Active genes in budding yeast display enhanced in vivo accessibility to foreign DNA methylases: a novel in vivo probe for chromatin structure of yeast

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Unlike higher eukaryotes, where an inverse correlation has been generally observed between gene expression and methylation of CpG sites, the budding yeast *Saccharomyces cerevisiae* lacks DNA methylation. Gene regulatory mechanisms can function independently of DNA methylation in yeast, and yeast strains expressing foreign DNA methylases that modify adenine and CpG residues have been found to be viable. We have used such strains to determine whether the transcriptional status of genes can influence the level of their DNA methylation in vivo. Several genes were tested, for example, *GAL1*, *-7*, and *-10*, *PHO5*, *HMRa* and *HMLα*, and *STE2* and *STE3*. Surprisingly, we found that all the genes displayed severalfold more methylation in the expressed state as compared to the repressed state. This procedure serves as a novel in vivo probe for the chromatin structure of yeast and potentially for higher eukaryotes.

[Key Words: In vivo DNA methylation; gene expression; budding yeast; foreign methylases]

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The budding yeast *Saccharomyces cerevisiae* and higher eukaryotes share several common features of chromatin structure, including nucleosomal organization, folding of chromatin into higher-order structures, as well as the conservation observed for transcriptional machinery (Weintraub 1985; Guarente 1988; Lowary and Widom 1989; Perez-Ortin et al. 1989). Although the budding yeast is thought to lack H1 histone (Perez-Ortin et al. 1989), a functional H1 homolog was demonstrated recently (Lowary and Widom 1989). In addition, active genes in yeast and higher eukaryotes display similar functional features, i.e., enhanced sensitivity to single-stranded endonuclease S1 and DNase I at sites flanking the active genes (for reviews, see Weintraub 1985; Perez-Ortin 1989), indicating an alteration of nucleosomal organization in the promoter and coding regions upon gene activation.

Despite these similarities, yeast and higher eukaryotes differ in one major respect: higher eukaryotes display methylation of cytosine residues in the CpG sequences in DNA (Holliday and Pugh 1975; McGhee and Ginder 1979; Cedar 1988; Razin and Riggs 1980; Felsenfeld and McGhee 1982; Holliday 1987), whereas the budding yeast is shown to lack this modification (Proffitt et al. 1984). More importantly, an inverse correlation has generally been observed between CpG methylation and gene expression in vertebrates and plants, leading to the hypothesis that gene expression is regulated by cytosine

methylation in higher eukaryotes. Because DNA methylation does not exist in yeast, mechanisms of gene activation and repression in yeast must operate independently of DNA methylation. Expression of the *Escherichia coli dam* methylase, which methylates adenine residues in GATC sites, and *Bacillus subtilis* SPR phage cytosine methylase, which methylates the cytosine residues in the *HpaII* (and also *HaeIII* and *BstNI*) sites, do not confer lethality to yeast (Hoekstra and Malone 1986; Feher et al. 1988).

As a way to monitor chromatin changes directly in vivo, we have exploited the yeast system by expressing foreign DNA methylases. We tested whether the transcriptional status of a gene modulates the level of methylation. We found that transcriptionally active loci are methylated more efficiently in their promoter and coding regions than are inactive genes. Thus, methylation of DNA in chromatin, especially by *dam* methylase, can be used as a novel and powerful probe for studying alterations in chromatin structure. This novel experimental approach should supplement the DNase I mapping technique and also be potentially applicable to the study of chromatin structure in higher eukaryotes.

Results

In the first set of experiments, we used yeast strains in which the *E. coli dam* methylase gene was chromosomal.

ally integrated at the *URA3* locus (Hoekstra and Malone 1986) and expressed under the control of the bacterial promoter. Thus, the adenine residue in GATC sites in chromatin is methylated. In the second set of experiments, the gene for the *B. subtilis* SPR phage cytosine methylase was expressed in yeast strains (Kiss and Baldauf 1983; Feher et al. 1988). Previously, it was noted that radiation-sensitive (*rad3-2*) mutant strains, which are defective in DNA excision repair, have 20–30% higher levels of DNA methylation than *RAD3*⁺ strains, presumably because the excision-repair pathway in *RAD3*⁺ strains removes the N⁶-methyladenine and 5-methylcytosine residues (Hoekstra and Malone 1986; Feher et al. 1988). Therefore, *rad3-2* strains were employed in this study.

dam methylation of the *GAL1-10* divergent promoter

The transcriptional activity of *GAL1* and *GAL10* genes, whose expression is under the control of the *GAL1-10* divergent promoter (Johnston and Davis 1984), can be altered by the kind of sugar used for growth of yeast culture. These genes are repressed by glucose, highly induced by galactose, or potentially active in raffinose medium. The potentially active state is defined as lacking a lag period in induction upon addition of galactose to the raffinose-grown cells. Methylation of *Sau3AI* (GATC) sites was quantitated by digesting the purified DNA with *MboI* and *DpnI*. *MboI* digests only unmethylated GATC sites, and *DpnI* only dimethylated sites (methylated on both strands), whereas *Sau3AI* cleaves irrespective of methylation.

