The EMBO Journal Vol.16 No.21 pp.6495–6509, 1997

Direct evidence for *SIR2* modulation of chromatin structure in yeast rDNA

Christian E.Fritze[1], Kristin Verschueren[2], Randy Strich[3] and Rochelle Easton Esposito[4]

Department of Molecular Genetics and Cell Biology, The University of Chicago, 920 E. 58th Street, Chicago, IL 60637, USA

[1]Present address: Department of Cell Biology, The Scripps Research Institute, 10550 N. Torrey Pines Rd, La Jolla, CA 92037, USA
[2]Present address: Celgen, Herestraat 49, 3000 Leuven, Belgium
[3]Present address: Fox Chase Cancer Center, 7701 Burholme Ave, Philadelphia, PA 19111, USA

[4]Corresponding author

The yeast *SIR2* gene maintains inactive chromatin domains required for transcriptional repression at the silent mating-type loci and telomeres. We previously demonstrated that *SIR2* also acts to repress mitotic and meiotic recombination between the tandem ribosomal RNA gene array (rDNA). Here we address whether rDNA chromatin structure is altered by loss of *SIR2* function by *in vitro* and *in vivo* assays of sensitivity to micrococcal nuclease and *dam* methyltransferase, respectively, and present the first chromatin study that maps sites of *SIR2* action within the rDNA locus. Control studies at the *MAT*α locus also revealed a previously undetected MNase-sensitive site at the a1-α2 divergent promoter which is protected in *sir2* mutant cells by the derepressed a1-α2 regulator. In rDNA, *SIR2* is required for a more closed chromatin structure in two regions: SRR1, the major *SIR*-Responsive Region in the non-transcribed spacer, and SRR2, in the 18S rRNA coding region. None of the changes in rDNA detected in *sir2* mutants are due to the presence of the a1-α2 repressor. Reduced recombination in the rDNA correlates with a small, reproducible transcriptional silencing position effect. Deletion and overexpression studies demonstrate that *SIR2*, but not *SIR1*, *SIR3* or *SIR4*, is required for this rDNA position effect. Significantly, rDNA transcriptional silencing and rDNA chromatin accessibility respond to *SIR2* dosage, indicating that *SIR2* is a limiting component required for chromatin modeling in rDNA.

Keywords: chromatin/rDNA/silencing/Sir2

Introduction

Eukaryotic organisms maintain stable genomes despite the presence of repeated DNA sequences and efficient homologous recombination. The absence of frequent deletions and translocations due to exchange between repeats, particularly in meiosis where recombination is elevated, suggests the existence of mechanisms to suppress such recombination. Indeed, the amount of meiotic recombination per unit length DNA falls dramatically as the size and repetitive DNA content of eukaryotic genomes increases. For example, although human cells contain ~150 times more DNA per haploid genome than yeast, both species exhibit similar numbers of reciprocal exchanges per meiosis (Lewin, 1990). The basis for this decline in recombination frequencies is not well understood, but may be explained by such diverse mechanisms as changes in chromosome compaction and altered distribution and/or reduced frequency of recombination initiation sites in the genome.

Our laboratory has investigated the pathways of recombination in diverse regions of the genome. Of particular interest is the phenomenon of recombination suppression in yeast, where reciprocal exchange between the ~120 tandem repeats of ribosomal RNA genes (rDNA) is ~100-fold less frequent in meiosis than exchange between unique genes (Petes and Botstein, 1977; Petes, 1979; Zamb and Petes, 1982). Paradoxically, rDNA sequences have been isolated (*HOT1*) that when removed from the context of the repeated array actually stimulate recombination between artificial duplications, suggesting that reduced recombination in the rDNA array is not due to a lack of recombination initiation sites (Keil and Roeder, 1984; Voelkel-Meiman *et al.*, 1987). Moreover, many properties of recombination appear to be similar to exchange in non-rDNA (Ozenberger and Roeder, 1991). Mutations selected for defects in *HOT1*-mediated recombination outside the rDNA display reduced rDNA recombination, strongly suggesting that *HOT1* enhances recombination in its natural context (Lin and Keil, 1991). In contrast to the *cis*-acting *HOT1* element, several *trans*-acting factors have been identified that are specifically required to maintain low levels of exchange in these regions (Christman *et al.*, 1988; Aguilera and Klein, 1990; Keil and Lin, 1991; Lin and Keil, 1991; Keil and McWilliams, 1993; Huang and Keil, 1995). For example, inactivation of either Topoisomerase I or II dramatically increases the frequency of mitotic recombination in the array, but not elsewhere in the genome (Christman *et al.*, 1988). Lack of Topoisomerase III function has a similar but broader effect, increasing exchange between rDNA genes and Ty delta sequences (Wallis *et al.*, 1989; Gangloff *et al.*, 1994, 1996). Our studies have demonstrated that one of the genes involved in transcriptional silencing (*SIR2*) suppresses mitotic and meiotic rDNA recombination 10- to 15-fold, while the other *SIR* genes (*SIR1*, *SIR3* or *SIR4*) have no effect on exchange in the array (Gottlieb, 1989; Gottlieb and Esposito, 1989). *SIR2* has been shown to be dispensable for *HOT1*'s ability to stimulate artificial repeat recombination (Gottlieb, 1989), but it is not clear whether *SIR2* influences *HOT1*-mediated recombination within the rDNA array.

The phenomenon of silencing in yeast is characterized by the repression of gene expression in a region-specific

but promoter-non-specific manner. Silencing has been observed in yeast at the cryptic mating-type loci (reviewed in Laurenson and Rine, 1992) and at telomeres (where it is designated telomeric position effect; Gottschling *et al.*, 1990). In both cases, repression is associated with specialized chromatin structures whose integrity depends upon a complex combination of *cis*-acting sites, several shared *trans*-acting factors, and histones. The *SIR1*, *SIR2*, *SIR3* and *SIR4* loci were the first genes identified as required for silencing (Haber and George, 1979; Klar *et al.*, 1979; Ivy *et al.*, 1986; Rine and Herskowitz, 1987). These genes prevent the expression of mating information contained at the cryptic *HM* loci and regulate the directionality of gene conversion events from the silent loci to the expressed *MAT* locus. The products of the *SIR2*, *SIR3* and *SIR4* loci are now known to interact with one another as well as histones H3 and H4 to form productive 'silencing complexes' at the *HM* loci and telomeres (Moretti *et al.*, 1994; Hecht *et al.*, 1995, 1996; Strahl *et al.*, 1997). Transcriptional silencing at each of the *HM* loci also depends upon a pair of *cis*-acting elements, designated silencers, which are themselves made up of partially redundant regulatory sites (Brand *et al.*, 1985, 1987) and contain an ARS element, RAP1-binding site (Shore *et al.*, 1987) and ABF1-binding site (Diffley and Stillman, 1988). Telomeres also contain ARS elements and a RAP1-binding site (Brand *et al.*, 1987; Buchman *et al.*, 1988), although the ARS elements are contained in the Y′ regions which are dispensable for telomeric silencing.

The idea that expression of silenced loci is regulated by chromatin structure stems primarily from findings that: (i) mutations which abolish silencing produce more open chromatin configurations at the derepressed loci (Nasmyth, 1982; Gottschling, 1992; Singh and Klar, 1992); and (ii) loss of silencing can be generated by histone mutations (Kayne *et al.*, 1988; Thompson *et al.*, 1994b) or mutations which affect histone modifications (Braunstein *et al.*, 1993). Studies by Nasmyth first demonstrated that *SIR* function is required for the formation of a specialized chromatin structure at the *HM* loci which is not observed at *MAT* (Nasmyth, 1982). Since then, expression in yeast of *Escherichia coli dam* methyltransferase has shown that repression at the cryptic mating-type loci and telomeres is tightly correlated with the presence of a methyltransferase-resistant chromatin structure (Gottschling, 1992; Singh and Klar, 1992). These findings demonstrate that chromatin structure at repressed loci can restrict access by *dam* methyltransferase and, by implication other important proteins, to the DNA.

In this study we have investigated the basis of *SIR2*'s suppression of rDNA exchange. We previously proposed that *SIR2* limits the accessibility of the rDNA array to the general recombination machinery, based on the finding that *RAD50* and *RAD52*, two genes not normally required for rDNA recombination, are needed for the elevated levels exhibited in the *sir2* mutant background (Gottlieb *et al.*, 1989). *SIR2*'s previously defined role in silencing at the cryptic mating type loci further suggested that this limited accessibility might be dependent upon a specialized chromatin structure dependent upon Sir2p which excludes the general recombination system in mitosis and meiosis. Here we report direct evidence that rDNA chromatin structure is responsive to the presence of *SIR2*. Furthermore, we demonstrate that rDNA chromatin structure and an associated rDNA position effect are responsive to *SIR2* dosage. Thus, Sir2p is a limiting component for silencing and recombination in the rDNA, unlike the other known contexts of *SIR* activity. These findings complement several very recent reports of other rDNA position effects, including Ty transposition into the non-transcribed spacer region, effects on transcription, and localization of Sir2p to the nucleolus (Bryk *et al.*, 1997; Gotta *et al.*, 1997; Smith and Boeke, 1997) (see Discussion). Together, these findings indicate that *SIR2* reduces the accessibility of rDNA sequences by regulating chromatin structure in the rDNA, presumably by altering the proportion of rDNA repeats bound by nucleosomes or regulating an aspect of higher-order chromatin configuration. The data strongly support the idea that *SIR2*-dependent alterations in chromatin structure are responsible for both suppression of recombination and transcriptional silencing in the rDNA.

