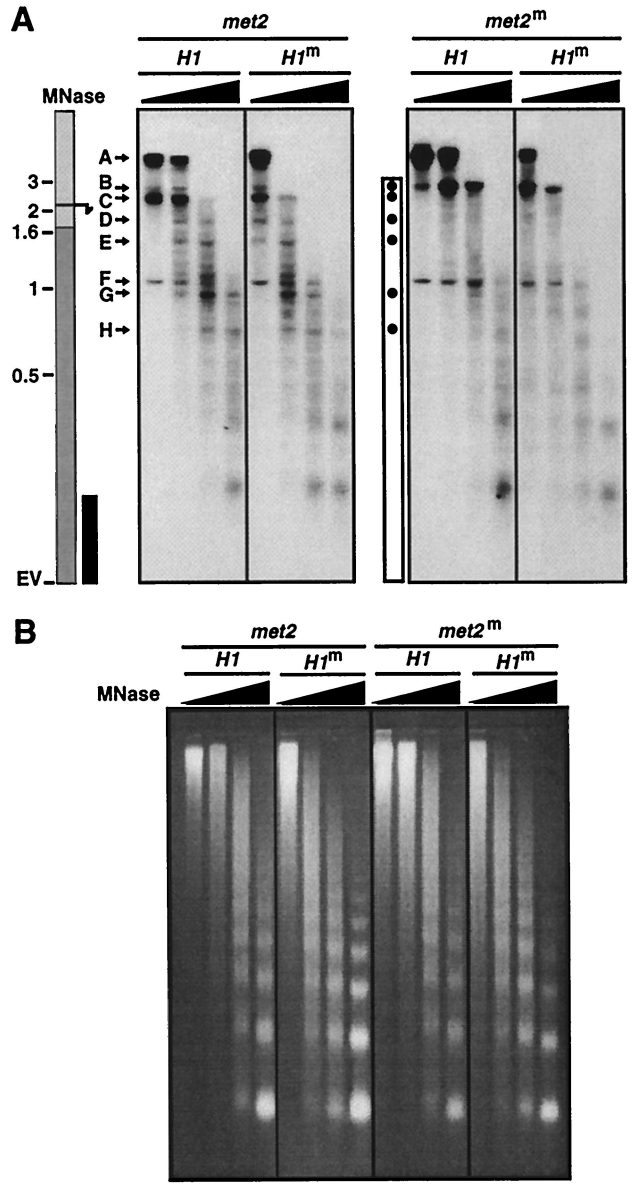

FIG. 4. Comparison of the methylation-associated chromatin modifications in *H1*-silenced and wild-type strains. (A) MNase analysis of the chromatin of the *met2* gene was performed in two strains harboring the unmethylated allele of *met2* (*met2*) and two strains harboring the methylated allele of this gene (*met2*^{m}). One strain of each type harbored the silenced allele of *H1* (*H1*^{m}); the other harbored the wild-type allele (*H1*). Protoplasts were incubated with increasing amounts of MNase and subjected to indirect end-labeling analysis. Samples were loaded on the same gel. The long vertical box at the left represents the *met2* gene, with the transcription start site (arrow), ORF (dark gray box), position of the *Eco*RV site (EV), and size markers (in kilobases) indicated. The black vertical box indicates the probe used for hybridization. A to H indicate the eight major bands obtained when *met2* was unmethylated (*met2*,*H1*; *met2*,*H1*^{m}). Band A corresponds to the *Eco*RV fragment carrying the *met2* gene. The white vertical box indicates the methylated region of *met2*. Black dots indicate the positions of bands that changed when *met2* was methylated (*met*^{m},*H1*; *met2*^{m},*H1*^{m}). (B) Ethidium bromide staining of the gel used for hybridization shown in panel A.

following germination. They suddenly stopped growing within a 6- to 13-day range, while wild-type strains continued to grow normally (Fig. 7). Short life span always cosegregated with the silenced *H1* gene in the progeny of crosses between an *H1*-silenced strain and the wild type. The 6- to 13-day delay preceding the sudden growth arrest was not shortened after one or two generations of intercrossing between *H1*-silenced strains, showing that passage through the sexual cycle results in a resetting of the life length of *H1*-silenced strains.

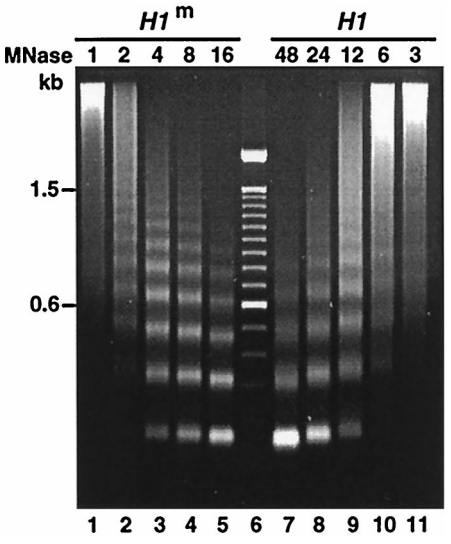

FIG. 5. Nucleosome repeat ladder obtained after chromatin digestion with MNase of one strain harboring the silenced allele of *H1* (*H1*^{m}) and one strain harboring the wild-type allele (*H1*). In the central lane is the size marker ladder (100-bp ladder; GIBCO BRL). The relative amounts of MNase used are indicated above the lanes.