The extent of dimethylation of a unique *Sau3AI* site, located 15 bp upstream of the TATA box and within the essential *GAL* promoter region (Johnston and Davis 1984) in the *EcoRI*–*AvaI* fragment (Fig. 1A), was estimated from the fraction of the 0.85-kb fragment digested by *DpnII*. It was found to be 3.5, 45.0, or 3.5%, respectively, for cells grown in glucose, galactose, or raffinose (Fig. 1B, lanes 4, 8, and 12, respectively). Thus, an increased level of dimethylation occurred in the expressed state. The *MboI* digestion pattern (lanes 3, 7, and 11) also supported this conclusion. The fact that *MboI* cuts only unmethylated and not hemi- and dimethylated DNA allows a direct quantitation of the level of hemi- and dimethylation. The level of hemimethylation was estimated by subtracting the level of dimethylation estimated from *DpnI* digests from that of hemi- + dimethylation obtained from *MboI* digests. Interestingly, a similar level of hemimethylation (Table 1) was observed for growth in glucose (24%), raffinose (25%), and galactose (27%). Thus, the significant increase is observed only for dimethylation in expressed state.

Methylation of the second *Sau3AI* site located in the *GAL1* coding region (Fig. 1A) was also investigated. This *Sau3AI* site overlaps the *XbaI* site in the sequence 5'-TCTAGATC-3', whose digestion with *XbaI* is blocked by methylation of the *Sau3AI* site. As judged by the fraction of the 1.91-kb *EcoRI* fragment remaining uncut by

XbaI (Fig. 1C), the *Sau3AI* site in the *GAL1* coding region was methylated (hemi- + dimethylation in this case) six- to ninefold more in DNA isolated from cells grown in galactose (lane 2) than in glucose (lane 1) or raffinose (lane 3). Thus, sites located both in the coding and the promoter regions (as well as several other sites in the *GAL1* coding region; data not shown) are methylated at an increased level in expressed state.

In principle, a slower growth rate of cells in galactose medium may allow *dam* methylase to act for a longer time in each cell cycle, providing a trivial explanation for the observed higher level of methylation. However, this possibility is unlikely because the generation time was quite similar in medium containing glucose (101 min), raffinose (114 min), or galactose (120 min) (data not shown). Furthermore, when the *GAL* genes were induced by independent genetical means, the active genes again displayed increased levels of methylation, as shown below.

These results indicated that *GAL1-10* chromatin is more accessible to *dam* methylation in its expressed state than in its unexpressed state. If this is indeed the case, then a higher level of methylation should be expected in strains allowing expression by other means. For this purpose, gal-constitutive strains were employed that express *GAL1*, *GAL7*, and *GAL10* genes in the absence of the galactose inducer. Genetic crosses were carried out between the *rad3-2*, *dam*⁺, and the gal-constitutive *GAL4*^c and *gal80Δ* strains to obtain strains of the required genotype (Nogi et al. 1977; Mylin et al. 1989). *GAL4*^c carries a carboxy-terminal deletion in the *GAL4* activator protein that abolishes its repressive interaction with *GAL80*, while *gal80Δ* has the *GAL80* repressor gene deleted (Nogi et al. 1977; Mylin et al. 1989). Five *rad3-2*, *dam*⁺ segregants from each cross were grown in medium containing glucose or raffinose, and the level of methylation of the *Sau3AI* site overlapping the *XbaI* site in the *GAL1* coding region was first determined by *EcoRI*–*XbaI* double digestion. Subsequently, the *GAL4*^c and *gal80Δ* genotype of each segregant was determined by assaying for constitutive expression of *GAL10*-encoded uridine diphosphoglucose 4-epimerase activity. Results from both crosses showed that only the segregants with a high level of methylation, two from the *GAL4*^c cross and all five from the *gal80Δ* cross, also exhibited constitutive epimerase expression (Fig. 2; Table 2), thus satisfying the prediction. Moreover, the specific epimerase activity of the *dam*⁺ constitutive segregants was comparable with their respective constitutive parent strains, which lack *dam* methylase gene, indicating that methylation had no discernable effect on expression of the *GAL10* gene (Table 2).

In addition to *GAL1* and *GAL10*, the linked and coregulated *GAL7* gene was also found to be more methylated when expressed than when not expressed (data not shown). To address further the question of the generality of the increased methylation of expressed genes in yeast, we analyzed methylation of mating-type loci, the phosphatase genes *PHO5* and *PHO3*, and the cell-type-specific gene *STE2*.