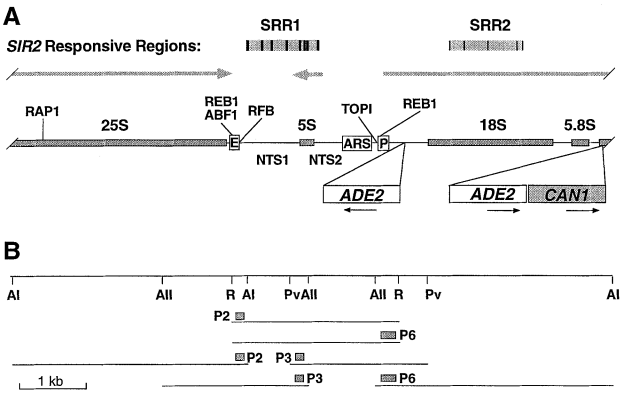

Fig. 1. (**A**) Integration of the *ADE2-CAN1* and *ADE2* markers into the rDNA array. The *ADE2-CAN1* marker was integrated at the *Kpn*I site in the rDNA of CFY558 and related strains. The unrelated strain REE1285 contains only a single *ADE2* insert at the *Hin*dIII site near the 5′ end of the 18S coding region. Arrows within the *ADE2* and *CAN1* genes indicate transcriptional orientation. *ADE2* and *CAN1* genes not to scale. Hash marks and gray shaded regions at the top of the figure mark the positions of the major *SIR2*-Responsive Regions (SRR1 and SRR2) detected by MNase (see Figures 6 and 8B). E, enhancer; RFB, replication fork barrier; P, promoter; ARS, matches to the ARS consensus sequence; TOPI, REB1, ABF1, RAP1, Topoisomerase I, REB1, ABF1 and RAP1 consensus binding sites. The HOT1 element is composed of the enhancer and promoter regions (E + P). (**B**) Strategy for mapping rDNA chromatin with MNase. The restriction fragments analyzed by micrococcal nuclease assays are indicated (thin lines) together with the position of probes used for indirect end-labeling (gray boxes). Restriction sites are *Ava*I (AI), *Ava*II (AII), *Eco*RI (R) and *Pvu*II (Pv). Not all restriction sites for these enzymes are shown.

Results

Identification of a position effect in the rDNA regulated by SIR2

To address whether *SIR2* regulates gene expression in the rDNA, a double *ADE2-CAN1* marker was constructed allowing selection for the presence of the marker or counter selection for its excision from rDNA. The marker was integrated into the rDNA array (Figure 1) of strains containing disruptions of the chromosomal *ADE2* and *CAN1* loci. Expression of *ADE2* allows growth on adenine omission medium (–ADE) and gives white colonies on either –ADE or COM (complete) medium. Loss or reduced

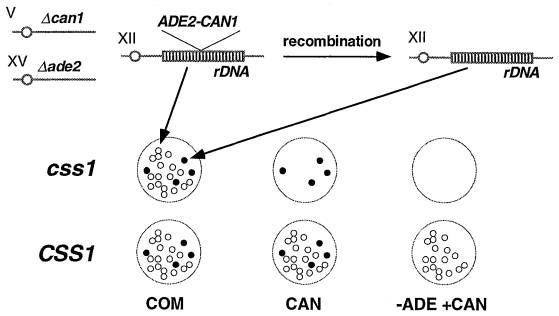

Fig. 2. Diagrammatic summary of CAN plating phenotypes of *rDNA::ADE2-CAN1* populations. Open and filled circles represent white and red colonies, respectively.

expression of the *ADE2* gene is detected by the appearance of red or pink colonies, respectively, on COM medium due to the accumulation of an adenine biosynthetic intermediate when *ADE2* is blocked (Roman, 1956). *CAN1* confers dominant sensitivity to the arginine analog canavanine (Can^S) (Grenson *et al.*, 1966; Hoffmann, 1985). Loss of *CAN1* function allows resistance to canavanine (Can^r). As with all unique markers inserted into the rDNA, a fraction of cells in an unselected culture lose the integrated marker via genetic recombination between rDNA repeat units flanking the insertion. Thus, plating cells containing the *rDNA::ADE2-CAN1* marker onto media containing canavanine (CAN media) should only permit growth of red Can^r colonies which have lost the rDNA marker by recombination. These red Can^r recombinants should not grow on media with canavanine but lacking adenine, since they are adenine auxotrophs.

During the course of our studies with the *ADE2-CAN1* marker we discovered allelic variants of a single locus, independent of *CAN1*, affecting the resistance of cells to canavanine. The dominant allele, which we called *CSS1*, confers resistance to canavanine, while the recessive allele, *css1*, results in super-sensitivity. The *CSS1* gene was cloned and shown to be an essential gene; its detailed characterization will be presented elsewhere (C.Fritze and R.Esposito, in preparation). Recent work by others indicates that it encodes a ubiquitin ligase, suggesting that super-sensitivity of the mutant is due to a failure of protein turnover and the accumulation of canavanyl-containing proteins. The present study took advantage of the behavior of each of these alleles to demonstrate in different assays that *SIR2* reduces gene expression in the rDNA. For example, as described further below, the combination of *CSS1* and *SIR2* together led us initially to detect silencing of the *CAN1* marker in rDNA, since the level of canavanine resistance resulting from the presence of both wild-type alleles permits sufficient growth on canavanine-containing medium to detect normal colonies even in the presence of the wild-type *CAN1* gene encoding sensitivity. The *css1* allele, on the other hand, proved very useful in examining *SIR2* dosage effects. Strains containing this allele, which are exquisitely sensitive to CAN-containing medium, facilitate the detection of increased *CAN1* gene silencing in rDNA, resulting in canavanine resistance beyond the already high level seen in *CSS1 SIR2* strains.

Figure 2 summarizes the phenotypes of each of the *CSS1* alleles with respect to the *ADE2-CAN* marker in rDNA. Strains carrying *css1* and the *ADE2-CAN1* marker which are super-sensitive to CAN, give no background colony growth on medium containing canavanine. Only rare red recombinants which have excised the marker and are now *can1 ade2* grow on this medium. Strains containing *CSS1*, on the other hand (including W303), exhibit a slow-growing white Can^r phenotype with occasional red Can^r papillae on CAN media. In different *CSSI* backgrounds, 25–100% of colony forming units (cfu) are capable of growth on –ADE+CAN media despite the presence of *ADE2-CAN1* in the rDNA (see also Figure 3, strain 2). Red colonies generate only stable red colonies when replated, while white Can^r colonies generate predominantly white colonies. Loss of *ADE2-CAN1* from rDNA in red colonies and its presence in white colonies was confirmed by Southern analysis (unpublished data). These findings initially suggested to us that the *CAN1* gene in the *rDNA::ADE2-CAN1* marker might be silenced. However, the *CAN1* gene in the rDNA marker could not be fully silenced, since wild-type cells were still responsive to canavanine with respect to viability, i.e. viability decreased as the level of canavanine in the plating media was increased (unpublished data). As reported in more detail below, this effect on *CAN1* expression is only observed when the marker is integrated in rDNA, and not in non-rDNA. Thus, the phenotype seen in *CSS1* strains is not simply due to the presence of an independent suppressor of canavanine sensitivity in this strain, but reflects properties of rDNA and the presence of other factors (e.g. *SIR2*) on expression.

To determine whether any of the *SIR* genes are required for the reduced expression of the *CAN1* marker in the rDNA, each of the four *SIR* genes was individually deleted or expressed in high copy in the parental strain containing *ADE2-CAN1* inserted in the rDNA. In a wild-type strain nearly all cells form colonies on media containing canavanine, and the majority of colonies on CAN media are white, indicating the presence of the *rDNA::ADE2-CAN1* marker (Figure 3, strain 2). However, in the Δ*sir2* strain, white Can^r colonies indicative of reduced *CAN1* expression are reduced 10- to 100-fold (Figure 3, strain 4). This phenotype is more clearly visible when *SIR2* and Δ*sir2* strains are compared on –ADE+CAN medium which excludes the growth of red Can^r recombinants caused by increased rDNA recombination in the Δ*sir2* strain (Gottlieb and Esposito, 1989). These results demonstrate that *SIR2* function is required for reduced expression of the *CAN1* marker in rDNA. In contrast to *SIR2*, loss of *SIR4* (Figure 3, strain 6), *SIR1* or *SIR3* (unpublished data) produced no detectable difference in expression of *ADE2* or *CAN1* as judged by colony color and plating efficiency on CAN media.

Dosage-dependent reduction of gene expression by SIR2

Since a *SIR2* strain displayed reduced expression of *CAN1* in *rDNA::ADE2-CAN1* as compared with an isogenic Δ*sir2* strain, we asked whether increased *SIR2* dosage further lowers *CAN1* expression. In order to detect any potential increase in *CAN1* repression by high-copy expression of *SIR2*, we employed an isogenic strain which lacks the *CSS1* episome to increase the canavanine-sensitivity of this background. Less than 1×10^{-4} cfu of the *css1-1* strain form colonies on –ADE+CAN media. (Figure 3,