DISCUSSION

By using MIP as a tool, we constructed *Ascobolus* strains in which the native *H1* gene was methylated and totally silenced, as shown by the complete absence of histone H1. This indicates

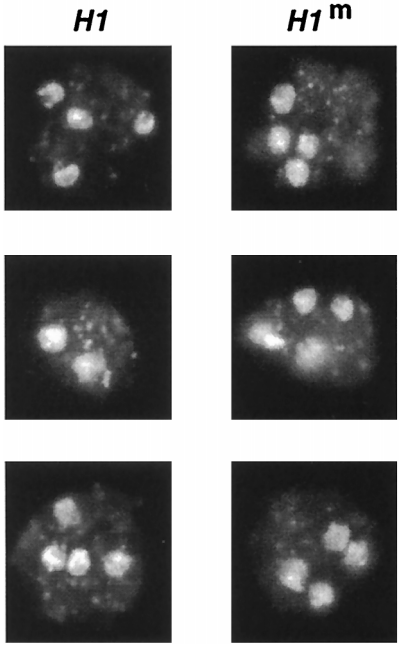

FIG. 6. Comparison of DAPI-stained nuclei in protoplasts from the wild-type strain (*H1*) and strains lacking H1 (*H1*^{m}).

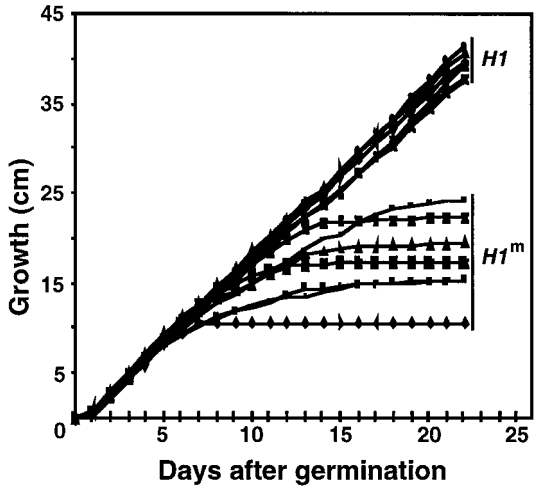

FIG. 7. Comparison of growth rates of six strains harboring the silenced copy of the *H1* gene ($H1^m$) and six wild-type strains (*H1*).

that in *Ascobolus* mycelium, the presence of H1 depends on the expression of a single copy of the *H1* gene. In this respect, *Ascobolus* behaves like other lower eukaryotes. We cannot exclude, however, the existence of one or more H1 histone variants in the sexual reproduction cycle of *Ascobolus*, as exists in animals in developmentally regulated systems.

The loss of H1 results in a general increase in MNase accessibility to chromatin. This effect indicates that the *Ascobolus* H1 protein is associated with a large fraction of the nuclear chromatin and behaves in that respect as expected for a linker histone. This conclusion is reinforced by the fact that a considerable fraction of the methylated genome is protected by H1 against hypermethylation.

Our study provides definitive evidence that at least in *Ascobolus*, histone H1 does not play a role in vivo in mediating the effects of DNA methylation on chromatin structure and gene expression. The main conclusion of this study is that histone H1 does not mediate methylation-associated gene silencing in *Ascobolus*, since strains lacking H1 can still undergo MIP and gene silencing. This first point is in line with in vitro observations made by Nan et al. (33) suggesting that histone H1 may not be involved in gene repression mediated by methylation. These authors showed that when accessing its binding sites, rat MeCP2, which acts as a transcriptional repressor, can displace a large fraction of histone H1 from methylated chromatin. Our study of the *met2* region suggests three other conclusions. (i) H1 does not play a role in nucleosomal positioning, as indicated by the fact that the loss of H1 did not lead to any detectable change in the nucleosomal pattern over the *met2* region. (ii) The chromatin changes associated with methylation are not dependent on histone H1. We showed that *Ascobolus* strains lacking histone H1 retained the chromatin modifications associated with the methylated *met2* region. (iii) H1 is present in both methylated and nonmethylated chromatin, as indicated by the observation that similar increases in chromatin accessibility were observed in the different strains lacking H1, independently of the methylation state. This finding is consistent with observations made in vitro showing that H1 does not bind preferentially to methylated DNA (5, 35).