A

GAL 1-10 Divergent Promoter

B

C

Figure 1. Southern blot analysis of *dam* methylation of the *Sau3AI* sites in the *GAL1-10* divergent promoter and *GAL1* coding region. (A) A partial restriction map of the *GAL1-10* divergent promoter. The two *Sau3AI* sites, whose level of methylation was tested, map within the 1.91-kb *EcoRI* fragment. The promoter *Sau3AI* site is located 0.64 kb from the *EcoRI* site on its left and is within a region required for transcription of both *GAL1* and *GAL10*. The other *Sau3AI* site overlaps the *XbaI* site in the sequence 5'-TCTAGATC-3', located 1.09 kb to the right of the left *EcoRI* site. The 685-bp *EcoRI*-*BamHI* fragment, isolated from the plasmid *pBM150*, was used as the probe for indirect end labeling. (B) Methylation of the *Sau3AI* site in the *GAL1-10* promoter. DNA isolated from cells grown in medium containing glucose (lanes 1–4), galactose (lanes 5–8), or raffinose (lanes 9–12) was digested first with *EcoRI* and *AvaI*, then with *Sau3AI* (lanes 2, 6, and 10), *MboI* (lanes 3, 7, and 11), *DpnI* (lanes 4, 8, and 12), or not subsequently digested (lanes 1, 5, and 9). (C) Methylation of the *Sau3AI* site in the *GAL1* coding region. DNA from cells grown in medium containing glucose (lane 1), galactose (lane 2), or raffinose (lane 3) was digested with *EcoRI* and *XbaI*. Strain used was K750 (Table 4). Nearly three times more DNA was loaded in lane 3 as compared to lanes 1 and 2, which can account for greater intensity of the signal in the lower band. Symbols S, M, and D represent *Sau3AI*, *MboI*, and *DpnI*, respectively.

dam methylation of mating-type genes

Strain K750 was constructed to study methylation of the mating-type loci. This strain contains a temperature-sensitive mutation in the *SIR3/MAR2* gene, which is one of the negative regulators of the silent cassettes (Haber and George 1979; Klar et al. 1979; Rine et al. 1979; Rine and Herskowitz 1987). The silent mating-type cassettes *HMLα* and *HMRα* are transcriptionally repressed at 25°C

but active at 35°C in the *sir3^{ts}* mutant. Results of *MboI* digests showed that while nearly all of the 4.92-kb *Hin*-*dIII* fragment containing *HMRα* was digested in DNA isolated from the repressed state (Fig. 3A, lane 1), a noticeable amount remained uncut (representing hemi- + dimethylation at all sites in the fragment) in the expressed state (Fig. 3A, lane 5). Other noteworthy changes were at sites located in the structural region of the *a1* and one each in the vicinity of E and I elements (compare

Table 1. Quantitation of levels of hemi- and dimethylation of the *Sau3AI* site in the *GAL1-10* promoter

Growth medium	(A) Hemi + dimethylation (%) (<i>MboI</i> lanes)	(B) Dimethylation (%) (<i>DpnI</i> lanes)	Hemi-methylation (%) (A) minus (B)
Glucose	27.5 (72.5) ^a	3.5	24
Galactose	72 (28)	45	27
Raffinose	28.5 (71.5)	3.5	25

Data shown in Fig. 1B were quantitated by densitometry.

^aThe figures in parentheses give the value of unmethylated DNA.

DpnI lanes 2 and 6) that are known to function as *cis*-acting silencer elements (Nasmyth et al. 1980; Abraham et al. 1984; Feldman et al. 1984). It is noteworthy that several sites past the E region remained largely unaffected by the state of *HMR* expression. An apparent increase in the *DpnI*-resistant *HindIII* fragment in the ex-

pressed state may result from the slightly higher amount of DNA loaded in lane 6 as compared with that in lane 2.

Similarly, the site c located near the I element of *HMLα* showed greatly enhanced methylation in the expressed state (compare lanes 2 and 6, Fig. 3B). Site b, located past c site, remained nearly fully unmethylated under both conditions (lanes 1 and 5). Our data provide no information on the level of methylation of site a because it is inherently resistant to digestion even with *Sau3AI*.