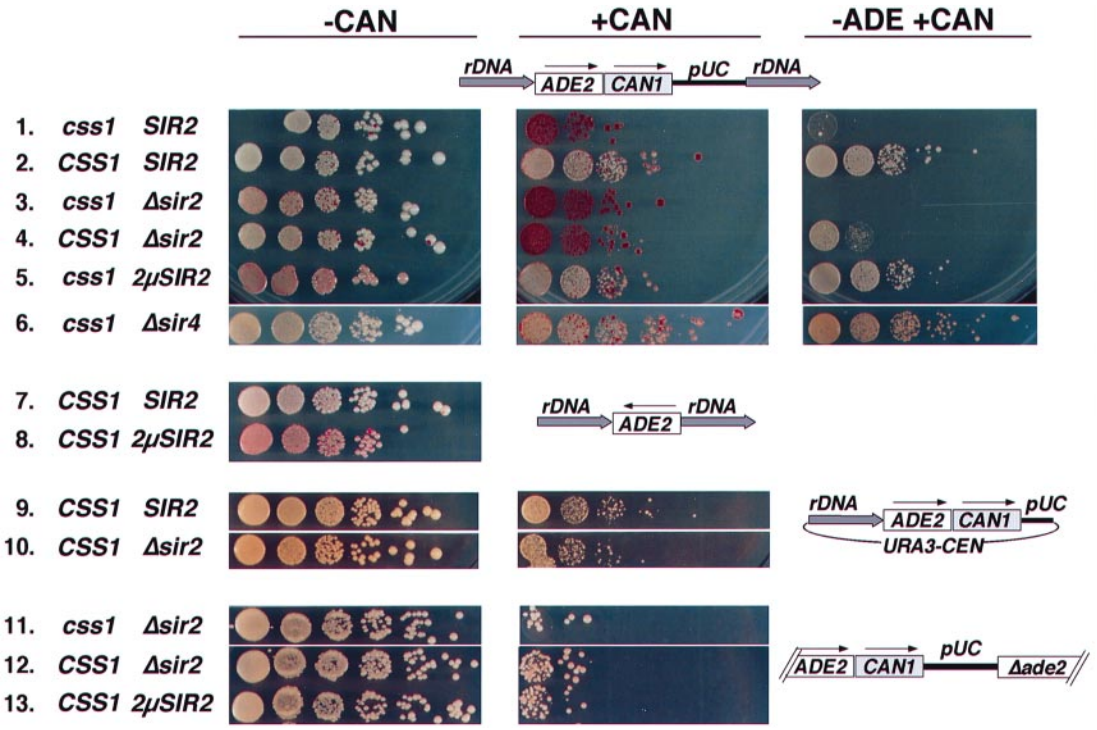

Fig. 3. Effect of *SIR2* on expression on the *ADE2-CAN1* marker in the rDNA. Log phase cultures in liquid medium were 10-fold serially diluted and plated in parallel on the indicated media. Strains 1–6 carry a single copy of *ADE2-CAN1* at the unique *Kpn*I site in the rDNA. Strains 7 and 8 carry a single *ADE2* insertion at the *Hin*dIII site in the 35S rRNA coding region. Strains 9 and 10 contain no rDNA insertions, but carry a centromeric plasmid with a single copy of rDNA and the *ADE2-CAN1* marker. Strains 11–13 carry no rDNA insertions and have the *ADE2-CAN1* marker integrated at the Δ*ade2* locus. Media is –URA for samples 1–8 and –LYS –URA for samples 9–13 to select for plasmid retention. Strain 1: CFY559 [YCp-*URA3*], strain 2: CFY559 [YCp-*URA3-CSS1*], strain 3: CFY559Δsir2 [YCp-*URA3*], strain 4: CFY559Δsir2 [YCp-*URA3-CSS1*], strain 5: CFY559Δsir2 [YEp-*URA3-SIR2*], strain 6: CFY520Δsir4 [YCp-*URA3-CSS1*], strain 7: REE1285 [YEp-*URA3*], strain 8: REE1285 [YEp-*URA3-SIR2*], strain 9: CFY558 [YCp-*URA3-rDNA-ADE2-CAN1*, YCp-*LYS2-CSS1*], strain 10: CFY558Δsir2 [YCp-*URA3-rDNA-ADE2-CAN1*, YCp-*LYS2-CSS1*]. Strain 11: CFY570 [YEp-*URA3*, YCp-*LYS2*], strain 12: CFY570 [YEp-*URA3*, YCp-*LYS2-CSS1*], strain 13: CFY570 [YEp-*URA3-SIR2*, YCp-*LYS2-CSS1*].

strain 1). Significantly, introduction of a multicopy *SIR2* plasmid into this strain restored 100% plating efficiency on –ADE+CAN media, indicating that *2*μ*SIR2* reduces *CAN1* expression below the level observed with wild-type *SIR2*. Additionally, high-copy expression of *SIR2* resulted in pink colonies, indicating that the *ADE2* marker is now also poorly expressed under conditions of high *SIR2* dosage (Figure 3, strain 5). Repression by *SIR2* is not specific to the particular *ADE2-CAN1* construction, since pink colonies, indicating lowered expression, also result from transformation of *2*μ*SIR2* into strain REE1285 (Figure 3, strain 8), which bears a different *ADE2* insert (at the *Hin*dIII site in the 35S gene; Figure 1). The absence of colony color differences between *SIR2* and Δ*sir2* genotypes, in light of observable changes in canavanine-resistance in the same strains, indicates that the threshold of expression for detecting a phenotypic difference is not the same for the *ADE2* and *CAN1* markers.

Since overexpression of *SIR2* represses *ADE2* and further reduces *CAN1* expression in the rDNA, we determined if this hyper-repressed phenotype requires the other *SIR* genes by overexpressing *SIR2* in the original panel of *SIR* deletion strains. The absence of the other *SIR* products does not alter the pink colony color indicative of reduced *ADE2* expression in the *rDNA::ADE2-CAN1* marker, nor does it alter the CAN plating phenotype of *2*μ*SIR2* strains (Fritze, 1994). *SIR1*, *SIR3* and *SIR4* are thus dispensable for the reduced expression of the *rDNA::ADE2-CAN1* marker in both wild-type and *2*μ*SIR2* strains. To confirm that *SIR2* repression is specific to the rDNA, we showed that colony color or canavanine sensitivity is unaffected by increased dosage of *SIR2* when the *ADE2-CAN1* marker is maintained on a plasmid or integrated elsewhere in the genome (Figure 3, strains 9–13). These studies thus demonstrate that: (i) *SIR2* represses both *ADE2* and *CAN1* in the rDNA; (ii) repression of *CAN1* and *ADE2* responds to *SIR2* gene dosage; and (iii) *SIR2*-dependent repression is independent of the *SIR1*, *SIR3* or *SIR4* functions. Although a correlation between increased *SIR2* dosage and hypoacetylation of histone pools has been shown (Braunstein *et al.*, 1993), these results constitute the first demonstration in yeast of an association between *SIR2* overexpression and reduced gene expression.

SIR2 silencing in the rDNA is associated with a small reduction in ADE2 and CAN1 RNA levels

We confirmed that repression of *rDNA::ADE2-CAN1* correlated with a reduction in transcription using S1 nuclease protection assays to quantitate precisely steady-state levels of *CAN1* RNA in *SIR2* and Δ*sir2* cells. Unexpectedly, Pol I read-through transcripts were detected which originated in the rDNA from the upstream 35S rRNA promoter, extending through the *ADE2* and *CAN1* markers (see Materials and methods). We determined that *SIR2*-dependent silencing of the genes inserted in the rDNA is not

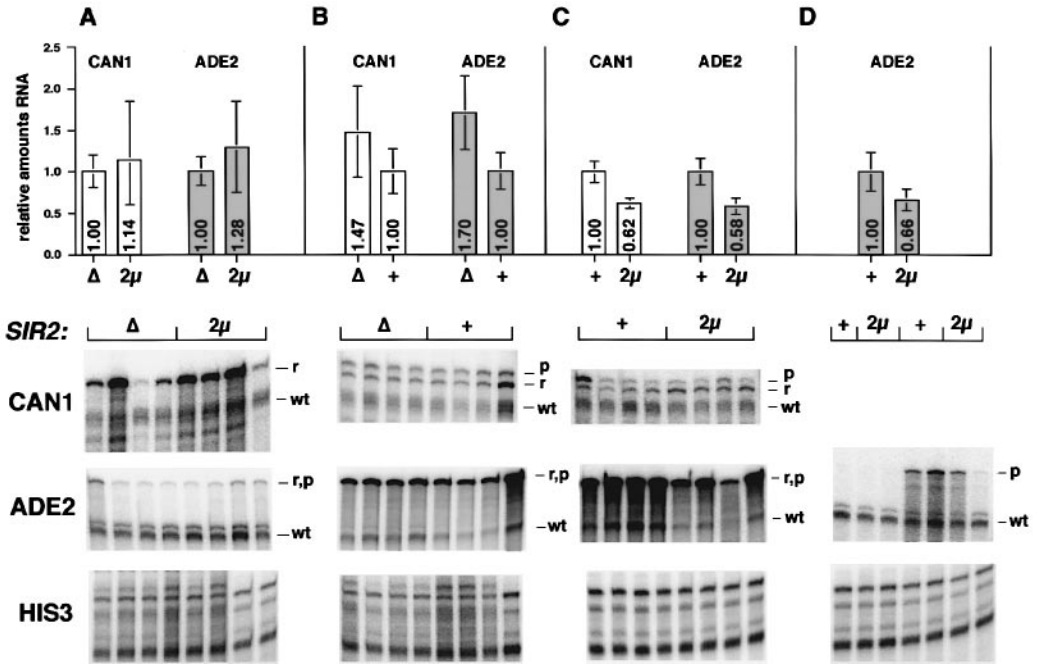

Fig. 4. Transcription of rDNA markers in the presence or absence of *SIR2*. Equal amounts of total RNA from duplicate samples prepared from independent colony isolates were analyzed with *CAN1*, *ADE2* and *HIS3* probes by S1 nuclease protection analysis as described. Arrows labeled p, r and wt indicate positions of residual probe, read-through transcript and wild-type transcript, respectively. RNA amounts were quantitated by phosphorimagery as described in Materials and methods. To control for loading variation, the *CAN1* and *ADE2* RNAs were normalized to the cluster of bands as shown above, which were quantitated as a unit, representing different transcription termination sites detected by the *3′-HIS3* probe. Each bar graph represents quantitation for the S1 experiment shown below it. Error bars represent the standard deviation of the data. (**A**) Transcription of the *ADE2-CAN1* marker integrated outside the rDNA at the Δ*ade2* locus. Δsir2 = CFY570 [YEp-*URA3*], 2 μSIR2 = CFY570 [YEp-*URA3-SIR2*]. (**B**) Transcription of the *rDNA::ADE2-CAN1* marker in *SIR2* versus Δ*sir2* strains. Δ*sir2* = CFY559Δsir2 [YCp-*URA3-CSS1*], *SIR2* = CFY559[YCp-*URA3-CSS1*]. (**C**) Transcription of *rDNA::ADE2-CAN1* in *SIR2* versus *2*μ*SIR2* strains. *SIR2* = CFY559 [YEp-*URA3*], *2*μ*SIR2* = CFY559[YEp-*URA3-SIR2*]. (**D**) Transcription of an *ADE2* insertion which produces no read-through RNA. In lanes 1–3, *SIR2* = CX100-2C[YEp-*URA3*], *2*μ*SIR2* = CX100-2C[YEp-*URA3-SIR2*]. In lanes 4–7, *SIR2* = CX100-6D[YEp-*URA3*], *2*μ*SIR2* = CX100-6D[YEp-*URA3-SIR2*].

dependent on the anomalous transcripts by examining silencing in strains in which *ADE2* was integrated into the rDNA in the opposite orientation from the 35S transcript to eliminate formation of read-through transcripts from the upstream 35S promoter (see Figure 1). Only RNAs with the wild-type RNA start site were transcribed in the single marker *rDNA::ADE2* integrant, and high-copy *SIR2* still reduced expression of the *ADE2* marker (Figure 3, strain 8). Therefore, our analysis of transcript levels focused on the wild-type *ADE2* and *CAN1* messages. The presence of a single copy of *SIR2* results in a small but measurable average decrease in *ADE2* and *CAN1* RNA levels, which was observed in several independent trials of this experiment (Figure 4B). High-copy *SIR2* expression further reduces the level of *CAN1* and *ADE2* mRNAs ~40% as compared with wild-type (Figure 4C). The same trend was discerned under conditions of increased *SIR2* dosage with *ADE2* RNA levels in the integrant containing the independent *ADE2* insertion in opposite orientation to the 35S transcript which produces only wild-type transcripts (Figure 4D). No *SIR2*-dependent changes in *ADE2-CAN1* RNA levels are seen when *ADE2-CAN1* is located outside the rDNA at the *ADE2* locus (Figure 4A.) These data indicate that the amount of *ADE2-CAN1* marker transcription in the rDNA varies inversely with increasing *SIR2* dosage, in parallel with *SIR2*'s phenotypic effects on gene expression.