Histone H1 is required for the normal vegetative life span of *Ascobolus*. Strains devoid of H1 suddenly stopped growing between 6 and 13 days after germination, i.e., after more than 50 division cycles. The short-life-span phenotype was not observed in *Tetrahymena* and *S. cerevisiae* (37, 61), which, unlike *Ascobolus*, do not display a metazoan-like histone H1. Such a phenotype also was not found in work performed with *A. nidulans*, although in this fungus H1 exhibits a metazoan-like tripartite structure (40). The effect found in *Ascobolus* may be accounted for by specific chromatin changes resulting from the loss of H1 that could repress genes essential to growth. Indeed, in *Tetrahymena*, although the loss of histone H1 has no detectable effect on viability and growth, it can result in the repression of some genes (50). The delay observed in *Ascobolus* before the arrest of growth suggests that the loss of H1 triggers the progressive accumulation of events that would lead eventually to the inability of the nuclei to divide further. The nature of these events is unknown. In the first hypothesis, one or several control genes ensuring the fidelity of the information flow, such as genes encoding chaperones, could be repressed concomitantly with the loss of H1. This would create a cascade of events that could indirectly lead to the arrest of growth. In a second hypothesis, a gene(s) essential to growth could be progressively repressed. Since strains lacking H1 display hypermethylation, methylation per se might be directly involved by spreading progressively toward essential genes, thus repressing them. This seems unlikely, however, since strains lacking H1 do not experience de novo methylation of nonmethylated genes, and neither methylation nor chromatin changes spread from hypermethylated regions to flanking sequences. Independently of methylation, the loss of H1 could be responsible for the progressive occurrence of chromatin changes in some essential genes. An attractive hypothesis comes from the data of Wolffe and colleagues showing that in vitro, the nonhistone linker protein HMG1 can replace histone H1 in chromatin (34, 55). Indeed, HMG1 was shown, also in vitro, to reversibly inhibit transcription by RNA polymerase II by interacting with the TATA-binding protein, suggesting that HMG1 is likely to affect the basal transcriptional machinery (11). Thus, it may be that the chromatin of strains lacking H1 becomes progressively enriched in HMG1, during early mycelial growth, reaching a stage at which the genome, or some critical genes, become silenced. Interestingly, strains lacking H1 undergo normal sexual reproduction if they are intercrossed before the arrest of growth, and the progeny displays the same 6- to 13-day delay before the arrest of growth. To account for this resetting of the life length expectancy through sexual reproduction, we must assume that expression of the critical gene(s) during this phase of the life cycle is not affected by the postulated changes in the chromatin control of gene expression.

Interestingly, the loss of histone H1 results in hypermethylation of chromosomal regions previously methylated by MIP. Since close to 100% of the C's belonging to CpG dinucleotides are methylated by MIP while other C's are less densely methylated (15), hypermethylation must mainly concern C's not belonging to CpG sites. Previous results suggest that two distinct mechanisms acting at CpG and non-CpG sites underlie maintenance of methylation in *Ascobolus* (12, 15). The first mechanism would consist in the methylation of CpG sites according to the classical maintenance model (19, 44). The second mechanism would result in the methylation of neighboring C's. It is known that in mammalian cells, in which methylation occurs almost exclusively at CpG sites the methyltransferase localizes to the chromosomal replication complex (28) and maintenance methylation takes place less than 1 min after replication (17). By contrast, chromatin assembly takes 10 to 20 min (8), histone deposition occurs in stages, and it is not until a complete histone octamer is assembled with DNA that histone H1 is stably sequestered (60). If this scenario also holds

for *Ascobolus*, the following model could explain our observations. Methylation at CpG sites would proceed in a first short step associated with DNA replication. The second mechanism directing methylation at non-CpG sites would occur secondarily, during chromatin assembly, and would be hindered by H1 interacting with DNA. In absence of H1, secondary methylation could not be blocked, resulting in hypermethylation. In keeping with this hypothesis is the observation that the variant human histone H1e can inhibit in vitro enzymatic DNA methylation (62).

ACKNOWLEDGMENTS

We are grateful to Annie Grégoire for help with some experiments and Solange Dehan for preparing all media. We thank Claudio Scazzocchio and colleagues, who kindly provided the *A. nidulans H1* gene and data prior to publication. We thank J. Desgrès and A. Costa, who kindly determined the 5-methylcytosine contents of *Ascobolus* strains by high-performance liquid chromatography analyses of deoxyribonucleosides obtained after enzymatic hydrolysis of DNA. We thank Geneviève Almouzni, Vincent Colot, and Allyson Holmes for critical reading of the manuscript and members of the laboratories for discussions.

J.L.B. was a recipient of a fellowship from the French Ministère des Affaires Etrangères followed by a fellowship from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina. This work was supported by grants from the Association pour la Recherche sur le Cancer (contracts 6200 and 9554) and the European Union (contract BIO4-96-0253).

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