The *dam* methylase gene is derived from *E. coli*, which grows better at higher temperature (37°C). Therefore, the methylase activity may work inherently better at 35°C as compared with that at 25°C. However, we found that only specific sites, not all the sites, exhibit increased dimethylation in *HMLα* and *HMRα* ruling out the possibility that growth at higher temperature leads to generally greater methylation in the experiment presented above. Second, the mating test is known to be a very sensitive assay of silent gene expression. Using this test, *sir3^{ts}* was found to be a tight mutation, as the mutant mates well at the permissive temperature (indicating full repression of *HM* loci), but is fully sterile at the restrictive temperature. Thus, we presume that the level of methylation reflects the difference in the state of expression. In addition, methylation of sites in another constitutively expressed mating-type cassette *MATa* was assayed as an internal control. Results (Fig. 3C) indicated that the *MATa* locus exhibited a similar high level (about 90%) of methylation of the *Sau3AI* site in the *aI* region at both temperatures, as evidenced by a lack of digestion of the 3.5-kb fragment to 3.0 kb by *MboI*. In addition, the site lying outside the Z region remained largely unmethylated at both temperatures, as indicated by complete digestion of the 4.2-kb *HindIII* fragment by *MboI*.

Strain K750 exhibited complete sterility because it failed to mate with an α tester strain at 35°C. We presume that the normally silent cassettes are indeed turned on at 35°C and, seemingly, methylation does not affect their expression.

The indirect end-labeling approach can only allow quantitation of methylation of the site immediately flanking the probe and the proportion of molecules that are methylated (hemi- or full) at all sites (*MboI*-resistant) or are not fully methylated at all sites (*DpnI*-resistant). Because of the presence of multiple sites in *HMRα*, the level of methylation of individual sites near E and I cannot be quantitated (Fig. 3A). In addition, the apparently small differences observed may be explained by a tem-

A *GAL4^c* × wt cross.

B *gal80Δ* × wt cross.

Figure 2. Southern blot analysis of *dam* methylation of the *Sau3AI* site in the *GAL1* coding region and its relation to gal-constitutivity. (A) *EcoRI-XbaI*-digested DNA from five *rad3-2*, *dam⁺* segregants of a cross between strains SC99 (*Gal4^c*) and K750 (*GAL⁺*) grown in glucose (lanes 1–5) or raffinose (lanes 6–10). (B) *EcoRI-XbaI*-digested DNA from five *rad3-2*, *dam⁺* segregants of a cross between strains SC285 (*Gal80Δ*) and JS1 grown in glucose (lanes 1–5) or raffinose (lanes 6–10). A smaller amount of DNA was loaded in lane 2 and longer exposures did not show any other bands. See Table 4 for genotypes of the strains used.

Table 2. Specific activities of epimerase in the segregants from the crosses involving *Gal*-constitutive strains

	Segregant	Epimerase activity (expressed in nmole/mg · hr) ^a		
		glucose	raffinose	galactose
Cross	1C	<5	188.2	ND ^b
<i>GAL</i> ⁺ (K750) × <i>GAL</i> ^{4c} (Sc99)	2B	<5	102.9	ND
	7B	<5	<5	ND
	9C	<5	<5	ND
	14B	<5	<5	ND
	5D	302.9	417.3	ND
<i>JS1</i> × <i>gal80Δ</i> (Sc285)	8D	171.6	218.3	ND
	13D	410.4	745.4	ND
	15B	113.7	250	ND
	20C	133.5	1075	ND
Control strains				
	Sc99	60.7	74.9	ND
	Sc285	222	168.7	ND
	K750	<5	<5	49.6

^aAverage of two measurements.^b(ND) Not determined.

perature effect in *sit3^{ts}* strains. To address both of these points, we investigated individual methylation-sensitive sites, namely the *AlwI* site in the E region and the *BclI* site in the I region in a *mar2Δ* strain. The important result is that both sites were insensitive to digestion in the expressed (*mar2Δ*) state, indicating a high level (nearly 100%) of methylation. Under the repressed state, about 15% of DNA was unmethylated at the *BclI* site and 40% at the *AlwI* site.

PHO5 and PHO3 genes

PHO5 is repressed at high concentrations of P_i (inorganic phosphate) while the adjoining gene *PHO3* is expressed constitutively (Meyhack et al. 1982). The striking result was that sites located in the promoter (0.34 kb) and coding region (1.65 kb) of *PHO5* were only slightly methylated (~5% digestion by *DpnI*) under the repressed state (Fig. 4, lane 2). They became nearly completely dimethylated (~90%) under the expressed state (lane 6). [Although the band intensities of lane 6 are weaker than that in lane 5, the amount of DNA loaded (as judged by ethidium staining) was very similar. Moreover, the fact that the *DpnI* digestion pattern appeared very similar to the *Sau3AI* pattern (lane 4) and that longer exposures did not indicate slower migrating bands in lane 6 (data not shown) indicated that the sites in *PHO5* gene were indeed nearly completely methylated under expressed state. Furthermore, the critical aspect of the data comprises comparison of intensities of different fragments displayed in the same lane with each other.] *PHO3* also exhibited subtle changes that were less pronounced than *PHO5* gene. Two sites within the coding region of *PHO3* appeared to be less cut with *MboI*, while the other two sites in the 3'-flanking sequences are slightly more digested in low P_i medium.