Loss of SIR2 function alters chromatin structure at both HMR and MAT

SIR2-specific features in rDNA chromatin were monitored by MNase cleavage and indirect end-labeling (Wu, 1980; see also Materials and methods). As a control for our studies of MNase sensitivity in rDNA, the same chromatin preparations used for the analysis of rDNA were used to visualize chromatin features at *HMR* and *MAT*. It has previously been demonstrated (Nasmyth, 1982) that loss of *SIR2* function causes significant changes in the MNase cleavage pattern of silent mating-type chromatin (*HML* and *HMR*), but not of chromatin at the actively transcribed *MAT* locus. Here, in agreement with the earlier studies of Nasmyth (1982), the MNase cleavage profiles of *HMR**a*** chromatin prepared from *SIR2* and Δ*sir2* cells are significantly different across the X region and near the promoter required for **a**1 and **a**2 transcription (Figure 5A), but remain unchanged at the *MAT*α HO cleavage site at the Y/Z junction (Figure 5B). Unexpectedly, however, our data revealed a previously undetected *SIR2*-dependent change at *MAT*, specifically in the promoter region of *MAT*α. This site is cleaved by MNase in the *SIR2* but not *sir2* strain (Figure 5B, lanes 1–10).

Transcription at *MAT*α*1*, in contrast to *MAT**a***, is repressed by the **a**1-α2 repressor in diploid cells via binding of the repressor to an operator site within the *MAT*α*1* promoter (reviewed in Herskowitz, 1990). Because silent mating-cassette derepression in *sir2* cells permits

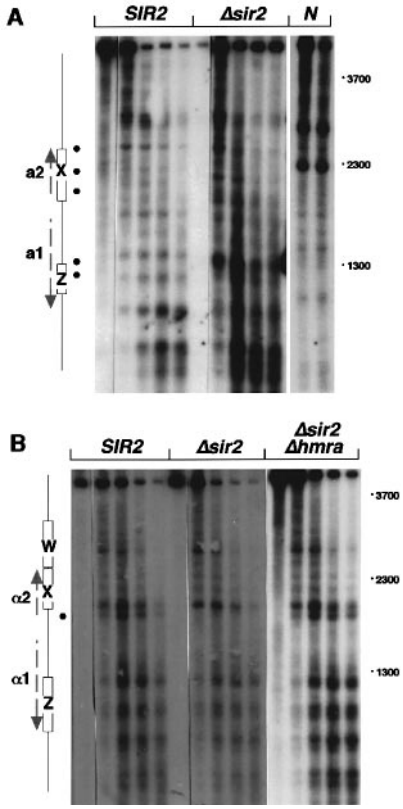

Fig. 5. (**A**) Micrococcal nuclease cleavage of *HMR**a*** chromatin in *SIR2* and Δ*sir2*. Chromatin from *SIR2* (strain YK18, lanes 1–5), and Δ*sir2* (strain YK4, lanes 6–10) strains, or naked DNA (lanes 11–12) was partially digested with micrococcal nuclease, treated with proteinase K and phenol:chloroform to remove protein, digested with *Hin*dIII, transferred to membrane and hybridized to a *Bam*HI–*Pst*I *HMR**a*** probe as described in Materials and methods. ● indicates positions at which MNase cleavage differs between *SIR2* and Δ*sir2*. X and Z represent sequence elements at *HMR**a***, arrowheads show the **a**1 and **a**2 transcripts. Numbers to the right of the figure correspond to the size in bp of markers co-electrophoresed alongside the sample lanes. (**B**) MNase cleavage of *MAT*α chromatin. MNase digestion and other treatments as described above, but with hybridization to a *Hin*dIII–*Nru*I probe to *MAT*α. Lanes 1–5 are nuclei from *SIR2* (YK18), lanes 6–10 from Δ*sir2* (YK4) and lanes 11–15 from Δ*sir2* in which *HMR**a*** has been deleted/disrupted (YK52). ● at left edge of photograph indicates the position of a band which appears in chromatin from *SIR2* and Δ*sir2 hmra::URA3* nuclei but not Δ*sir2* nuclei. W, X and Z represent sequence elements at *MAT*α; arrowheads show the α1 and α2 transcripts. Naked DNA profiles show MNase cleavage sites at the same positions as lanes 1–5 and 11–15 (Nasmyth, 1982; also unpublished data).

formation of the **a**1-α2 repressor, we tested whether the change in chromatin structure in the promoter region of *MAT**a*** depends on the presence of this repressor. Disruption of *HMR**a*** to eliminate the **a**1-α2 repressor showed that the promoter site at *MAT*α is still cleaved in a *sir2* strain lacking the **a**1-α2 repressor (Figure 5B, lanes 11–15). Therefore, the protection of a MNase cleavage site in *MAT*α *sir2* chromatin is indeed dependent upon the presence of the **a**1-α2 repressor, perhaps due to binding of the repressor to its target sequence in the *MAT*α*1* promoter.

SIR2 reduces accessibility of rDNA chromatin to nuclease digestion in the non-transcribed spacer

A physical map of the rDNA repeat unit with relevant restriction sites and sequence elements is shown in Figure

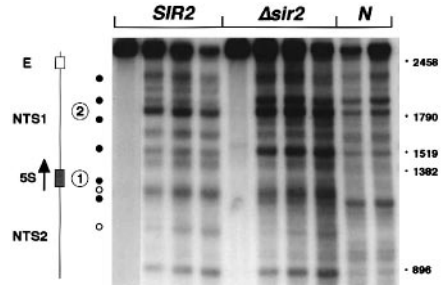

Fig. 6. MNase cleavage sites in chromatin of the rDNA non-transcribed spacer (NTS). MNase digestion and other treatments as described in Figure 5, but digestion with *Eco*RI and hybridization to probe P6. MNase concentration increases from left to right within each series; lanes 1 and 5 were not treated with MNase. The line diagram at left depicts features of the rDNA gene in this region. ● and ○ indicate MNase cleavages identified as sites of chromatin-specific protection and enhancement, respectively (see text). These sites constitute SRR1. E, enhancer; gray box and arrowhead labeled 5S represent the positions of coding region and transcript for the 5S rRNA gene. Circled numbers 1 and 2 denote *Dpn*I restriction sites referred to later in the text.

1. Each rDNA repeat consists of 9080 bp of DNA and gives rise to two primary transcripts: a 5S rRNA (Pol III) and a 35S precursor RNA (Pol I) which is processed into 5.8S, 18S and 25S rRNA species. Adjacent 35S coding regions are separated by a non-transcribed spacer (NTS) which is sub-divided by the centrally located 5S gene into two ~1 kb regions, NTS1 and NTS2 (reviewed in Planta and Raue, 1988). NTS1 contains an enhancer for Pol I transcription (E), while NTS2 contains a promoter (P), an *ARS* element that has been shown to function as an origin of replication *in vivo* (Skryabin *et al.*, 1984; Linskens and Huberman, 1988), and a binding site for Topoisomerase I (TOPI) (Bonven *et al.*, 1985). A consensus binding site for the transcription factor *RAP1* is present in the 25S rRNA coding sequence (Buchman *et al.*, 1988). The *HOT1* recombination initiator is composed of the enhancer and promoter for 35S transcription. The NTS1 element also contains a replication fork barrier near the end of the 35S transcription unit which impedes the progress of replication forks which travel in the opposite direction than 35S transcription (Brewer and Fangman, 1988).

Our analysis of MNase sensitivity in the rDNA NTS is presented in Figure 6. The MNase cleavage pattern of wild-type (*SIR2*) chromatin differed from naked DNA at eight distinct positions within the NTS1–5S–NTS2 region. Six of these positions are cleaved with reduced efficiency in chromatin as opposed to naked DNA and represent sites of chromatin-specific protection from MNase (Figure 6, closed circles). The remaining two positions constitute cleavage sites in chromatin which are not present in naked DNA and thus define chromatin-specific enhanced cleavages (Figure 6, open circles).