In vivo accessibility of yeast chromatin to *B. subtilis* phage SPR methylase

We used strains containing the plasmid-borne *B. subtilis* phage SPR methylase gene (Kiss and Baldauf 1983) expressed under the control of the *GAL1* promoter (Feher et al. 1988). This methylase modifies both the C residues of the *HpaII* site, CCGG, and the internal C residue in the sequences GGCC and CCA/TGG (Kiss and Baldauf 1983). Methylation of the internal C in CCGG site confers resistance to digestion with *HpaII*. When either or both C residues are methylated, *MspI* cannot cleave the site (Keshet and Cedar 1983). Because the protocol required expression of the methylase gene by growing cells in galactose, methylation of the *GAL1-10* system could not be analyzed. Instead, we subjected the cell-type-specific genes *STE2* and *STE3* to this analysis (Sprague et al. 1983; Nakayama et al. 1985). A lack of digestion of the 0.92-kb fragment at the sequence 5'-ACCGGC-3' by *MspI* and *HpaII* indicated complete methylation of both the C residues of the site located in the promoter in α cells that express the *STE2* gene (Fig. 5). However, in α cells not expressing the *STE2* gene, a significant fraction of the 0.92-kb fragment was digested by both *MspI* and *HpaII*, generating the 0.8-kb fragment, reflecting only a partial methylation of the same site. It is also clear that both Cs were methylated to different extents in α cells, as indicated by the difference in cleavage by *MspI* and *HpaII*.

Similarly, an analysis of the *STE3* gene, which is not expressed in α cells (Sprague et al. 1983; Nakayama et al. 1985), showed a nearly complete digestion of the 1.24-kb fragment by *MspI* (Fig. 6, lane 2). This result indicated a very low level of methylation (2–4%) in the promoter region. In contrast, about 15% of the fragment remained undigested and was, therefore, methylated in α cells that express the gene (Fig. 6, lane 5). The same site was completely digested with *HpaII* (Fig. 6, lanes 3 and 6) under

Figure 3. Southern blot analysis of *dam* methylation of mating-type loci in a *sir3^{ts}* strain (K750; A, B, and C) and the *mar2Δ* strain (D). (A) *HMRa*. DNA from cells grown at indicated temperatures was digested with *HindIII* alone (lane 3), *HindIII* plus *Sau3AI* (lane 4), *HindIII* plus *MboI* (lanes 1 and 5) or *HindIII* plus *DpnI* (lanes 2 and 6). (B) *HMLα*. DNA was digested with *HindIII* and *XbaI* (lane 3) followed by digestion with *Sau3AI* (lane 4), *MboI* (lanes 1 and 5), or *DpnI* (lanes 2 and 6). (C) *MATa*. DNA was digested with *HindIII* alone, *HindIII* plus *Sau3AI*, *HindIII* plus *MboI* as indicated. (D) *HMRa*. DNA from *Mar⁺* (lanes 1, 3, and 5) and *mar2Δ* (lanes 2 and 4) strains was digested with *HindIII* alone (lane 5), *HindIII* plus *BclI* (lanes 1 and 2) or *HindIII* plus *AlwI* (lanes 3 and 4). The probes used for indirect end labeling were the *HMRa* (centromere)-proximal *HindIII*–*EcoRI* fragment for A and D, the *HMLα*-proximal *HindIII*–*PvuII* fragment for B, and the *MATa*-proximal *HindIII*–*EcoRI* fragment for C. 25°C and 35°C indicate the growth temperature of the culture.

Figure 4. Southern blot analysis of *dam* methylation of the linked genes *PHO5* and *PHO3*. DNA isolated from cells grown in medium containing high (lanes 1 and 2) or low (lanes 3–6) levels of P_i was digested with *Bam*HI followed by *Mbo*I (lanes 1 and 5), *Sau*3AI (lane 4), or *Dpn*I (lanes 2 and 6). The *Bam*HI–*Sal*I fragment was used as the probe for indirect end labeling. The arrow indicates the DNA fragment resulting from digestion at both *Sau*3AI sites contained within the *Bam*HI–*Sal*I fragment used as the probe, and the *Sau*3AI located in the *PHO5* coding region. In addition, the strain used also contains another *Sau*3AI site located close but distal to the 1.65-kb site. The site located at 0.34 kb is situated in the promoter and that at 1.65 kb is situated in the 3' end of the *PHO5* coding region.

both conditions, indicating a lack of methylation of the 3'C residue.

Discussion

Correlation between the level of methylation and gene expression

The most significant finding is that all the yeast genes we tested exhibited increased methylation in the expressed state (Tables 1 and 3), and sites within and outside of the coding sequences were affected. Our interpretation of the finding is that increased methylation reflects increased accessibility of the active chromatin to methylases in vivo. The most extensive study was carried out with the *GAL* genes that showed a greater than 10-fold increased level of dimethylation in the expressed state. However, the level of hemimethylation of the promoter site was not altered (Table 1). The basis for the high, unchanged level of hemimethylation of the *GAL1*-

10 promoter site is not known. It may reflect a consequence of replication, a time during which the chromatin template may become transiently unfolded and gets hemimethylated randomly on one or the other strand at a particular site, but not enough of a time window exists for dimethylation. This point needs to be resolved further.

A comment should be made regarding the differences in the level of methylation of *GAL* genes in wild-type cells grown in galactose-containing medium, as compared to those of constitutive *gal80Δ* and *GAL4^c* cells grown in glucose or raffinose medium. A lack of glucose repression (Table 2) and nearly complete methylation of the site in *GAL1* coding region (as well as the promoter region; data not shown) was observed in *gal80Δ* strains. *GAL4^c* strains are still repressible by glucose; the constitutivity ensues with growth in raffinose medium (Table 2). Consistent with this, Lohr and Hopper (1985) observed that *gal80Δ* strains exhibit greater *GAL1* transcription as well as greater DNase I digestion as compared to *GAL4^c* strains when grown in glucose-containing medium. Thus, in the *GAL* system, it appears that increased expression of the gene was correlated with enhanced levels of methylation.

Likewise, the highly regulated *PHO5* gene was only slightly (5%) methylated at the promoter site in the repressed state but was highly methylated (90%) upon activation (see Table 3). The adjoining *PHO3* gene also showed detectable but less pronounced changes. Although the *PHO3* gene is considered to be expressed constitutively (Meyhack et al. 1982), the observed subtle changes in methylation pattern in low P_i medium may reflect changes in the level of expression. Similarly, sites located near the important regulatory E and I regions of

Figure 5. Southern blot analysis of CpG methylation of the *STE2* gene. DNA prepared from isogenic strains JSa (SPR) and JSa (SPR) expressing a galactose-inducible *B. subtilis* phage SPR methylase was digested with *Msp*I or *Hpa*II as indicated. The line drawing shows the restriction map of *STE2*; *Hind*III sites in parentheses are based on the work of Nakayama et al. (1985), but our strain lacks these sites. The fragment represented by a filled in box was used as the probe for indirect end labeling. The open box indicates the open reading frame of the gene. The site being studied is located upstream of the 5' end of the coding region.

Figure 6. Southern blot analysis of CpG-methylation of the *STE3* gene. DNA isolated from isogenic strains J5a (SPR) (lanes 1–3) and J5a (SPR) (lanes 4–6) expressing the SPR methylase was digested with *HindIII*, followed by *MspI* (lanes 2 and 5) or *HpaII* (lanes 3 and 6). Lanes 1 and 4 display the control *HindIII* digests. The *HpaI*–*EcoRI* fragment of the *STE3* gene was used as the probe for indirect end labeling. The origin of the common band of ~700 bp seen in all the lanes is not known. The 1.24-kb site is located within the coding region, while that at 0.31 kb is situated upstream of the 5' end of the gene.

HML and *HMR* loci exhibited increased methylation when these normally cryptic loci were expressed in *sir3^{ts}* and *mar2Δ* strains.

An interesting finding of this study was that all of the sites showing altered levels of methylation in *HMLα* and *HMRα*, as also in case of *PHO3*, did not lie in a transcriptional unit or near the promoter. In fact, some lie in the 3' end of transcriptional units originating from an internal divergent promoter. These findings suggest that activation of both the silent cassettes might involve an unfolding or opening-up of a whole domain spanning the accessible sites, which, in turn, might increase their accessibility to the transcriptional apparatus. These results may be viewed in light of a recent report showing that the E and I regions can interact with one another and with a site within the Y region via the protein RAP1, thus forming the hypothetical repression loop (Hofmann et al. 1989). Activation of the cassettes might involve breaking these interactions, resulting in unfolding and greater accessibility of the region.

Another point concerns the extent of correlation between the level of methylation and the level of transcription of a particular gene. The genes we chose to study reflect a broad spectrum of the level of transcription. In general, our results are consistent with a direct correlation, given that *GAL* and *PHO5* genes are heavily transcribed and *STE2* and *HM* loci are transcribed inefficiently. Clearly, however, the correlation is not absolute. For example, a site in the weakly expressed *MATα* was

also highly methylated. A more likely explanation is that chromatin context effect may modulate the extent of methylation not only of each gene but also of each site. Another important question that is beyond the scope of this study is whether the act of transcription per se, as opposed to changed chromatin structure, or both features promote methylase accessibility. We note, however, that many sites whose methylation level was increased are outside of the transcription unit.