Chromatin prepared from the Δ*sir2* strain is more sensitive to MNase cleavage at all six sites of chromatin-specific protection, indicating that loss of *SIR2* function reduces the extent of protected chromatin in this region of the rDNA. The differences between Δ*sir2* and *SIR2* chromatin are restricted to a 0.9 kb region starting 300 bp downstream of the 5′ *Eco*RI site at the border of the enhancer and encompass the 5S rDNA coding region. This region represents the major location of *SIR2*-dependent alterations we detected in the entire rDNA, and is here

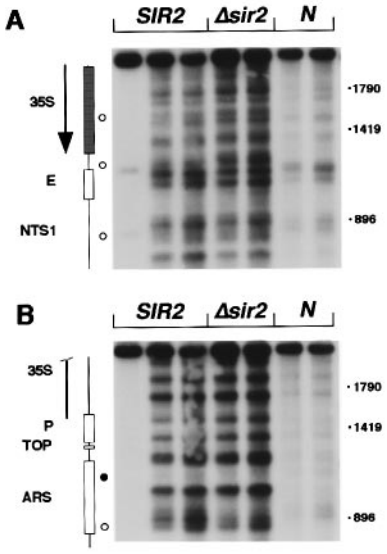

Fig. 7. (**A**) MNase sensitivity of chromatin in the rDNA enhancer region. MNase digestion and other treatments as described in the legend to Figure 5, but with *Ava*II digestion and hybridization to probe P3. Lanes 1–3 are nuclei from *SIR2*, lanes 4–5 from Δ*sir2* and lanes 6–7 are naked DNA. ○ indicate MNase cleavages identified as sites of chromatin-specific enhancement. The increased intensity of the band in lanes 4 and 5 near the edge of the enhancer (E) was not observed in two subsequent repetitions of this experiment. E, enhancer; gray box and arrowhead denote the 3′-end of the 25S coding region and 35S transcript, respectively. (**B**) MNase sensitivity of chromatin in the 35S rRNA promoter. MNase digestion and other treatments as described in Figure 5, but with *Pvu*II digestion and hybridization to probe P3. ● and ○ indicate MNase cleavages identified as sites of chromatin-specific protection and enhancement, respectively (see text). ARS, ARS consensus sequences; TOP, Topoisomerase I binding site; P, promoter; the heavy black line indicates the 5′ end of the 35S transcript.

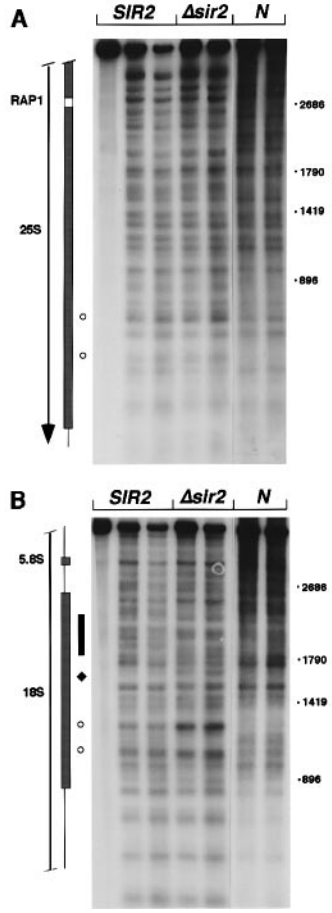

Fig. 8. (**A**) MNase sensitivity of the 25S rRNA coding region. MNase digestion and other treatments as described in Figure 5, but with *Ava*I digestion and hybridization to probe P2. Lanes 1–3 are nuclei from *SIR2*, lanes 4–5 from Δ*sir2* and lanes 6–7 are naked DNA. ○ indicate MNase cleavages identified as sites of chromatin-specific enhancement (see text). RAP1, RAP1 binding site; gray box, 25S rDNA coding region; arrowhead, 35S transcript. (**B**) MNase sensitivity of chromatin in the 18S and 5.8S rRNA coding regions. MNase digestion and other treatments as described in Figure 5, but with *Ava*I digestion and hybridization to probe P6. Lanes 1–3 are nuclei from *SIR2*, lanes 4–5 from *sir2* and lanes 6–7 are naked DNA. ○ indicate MNase cleavages identified as sites of chromatin-specific enhancement. ◆ indicates the position of a site whose cleavage is enhanced in Δ*sir2* chromatin as compared with both wild-type chromatin and naked DNA. The filled bar indicates the position of unclassified *sir2*-specific features. These sites constitute SRR2. Gray boxes, 18S and 5.8S rRNA coding regions; black line, 35S transcript.

designated SRR1 (*SIR2* Responsive Region 1). The same *SIR2*-dependent alterations representing SRR1 are also detected by mapping with respect to the *Eco*RI restriction site at the 5′ end of NTS1 (unpublished data). Loss of *SIR2* function does not lead to the elimination of all MNase-detectable chromatin structure in this region however, since the two sites of chromatin-specific enhanced cleavage (Figure 6, open circles), are not affected by the *SIR2* genotype. These data demonstrate that the loss of *SIR2* function either alters the structure of chromatin in SRR1 to allow increased MNase cleavage, or results in a decrease in the proportion of rDNA repeat units which are organized into a chromatin structure that protects the rDNA NTS from MNase cleavage.

Because NTS1 contains the Pol I enhancer and promoter that comprise the *HOT1* element as well as a number of other *cis*-acting sequences, chromatin features in these regions were examined more closely. No reproducible differences in MNase sensitivity were detected between *SIR2* and Δ*sir2* chromatin in the enhancer or promoter (Figure 7).

The 35S transcription unit contains few chromatin features as measured by MNase

The MNase cleavage profile of the chromatin preparations in the remainder of the rDNA repeat unit, including the 35S coding region, is similar to that of the naked DNA samples (Figure 8). In the 25S coding region, only two sites of chromatin-enhanced MNase cleavage are detected, and no changes between the MNase cleavage profile of *SIR2* and Δ*sir2* chromatin are visible (Figure 8A). In the 18S coding region a number of chromatin-specific features are detected (Figure 8B); however, these features are considerably weaker than the differences identified in the NTS. These included two sites of chromatin-enhanced MNase cleavage (Figure 8B, open circles) whose intensity increases by loss of *SIR2* function and a site at which chromatin is more readily cleaved in Δ*sir2* chromatin compared with both wild-type chromatin and naked DNA (Figure 8B, filled diamond). A series of complex *sir2*-dependent changes can also be seen which we could not unambiguously ascribe to chromatin-specific enhancement or protection (Figure 8B, filled square).

Overall, our analysis of the 35S rRNA region by MNase treatment revealed large stretches of the rDNA which are devoid of positioned nucleosomes, consistent with the high level of transcription in this region (Dammann *et al.*, 1993). Several chromatin-specific features occur in the 18S coding region, and at a subset of these sites chromatin prepared from Δ*sir2* cells is more readily cleaved by MNase than chromatin from *SIR2* cells. The *SIR2*-dependent chromatin alterations in the 18S region are designated SRR2 (*SIR2* responsive region 2).

sir2-specific changes in rDNA chromatin are independent of the a1-α2 repressor

As indicated earlier, derepression of the silent mating cassettes in a *sir2* background permits expression of the **a**1-α2 regulatory molecule. To determine whether the *sir2*-specific changes in rDNA NTS chromatin were a primary defect of Δ*sir2* or an indirect consequence of the presence of the **a**1-α2 repressor (as was found at *MAT*α), we eliminated the **a**1-α2 repressor from the Δ*sir2* strain by disrupting *HMR***a** as described earlier. Chromatin from the Δ*hmra* strain was visualized by MNase treatment as before. The *sir2*-dependent changes in NTS chromatin are still visible in the Δ*hmr***a** Δ*sir2* strain (unpublished data), indicating that they do not result indirectly from **a**1-α2 expression.

SIR2 restricts accessibility of the rDNA NTS to modifications in vivo

The *in vitro* analysis of purified nuclei described above demonstrates that loss of *SIR2* function increases the accessibility of MNase to chromosomal DNA in the rDNA NTS1/5S region, and to a lesser extent, in the 18S rRNA coding region. In order to determine whether these results reflect changes in rDNA chromatin accessibility *in vivo*, we examined whether sequences in the rDNA NTS1/5S region of *SIR2* and Δ*sir2* strains are differentially susceptible to methylation by heterologously expressed *E.coli dam* methyltransferase. Modification of adenosine residues in the sequence GATC by *dam* methyltransferase constitutes a silent, stable *in vivo* modification which can be easily detected by Southern analysis with restriction enzymes that discriminate between methylated and unmethylated GATC sequences (Gottschling, 1992; Singh and Klar, 1992). This assay measures directly the extent to which chromatin structure restricts *in vivo* access to the DNA template and thus provides a convenient method to extend and confirm the observations made with MNase *in vitro*.

Two GATC sites in the NTS1 were chosen for this analysis. The first GATC site (Position 1) lies near a site of chromatin-specific protection from MNase that is more efficiently cleaved in a Δ*sir2* background than in wild-type (see Figure 6). The second GATC site (Position 2) is in a region of NTS1 that is efficiently cleaved by MNase regardless of *SIR2* genotype (Figure 6). If *SIR2* regulates the accessibility of rDNA chromatin near Position 1, it should be more efficiently methylated in a Δ*sir2* strain than in wild-type and be detected by the presence of a 1331 bp *Eco*RI–*Dpn*I restriction fragment. Lack of methylation gives rise to a 1519 bp *Eco*RI–*Pvu*II fragment. The exclusive presence of the 1519 bp *Eco*RI–*Pvu*II fragment (Figure 9B, lane 4) and the 1331 bp *Eco*RI–*Sau*3A fragment (Figure 9B, lane 3) in DNA from a control strain lacking the *dam* integration confirms the presence of the restriction sites and demonstrates the lack of significant *Dpn*I cleavage in the absence of *dam* expression. Methylation of Position 2 gives rise to a