The limited amount of data for cytosine methylation of *STE2* and *STE3* genes also showed increased methylation of active genes. The different levels of methylation of adjoining C residues of the *HpaII* site in the *STE2* promoter in α cells may reflect their differential accessibility to methylation in chromatin, or differential substrate specificity of the enzyme. On the other hand, a significant level of methylation of the external, but not the inner, C residue of the *HpaII* site in the *STE3* promoter may occur because of the protection of the inner C residue by a bound protein in chromatin.

Methylation as a probe for accessible regions of chromatin

Our results show that in vivo methylation by a foreign methylase, especially *dam* methylase, can serve as a powerful probe for studying the in vivo accessibility of yeast genes during transcription, and possibly during replication and recombination. Since higher eukaryotes do not contain high levels of N⁶-methyladenine, this approach can be of potential use to study their in vivo chromatin structure. This novel in vivo experimental approach contrasts and complements the currently used semi-in vivo nick-translation (Levitt et al. 1979) and in vitro DNase I (Weintraub and Groudine 1976) approaches for studying chromatin structure. Our approach is simpler, and, also because it is in vivo in nature, is not subject to experimental artifacts.

We realize that our system is limited to the analysis of endogenously present GATC sites in the genome. Such sites may be incorporated in the region of interest by in vitro mutagenesis. On the other hand, presence of multiple GATC sites in a restriction fragment of interest makes the analysis difficult. One way to focus on a particular site is to use one of many "6-base-cutter" endonuclease activities available whose recognition sequence overlaps the GATC site and whose activity is sensitive to methylation. Another potential application of the technique is to exploit the acquired *DpnI* sensitivity of active genes to isolate differentially expressed loci from different cell types.

Correlation with previous studies on yeast chromatin

Several studies have been reported on the chromatin structure of different genes of yeast, especially for *PHO5*–*PHO3* and mating-type loci. Presence of an inducer independent DNase I hypersensitive site that is located 370 bp upstream of the *PHO5* gene has been demonstrated by Fascher et al. (1990). This site contains a major UAS element flanked by specifically positioned nucleo-

Table 3. Quantitation of levels of *dam* and cytosine methylation at different loci

Gene locus	<i>dam</i> methylation				CpG methylation	
	hemi-		di-		on ^a	off ^b
	on ^a	off ^b	on ^a	off ^b		
<i>GAL 1-10</i>						
promoter	27		45	3-5		ND
coding region		ND	~50	5-10		ND
<i>Mata</i>						
coding region (constitutive)			90			50 ^d
noncoding region			10			
<i>HMRa</i>						
E site	95-100 ^c	60 ^c				
I site	100 ^c	85 ^c				
<i>HMLa</i>						
near I region						
site c	25 ^c	1-2 ^c				ND
site b	0	0				
<i>PHO5</i>						
promoter	90	≤5				ND
coding region	90	≤5				
<i>STE2</i>						
promoter	15-25 ^d	0 ^d			100	30-60
<i>STE3</i>						
promoter					15	1-2

^aExpressed.^bUnexpressed.^cHemi- + dimethylation.^dData not shown.

(ND) Not determined.

somes. Induction of the gene under low P_i condition leads to removal of two nucleosomes upstream and two nucleosomes downstream of this site (Fascher et al. 1990). Accordingly, the *Sau3AI* site in that region also became accessible to methylation. We also found increased methylation of a site in the *PHO5* coding region where no DNase I hypersensitive site has been reported. In fact, this region is known to be covered with nucleosomes (Fascher et al. 1990). Thus, increased methylation of that site implies that DNA methylation is occurring in chromatin rather than on naked DNA.

In an interesting analysis of chromatin organization of the mating-type loci, Nasmyth (1982) showed that *MAR/SIR* genes are responsible for the loss of several DNase I sensitive sites located between E and I regions at the *HM* loci. These in vitro results are in accord with our in vivo results showing increased level of methylation of sites delimited by E and I regions. Altered chromatin structure of the *HM* loci in the expressed state was also indicated by the accessibility of these loci to *HO*-encoded endonuclease (Klar et al. 1981) and DNA repair (Terleth et al. 1989).

Role of DNA methylation in gene expression in higher eukaryotes

Most of our studies involved adenine methylation. The effect of adenine methylation on gene regulation in

higher eukaryotes is not known; however, CpG methylation is generally inversely correlated with gene expression in higher eukaryotes. Thus, our this study has no direct bearing on the question of the role of DNA methylation in gene expression in higher eukaryotes. It is now widely accepted that CpG methylation plays a role in maintenance of the inactive state of chromatin (for review, see Cedar 1988). The most direct link between site-specific demethylation and gene activation has been provided by Saluz et al. (1986) and Wilks et al. (1982) for the vitellogenin promoter. However, the issue of cause or consequence of methylation on gene expression is not clearly understood. It does remain an active area of research.