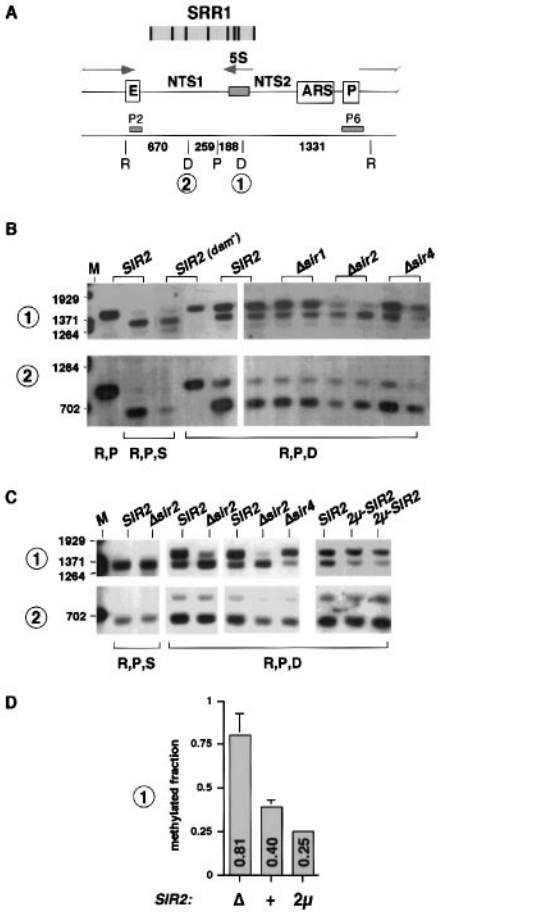

Fig. 9. Accessibility of rDNA chromatin to heterologously expressed *dam* methyltransferase. (**A**) Schematic map of the rDNA NTS. R, P and D mark the positions of *Eco*RI, *Pvu*II and *Dpn*I–*Sau*3AI restriction sites, with corresponding restriction fragment sizes indicated underneath. Circled numerals 1 and 2 refer to the *Dpn*I sites monitored in this experiment (also marked in Figure 6). SRR1, *SIR2* responsive region 1; E, enhancer; P, promoter; A, ARS consensus sequences. Boxes labeled P2 and P6 represent probe sequences. (**B**) Analysis of *dam* methylation in *SIR2*, Δ*sir1*, Δ*sir2* and Δ*sir4* strains. Genomic DNA was prepared from mid-log cultures in YPDA medium and digested with either *Eco*RI, *Pvu*II (R,P, lane 1), *Eco*RI, *Pvu*II and *Sau*3A (R,P,S, lanes 2, 3) or *Eco*RI, *Pvu*II and *Dpn*I (R,P,D, lanes 4–12). Methylation at Position 1 was detected with probe P6; methylation at Position 2 was detected after stripping the membrane and re-hybridization with probe P2. M, size standards; *SIR2*, DNA prepared from two independent isolates of UCC1021; *SIR2 (dam-)*, UCC1001; Δ*sir1*, UCC1044; Δ*sir2*, UCC1042; Δ*sir4*, UCC1045. Lanes 1–5 and 6–12 correspond to different exposures from two regions of the same membrane. (**C**) Analysis of *dam* methylation in *SIR2*, Δ*sir2* and 2μ*SIR2* strains. Duplicate *SIR2* and Δ*sir2* samples represent DNA samples purified from independent cultures on separate days. In lanes 9–11, DNA was prepared from mid-log cells grown in −URA medium and digested with *Eco*RI, *Pvu*II and *Dpn*I. *SIR2*, UCC1023 transformed with pRS426 (YEp-*URA3*); 2μ*SIR2*, independent isolates of UCC1023 transformed with pJR68 (YEp-*URA3*-*SIR2*). (**D**) Quantitation of results for Position 1 presented in (C). Band intensities were measured by scanning and densitometry as described. Values for isolates with the same genotype were expressed as the proportion of methylated sites to total and averaged.

670 bp *Eco*RI–*Dpn*I fragment. Its appearance is predicted to be unaffected by *SIR2* function. Analysis of DNA samples in the control *dam*$^-$ background once again confirms the restriction pattern and lack of background methylation/*Dpn*I cleavage (Figure 9B, lanes 3 and 4).

The ability of the *dam* methyltransferase to modify Position 1 and Position 2 in response to the *SIR* genotype is shown in Figure 9B (lanes 5–12) and Figure 9C. Less than 40% of GATC sites at Position 1 are methylated in a wild-type background, indicating restricted access to the sequence by *dam* methyltransferase. Loss of *SIR1* or *SIR4* function does not alter the pattern of *Dpn*I cleavage at Position 1 as compared with wild-type. However, when Position 1 is assayed in a Δ*sir2* background, >80% of sites were methylated. As predicted from the MNase studies, loss of *SIR2* function increases accessibility of this site to methyltransferase. The increased accessibility is due specifically to loss of *SIR2* function and not to derepression of the silent mating-cassettes since increased accessibility is not observed when the *SIR1* or *SIR4* gene is deleted. In contrast to Position 1, ~90% of sites at Position 2 are methylated regardless of *SIR2* genotype, as expected. Once again, loss of *SIR1* or *SIR4* does not significantly alter accessibility at Position 2. These results demonstrate that mutations in *SIR2* increase accessibility to the formerly protected Position 1 site *in vivo* as suggested by the MNase studies. Intriguingly, deletion of *SIR4* leads to a slight increase in protection of Position 1 in this experiment. This can be explained by the recent finding that delocalization of Sir2p from telomeres in a *sir4* background leads to increased accumulation of Sir2p in the nucleolus (Gotta *et al.*, 1997). In summary, *SIR2* function (but not *SIR1* or *SIR4* function) is required *in vivo* for the maintenance of a more closed rDNA chromatin structure. This finding parallels the results of previous genetic data that *SIR2* (but not *SIR1*, *SIR3* or *SIR4*) suppresses rDNA recombination (Gottlieb and Esposito, 1989) and agrees with our finding that *SIR2* is required for reducing expression of marker genes integrated into the rDNA array.

rDNA accessibility responds to increased dosage of SIR2

Since *SIR2* chromatin has a more closed configuration than Δ*sir2* chromatin, we examined whether increased *SIR2* dosage further reduces rDNA accessibility. The *dam* methyltransferase protection assay was thus repeated with a strain bearing a high-copy *SIR2* plasmid (2μ*SIR2*) (Figure 9C, lanes 9–11). In the *SIR2* background, the *Dpn*I site Position 1 in the NTS is methylated at 44% of GATC sites, in close agreement with the results from the previous experiment (38%). When *SIR2* dosage is increased via expression from a 2μ plasmid, only 25% of sites are methylated. Therefore, high-copy *SIR2* expression results in greater protection of rDNA near Position 1 as compared with a wild-type background. No effect is observed at Position 2. These data show that rDNA accessibility in the rDNA NTS decreases with increasing *SIR2* dosage, indicating that Sir2p is a limiting component or regulatory function required for closed chromatin structure in this region.

Discussion

A SIR2-dependent position effect in the rDNA

At the silenced HM mating loci and telomeres, *SIR2*, the other three *SIR* genes and additional factors are all required for the maintenance of specialized chromatin structures and inhibit transcription in a promoter-non-specific and position-dependent manner. As such, the *SIR* genes define a set of region-specific regulators that inhibit gene expression of a chromosomal 'neighborhood' as contrasted with repression of individual genes (reviewed in Laurenson and Rine, 1992).

In this study we have demonstrated that *SIR2* also regulates region-specific gene expression in the rDNA repeated array, as has recently been found by (Bryk *et al.*, 1997; Smith and Boeke, 1997). We also present the novel finding that *SIR2* function regulates chromatin structure by direct assays of MNase sensitivity and *in vivo dam* methyltransferase accessibility. At least two regions of the rDNA, designated SRR1 and SRR2, are affected by the presence of Sir2p, resulting in a more closed chromatin structure. In all nine positions where *SIR2*-dependent changes could be clearly identified, loss of *SIR2* function led to increased cleavage by MNase. The increased susceptibility of these regions to MNase *in vitro* reflects a change in chromatin structure at these sites which may result from one of a number of diverse mechanisms, including changes in nucleosome positioning or occupancy, DNA unwinding, association of DNA-binding proteins, or higher-order chromatin folding. To determine whether increased cleavage by MNase *in vitro* reflected increased exposure of the underlying DNA template, we assayed the accessibility of a putative *SIR2*-protected site in SRR1 to *dam* methyltransferase. The results of this analysis indicated that at this site *SIR2* protects the rDNA sequence from attack by methyltransferase, and by implication from access to other proteins which require interaction with the DNA.

Repression of gene expression by *SIR2* was not observed when marker genes were located outside the rDNA array (even when adjacent to a single rDNA repeat), indicating that repression was governed by positional context. Additionally, unlike other known contexts of *SIR* activity, chromatin structure and repression in the rDNA specifically responded to *SIR2* gene dosage, indicating that the *SIR2* gene product is a limiting component or regulatory factor which regulates these phenomena in the rDNA. The close association between *SIR2* dosage, rDNA chromatin changes and gene expression suggests that *SIR2's* effect on gene expression in the rDNA is mediated by changes in chromatin structure, specifically via the formation of a more closed chromatin configuration which may exclude transcription factors. The effect of *SIR2* in rDNA is to be contrasted with the situation at yeast telomeres, where position effect associated with repression and closed chromatin is specifically responsive to *SIR3* dosage (Renauld *et al.*, 1993).

Our result that *SIR2* causes pronounced chromatin changes at SRR1 is in good agreement with the recent findings that Sir2p localizes to the nucleolus and that rDNA NTS/5S sequences are enriched in immunocomplexes precipitated by anti-Sir2p antibodies (Gotta *et al.*, 1997). Taken together with our results, these data build a

compelling case that *SIR2*'s effects on chromatin structure, gene expression and recombination result from direct or indirect interaction primarily with the NTS1–5S–NTS2 region of the rDNA repeat unit.

SIR2-dependent chromatin changes and recombination in the rDNA

We had previously proposed that *SIR2*-mediated changes in rDNA chromatin structure may protect rDNA from general recombination functions, since crossing over in this 1 Mb region occurs 50- to 100-fold less frequently than expected based on physical distance (Petes and Botstein, 1977; Petes, 1979; Zamb and Petes, 1982). This was based on the finding that two genes dispensable for the low level of rDNA recombination in wild-type cells (*RAD50* and *RAD52*) are essential for the elevated recombination observed in a Δ*sir2* background (Gottlieb, 1989). Recently, a compelling body of evidence has developed demonstrating that DNA accessibility throughout the genome is critical in the initiation of general and site-specific recombination events. Recombination-initiating double-strand breaks (DSBs) have been found during meiosis, predominantly in the upstream regions of yeast genes (Wu and Lichten, 1994). During both mitosis and early meiosis, the DNA at these sites is more exposed than surrounding regions as judged by MNase and DNase I treatment. Events which increase or decrease the accessibility of the DSB sites lead to parallel increases or decreases in recombination initiation (Ohta *et al.*, 1994; Wu and Lichten, 1994). These results suggest that DNA accessibility determines the frequency with which the recombination machinery selects specific sites for recombination initiation. In site-specific systems, recombination initiation can also be regulated via changes in chromatin accessibility, as in yeast where *SIR* functions protect the silent mating-type cassettes from HO cleavage, and in mammals where chromatin structure plays a role in the activation of immunoglobulin gene rearrangements. In a similar manner, the ability of *SIR2* to regulate rDNA recombination may stem from its ability to regulate the accessibility of SRR1 and/or SRR2. *SIR2*-mediated changes in rDNA chromatin structure may also exclude recombination factors like *RAD50* and *RAD52*, thereby explaining why the products of both genes are dispensable for rDNA recombination in wild-type cells but are essential for the increased recombination observed in a Δ*sir2* background (Gottlieb, 1989). The identification of *SIR2*-responsive sites in rDNA chromatin now makes possible the detailed analysis of *cis*-acting sequences required for specialized chromatin structure and recombination suppression in the rDNA, focusing particularly on the sequences underlying SRR1 and SRR2. It is tempting to speculate that *SIR2* regulates chromatin structure in the rDNA by altering histone acetylation (as at the silent *HM* loci and telomeres; Braunstein *et al.*, 1993), but the effects of histone modifications on chromatin structure in the rDNA remain to be determined.

Does the position effect in the rDNA involve switching between heritable expression states?

Silencing by *SIR2* results in intermediate expression of *ADE2* and *CAN1* genes located in the rDNA array. Even in the presence of a multicopy *SIR2* plasmid, reduced expression of *ADE2* yields pink colonies (rather than red) and a 40% reduction in steady-state *ADE2* RNA levels as compared with wild-type. Repression of *CAN1* generates a similar decrease in *CAN1* RNA and leads to slow growth in the presence of canavanine. Expression of both markers fails to be completely extinguished by *SIR2* function. This result is perhaps not surprising, considering that the rDNA array is a region of vigorous and crucial transcriptional activity where stringent silencing would be deleterious. The colony color, CAN growth and RNA level data are compatible with a mode of silencing by *SIR2* which is of intermediate strength and uniform in the population. We considered whether this behavior may reflect mixtures of cells with high and low expression states. Transcriptional silencing of telomeric loci is typified by semi-stable, heritable switching between active and inactive expression states (Gottschling *et al.*, 1990), a characteristic which is shared with the *HM* loci under some conditions (Pillus and Rine, 1989; Mahoney *et al.*, 1991; Sussel *et al.*, 1993). When the *ADE2* locus is placed near a telomere, red/white sectors are formed. The same behavior is observed with an *HMR::ADE2* marker in the presence of *HMR* ARS^- mutations and certain alleles of *rap1* (Sussel *et al.*, 1993). However, this result contrasts with our findings in the rDNA, since *rDNA::ADE2* strains display uniform color regardless of *SIR2* genotype. Furthermore, while rapid switching between fully 'on' and 'off' *CAN1* expression states could account for slow growth in CAN media, one would expect that the repressed Can^r state would coincide with repression of the adjacent *ADE2* locus. Since Can^r colonies are white rather than red or pink, this description does not appear correct. Rather, we favor the view that rDNA silencing results either from a constant, intermediate level of silencing or rapid on/off switching, a model shared with others (Smith and Boeke, 1997).

The rDNA position effect may require fewer components than other silenced regions

Silencing in yeast has been shown to depend upon a large number of *cis*- and *trans*-acting factors, many of which are shared between silencing at the *HM* loci and telomeres. The differential requirements for individual *cis*- and *trans*-acting factors at *HML*, *HMR* and telomeres suggest that different combinations of multiple silencing components contribute to silencing at these regions (Laurenson and Rine, 1992). Thus far, the finding that nucleosomes at silenced loci are hypoacetylated in a *SIR*-dependent manner (Braunstein *et al.*, 1993) constitutes the only candidate biochemical role of the *SIR* proteins. While *SIR2* leads to reduced acetylation of cellular histone pools, *SIR2*, *SIR3* and *SIR4* are all required for maintenance of the hypoacetylated state. In contrast, we have shown here that of the four *SIR* genes, only *SIR2* is required for the three rDNA-specific phenomena we have described: suppression of recombination, suppression of gene expression, and regulation of chromatin structure. The *SIR1*, *SIR3* and *SIR4* functions are dispensable for each of these phenomena in the context of the rDNA. It is not known whether the recently identified *SIR2*-related protein family (Brachmann, 1995, #639; Derbyshire, 1996 #619) plays a role in rDNA silencing. At the *HM* loci and telomeres, a complete understanding of silencing has been complicated by the partially redundant nature of the silencing apparatus

Table I. Strains used in this study

Strain	Genotype	Origin
YK18	*MAT***a** *ade2 can1 cyh2 his4 leu2 lys1 lys2 spo13-1 trp1-1 tyr1-2*	S.Gottlieb
YK4	*MAT***a** *ade2 can1 cyh2 his4 leu2 lys1 lys2 sir2::LEU2 spo13-1trp1-1 tyr1-2*	S.Gottlieb
YK52	YK4 *hmra::URA3*	this study
REE1283(A–)	*MAT***a** *ade2-1 can1 his4-260 leu2-3,112 lys2ΔBX trp1-HIII ura3-52*	this study
CFY500	REE1283*(A–) rDNA:ADE2-CAN1*	this study
CFY520	*MAT***a** *ade2 can1 his4 leu2 lys2 css1-1 tyr1-2 ura3-52 rDNA:ADE2-CAN1*	this study
CFY520Δsir1	CFY520 Δ*sir1*	this study
CFY520Δsir2	CFY520 Δ*sir2*	this study
CFY520Δsir3	CFY520 Δ*sir3*	this study
CFY520Δsir4	CFY520 Δ*sir4*	this study
CFY543	CFY520 *CAN1*, no rDNA insert	this study
CFY557	CFY543 Δ*can1::hisG ade2::pΔADE2*	this study
CFY558	CFY557 Δ*ade2::hisG*	this study
CFY558Δsir2	CFY558 Δ*sir2::LEU2*	this study
CFY559	CFY558 *rDNA:ADE2-CAN1*	this study
CFY559Δsir2	CFY559 Δ*sir2::LEU2*	this study
CFY570	CFY558Δsir2 Δ*ade2:::pADECAN*	
REE1285	*MAT***a** *ade2-1 can1 his4-260 leu2-3,112 lys2ΔBX trp1-HIII ura3-52 rDNA:ADE2*	this study
REE1303	*MATα can1r cyhr leu2 lys2 his1-7 hom3-10 trp1 ura3 rDNA:ADE2*	S.Gottlieb
UCC1001	*MAT***a** *ura3-52 ade2-101 trp1Δ1 his3Δ200 leu2-Δ1 lys2-801adh4::URA3-TEL*	D.Gottschling
UCC1021	*MAT***a** *ura3-52 ade2-101 trp1Δ1 his3Δ200 leu2-Δ1 lys2-801::dam::LYS2 adh4::URA3-TEL*	D.Gottschling
UCC1044	UCC1021 Δ*sir1::HIS3*	D.Gottschling
UCC1042	UCC1021 Δ*sir2::HIS3*	D.Gottschling
UCC1045	UCC1021 Δ*sir4::HIS3*	D.Gottschling
UCC1023	*MAT***a** *ura3-52 ade2-101 trp1Δ1 his3Δ200 leu2-Δ1 lys2-801::dam::LYS2*	D.Gottschling
W303a/α	*MAT***a***/MATα leu2-,3-112/leu2,-3,112 ade2/ade2 his3-11,15/his3-11,15 can1-100/can1-100 trp1-1/trp1-1 ura3-1/ura3-1*	R.Rothstein

(Stone *et al.*, 1991; Sussel and Shore, 1991; Chien *et al.*, 1993; Thompson *et al.*, 1994a). The rDNA may present an alternative, simplified context for dissecting silencing, since silencing is potentially accomplished with fewer functions.

Additional biological consequences of SIR2-dependent chromatin structure in the rDNA

In addition to the suppression of recombination and gene expression, *SIR2*'s ability to regulate rDNA chromatin structure may have additional effects in the rDNA. Recent studies have shown that rRNA gene expression in *Saccharomyces cerevisiae* is regulated in part by modulating the proportion of rDNA units which are transcriptionally active at any one time (Dammann *et al.*, 1993). Using DNA and RNA cross-linking techniques in an elegant series of experiments, Dammann *et al*. demonstrated that active and inactive repeat units are found in the same cell, and the ratio between them varies according to growth phase. Inactive units were shown to be packaged along their length in consistently spaced nucleosomal arrays typical of bulk chromatin. Active units, on the other hand, are devoid of nucleosomes along their coding regions and display a modified nucleosomal structure in the NTS which is thought to reflect the binding of *trans*-acting proteins.

The correspondence between the involvement of *SIR2* in the regulation of rDNA chromatin structure and the finding that rDNA units are organized into functionally and structurally distinct chromatin configurations suggests the intriguing possibility that *SIR2* may also play a role in the regulation of the proportion of active and inactive rDNA transcription units. Both the MNase and methyltransferase procedures generate a cleavage profile for the mass average of rDNA in the assay. Moreover, because the rDNA unit is a repeated structure (~100 copies/haploid cell), this raises the possibility that the repeated array within a single cell contains a mixture of units with distinct cleavage profiles (and therefore distinct chromatin configurations). It is not clear whether *SIR2* function results in a more closed chromatin configuration in all or a subset of rDNA repeat units within one cell. Because of the relationship between the activity of rDNA transcription units and rDNA ARS function (Walmsley *et al.*, 1984; Saffer and Miller, 1986), *SIR2* may also play a role in the initiation of rDNA replication analogous to the established correspondence between ARS activity/timing and silencing at telomeres and the *HM* loci (Miller *et al.*, 1984; Saffer and Miller, 1986; Mahoney *et al.*, 1991; McNally and Rine, 1991; Ferguson and Fangman, 1992; Aparicio and Gottschling, 1994; Lucchini and Sogo, 1994).

Finally, recent evidence indicates that, under certain specialized circumstances, SIR3p and SIR4p are also localized to the nucleolus (Gotta *et al*., 1997; Kennedy *et al*., 1997), but this appears to be due primarily by virtue of their potential to interact with SIR2p. Intriguingly, these studies suggest that major redistribution of silencing factors to the rDNA also plays a significant role in senescence (Kennedy *et al*., 1997). Whether Sir2p plays an additional role in this process other than to aid in the redistribution of silencing factors is not yet clear.