In our system of yeast strains expressing foreign DNA methylases, methylation reflects the state of expression of yeast genes and is, therefore, a consequence of gene expression. A potential application of our study to higher eukaryotes may be in developing an in vivo *dam* methylation system for monitoring early changes in chromatin accessibility during changes in gene regulation.

Materials and methods

Strains and plasmids

The strains used in this study and their genotypes are listed in Table 4. The strains expressing *dam* methylase gene were obtained by crossing the original strains (provided by Bob Malone)

Table 4. *Strains used in this study*

Strain	Genotype	Source
K750	<i>MATa sir3^{ts}, cry1^t, his4 or -7, ade2, leu2, rad3-2, dam⁺, GAL⁺</i>	this work
JS1	<i>MATa, sir3^{ts}, cry1^t, ade2, leu2, rad3-2, dam⁺, gal⁻</i>	this work
SC99	<i>MATα, GAL4^c, his⁻, GAL⁺, ade⁻</i>	J. Hopper
SC285	<i>MATα, gal80Δ, ura3-52, leu2-3,112, ade1, MEL⁺ GAL⁺</i>	J. Hopper
9Ca	<i>MATa, his4 or -7, leu2, cry1^t, rad3-2, dam⁺, GAL⁺</i>	this work
9Cα	<i>MATα, his4 or -7, leu2, cry1^t, rad3-2, dam⁺, GAL⁺</i>	this work
JSα(SPR)	<i>MATa, his3-Δ200, leu2-Δ1, trp1-Δ1, tyr7-1, ura3-52, GAL⁺ (GAL1-SPR)</i>	this work
JSα(SPR)	<i>MATα:URA3, his3-Δ200, leu2-Δ1, trp1-Δ1, tyr7-1, ura3-52, GAL⁺ (GAL1-SPR)</i>	this work

containing the chromosomally integrated *dam* methylase gene with other suitable strains. The methylase gene in these strains is expressed constitutively under the control of its own promoter.

Growth conditions and genetic analysis

Standard procedures for growth and genetic analysis of yeast were followed according to Sherman et al. (1983). Cells were grown in YEP medium containing glucose (2%), galactose (2%), or raffinose (5%). To study methylation of mating-type loci, the strain K750 was grown to mid-log phase in YEPD medium at the permissive (25°C) or restrictive (35°C) temperatures.

For studying methylation of linked genes *PHO5-PHO3*, the strain K750 was grown in low and high P_i media to mid-to-late log phase. The low P_i medium was prepared according to Rubin (1974).

For studying cytosine methylation of yeast genes *STE2* and *STE3*, isogenic JSα (SPR) and JSα (SPR), strains expressing the *B. subtilis* SPR phage cytosine methylase under the control of *GAL1* promoter (Feher et al. 1988) were grown in YNB-Leu + raffinose. After growing to mid-log phase, the methylase was induced by adding galactose to a final concentration of 2% and further growth for 8–12 hr.

DNA preparation, gel electrophoresis, and Southern hybridization

The genomic DNA was prepared from yeast cells according to Abraham et al. (1984). After an overnight digestion with 15- to 20-fold excess of the appropriate enzyme, to ensure complete digestion 4–5 additional units of the enzyme were added and incubation continued for additional 4–6 hours. As a control for the completion of digestion 0.5 μg of the 643-bp *AvaI-PvuII* fragment of pBR322 DNA (isolated from *dam⁻* and *dam⁺* strains), which contains two *Sau3AI* sites, was added to the *MboI* and *DpnI* reactions. The digests were resolved by electrophoresis in 1–1.2% agarose gel in 1× TAE buffer. Complete digestion was indicated by the generation of 401- and 242-bp bands and the loss of the pBR322 643-bp band (data not shown). After blotting to Nytran membrane according to Southern (1975), overnight hybridization was done, followed by high-stringency washes and autoradiography at –70°C with an intensifying screen. Band intensities were quantitated by densitometry. Each set of experiments was repeated three times producing reproducible results. Percent dimethylation was estimated by calculating the intensity of the smaller band resulting from *DpnI* digestion in Figures 1 and 2 as a percentage of the sum of both the bands.

Scoring the *dam⁺* segregants

The *dam⁺* segregants were identified as their DNA gained sensitivity to digestion by *DpnI*. The genomic DNA from *dam⁻* segregants remained undigested by *DpnI*. The digests were electrophoresed in agarose gels and the DNA was visualized by staining with ethidium bromide.

Enzyme activities and protein estimation

The epimerase activity was measured by the two-step protocol of Kalckar et al. (1959). The protein estimation was done by Bradford Assay and the specific epimerase activity was expressed as nmole/mg·hr.

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