Materials and methods

Strains and media

A complete list of yeast strains used in this study is presented in Table I. Bacterial plasmids were maintained in *E.coli* DH5αF$^-$ (Woodcock *et al.*, 1989). Incubations at 30°C and 37°C were used to support growth of yeast and *E.coli*, respectively. Transformations of *E.coli* (Dower *et al.*, 1988) and *S.cerevisiae* (Schiestl and Gietz, 1989) were performed as described. Liquid and solid YPDA, SD, sporulation, dissection media

and media containing canavanine were as described (Sherman *et al.*, 1974). Selective media (supplemented minimal media, SMM) were made by supplementing minimal medium with liquid amino acid stock solutions as required to complement strain auxotrophies. Experiments requiring yeast strains with episomal plasmids were conducted with fresh transformants which were propagated under selective growth conditions except were stated. All gene disruptions and integrations were confirmed by Southern analysis (Southern, 1975), including the verification of single-copy insertions in the rDNA array.

Strain CFY543 was constructed by two-step gene replacement from a segregant of CFY520 which has lost its rDNA insert. This starting strain was transformed with pURACAN to uracil prototrophy. Integrants whose structure was confirmed by Southern analysis were plated on 5-FOA medium to select for loss of the *URA3* marker. Survivors were screened by Southern analysis and for Can^S phenotype. CFY556 was generated by disruption of the *CAN1* gene in strain CFY543 by a two-step process (Alani *et al.*, 1987) to generate CFY556. *CAN1* was disrupted by transformation with a linear fragment from pΔCAN1 (O.Aparicio) in which the central *CAN1 Hin*dIII fragment has been replaced by the *hisG-URA3-hisG* cassette. The *ADE2* locus of strain CFY556 was disrupted with the plasmid pΔADE2 (Aparicio *et al.*, 1991) by the same method to form CFY558. The intermediate containing the integration of the *hisG-URA3-hisG* cassette at the deleted *ADE2* locus was retained as strain CFY557. The *ADE2-CAN1* cassette was integrated into the rDNA of CFY558 to form CFY559 by linearizing pCAR1 at the unique *Sac*II site in the rDNA and transforming CFY558 to Ade^+. CFY559Δsir2 was generated form CFY559 by disrupting the *SIR2* gene with pJH103.1 (Ivy *et al.*, 1986). Strain CFY558Δsir2 is a mitotic segregant of CFY559Δsir2 that has lost the *ADE2-CAN1* insert from the rDNA. CFY570 was constructed by transformation of CFY558Δsir2 to Ade prototrophy with circular pADECAN plasmid. Southern analysis was used to screen transformants for isolates which had integrated the plasmid at the *ADE2* locus to form the structure depicted in Figure 1. CX200-2C and CX200-6D are independent Ura^+ Ade^+ meiotic segregants from a cross of CFY557 and REE1303. Both were isolated from tetrads with four normally segregating spores and were subsequently plated on medium containing 5-FOA to select for loss of the *hisG-URA3-hisG* cassette in the *ADE2* locus. REE1285 contains an integration of the 3.6 kb *ADE2* fragment at the *Hin*dIII site within the 35S coding region of the rDNA (originally constructed by R.Keil). The transcriptional orientation of *ADE2* is opposite to the 35S transcription unit.

The strains YK18 and YK4 are mitotic segregants of SG25-36D and G200, respectively, that have lost the *URA3* insert in the rDNA (Gottlieb and Esposito, 1989). YK52 is isogenic to YK4, but contains a disruption of *HMR**a*** (*hmr**a**::URA3*). *HMR**a*** was disrupted by transforming the strain YK4 to uracil prototrophy with a *Hin*dIII fragment derived from pCH4 that contains *HMR**a*** sequences in which the coding and flanking regions were deleted and replaced by the *URA3* gene. Disruption of the *HMR**a*** locus was confirmed by Southern analysis in transformants which mated as **a** cells (Southern, 1975).

Plasmids

A complete list of yeast strains used in this study is presented in Table II. The plasmid pCAR1 was employed to introduce the *ADE2-CAN1* marker into the rDNA. pCAR1 was constructed by subcloning a 9.1 kb rDNA *Kpn*I fragment from pKK1 (Gottlieb, 1989) into the *Kpn*I site of pUC19 (Yanisch *et al.*, 1985) to form pCF15. Two *Kpn*I sites are found in the published yeast rDNA sequences; the KK1 subclone contains only the *Kpn*I site near the 5′ end of the 26S coding region. A 3.6 kb *Bam*HI fragment containing *ADE2* from plasmid L909 (gift from R.Keil) was cloned into the *Bam*HI site of pCF15 to generate pCF16. Finally, a 4.1 kb *Xho*I *CAN1* fragment from plasmid pCAN1-2-1 (gift from J.Shuster) was ligated into the *Sal*I site of pCF16 to generate pCAR1. The 35S rRNA, *ADE2* and *CAN1* genes are all in the same transcriptional orientation. The *CAN1 Xho*I fragment contains ~2 kb of upstream sequence and the entire *CAN1* coding region. The downstream *Xho*I site is not in the published *CAN1* sequence (Hoffmann, 1985); we have mapped it to position 2202 where the published sequence indicates a 5/6 match for the *Xho*I recognition sequence (CTCAG as compared with CTCGAG).

The centromeric plasmid pCARCEN was constructed by co-transforming a *ura3* yeast strain with the large *Pvu*II fragment of pRS316 and pCAR1. Ura^+ transformants were rescued to *E.coli* and plasmid was purified to confirm that the small *Pvu*II fragment of pRS316 had been replaced with the *Pvu*II fragment of pCAR1 containing the rDNA, *ADE2* and *CAN1* inserts. pCACEN was generated by partial *Kpn*I digestion of pCARCEN and religation to remove the 9.1 kb rDNA repeat unit.

Table II. Yeast plasmids used in this study

Plasmid[a]	Marker	Origin
YCp:		
pRS316	*URA3*	Sikorski and Hieter (1989)
pRS317	*LYS2*	Sikorski and Hieter (1989)
pCSS1(16)	*URA3-CSS1*	this study
pCSS1(17)	*LYS2-CSS1*	this study
pCARCEN	*rDNA-ADE2-CAN1*	this study
pCACEN	*ADE2-CAN1*	this study
YEp:		
pRS426	*URA3*	Christianson *et al.* (1992)
pJH50	*LEU2-SIR1*	Ivy *et al.* (1986)
pJH423	*LEU2-SIR2*	Ivy *et al.* (1986)
pJR68	*URA3-SIR2*	J.Rine
pKAN63	*LEU2-SIR3*	Ivy *et al.* (1986)
pKAN59	*LEU2-SIR4*	Ivy *et al.* (1986)
YIp:		
pΔADE2	Δ*ade2::hisG-URA2-hisG*	O.Aparicio
pΔCAN1	Δ*can1::hisG-URA3-hisG*	H.Renauld
pCAR1	*rDNA-ADE2-CAN1*	this study
pADECAN	*ADE2-CAN1*	this study
pJI123.2	*sir1::LEU2*	Ivy *et al.* (1986)
pJH103.1	*sir2::LEU2*	Ivy *et al.* (1986)
pJH107.1	*sir3::LEU2*	Ivy *et al.* (1986)
pDM610.23	*sir4::LEU2*	Ivy *et al.* (1986)

[a]YCp, YEp and YIp denote yeast centromeric, multicopy and integrating plasmids.

Linear fragments from plasmids pJI23.2, pJH103.1, pJH107.1 and pDM610.23 (Ivy *et al.*, 1986) were used as described to disrupt the *SIR1*, *SIR2*, *SIR3* and *SIR4* genes respectively. The 2μ-based plasmids pJH50, pJH423, pKAN63 and pKAN59 (Ivy *et al.*, 1986) contained the *SIR1*, *SIR2*, *SIR3* and *SIR4* genes, respectively. These disruption and 2μ plasmids are marked with *LEU2*. The plasmid pJR68 was also used for multi-copy expression of *SIR2* (gift from J.Rine).

pCSS1(16) contains a 5.1 kb *Sal*I fragment encoding the *CSS1* gene in the *Sal*I site of the *URA3* marked centromeric vector pRS316. pCSS1(17) contains the same *CSS1*-complementing fragment as pCSS1(16) cloned into the LYS2-marked centromeric vector pRS317. pBE288 is the *Hin*dIII fragment containing the 5′ end of the *ADE2* coding region in the *Hin*dIII site of pVZ1 (Henikoff and Eghtedarzadeh, 1987).

pCH1 contains a 2458 bp *Eco*RI fragment containing the 5S rDNA gene and surrounding non-transcribed spacer sequences cloned into the *Eco*RI site of the pUC19 polylinker. pCH2 and pCH6 are derived from pCH1 and contain a 200 bp *Eco*RI–*Hin*dIII and a 250 bp *Sma*I fragment of pCH1, respectively, cloned into the polylinker of pUC19.

RNA analysis

Total yeast RNA was prepared by harvesting ~4×10^8 cells from mid-log cultures. Cell pellets were disrupted for 2.5 min on a vortex mixer in the presence of 0.4 g acid-washed glass beads, 0.2 ml YRLB (0.5 M NaCl, 0.2 M Tris, pH 7.5, 0.01 M EDTA, 1.0% SDS) and 0.3 ml phenol:chloroform. After addition of 0.3 ml phenol:chloroform and 0.3 ml YRLB, vortexing was continued for 1.5 min and phases were separated. The aqueous phase was re-extracted with 0.3 ml phenol:chloroform by vortexing for 1.5 min. The aqueous phase was precipitated in RNase-free tubes with 2 vols 100% EtOH containing 0.05% DEPC for >30 min at –20°C. RNA pellets were washed with DEPC-treated 70% EtOH, resuspended in DEPC-treated water, and stored at –80°C. Yield was judged by UV absorbance at 260 nm.

S1 nuclease protection assays were performed as described (Berk and Sharp, 1977) using 20 μg total RNA per sample and 20 000, 30 000 and 50 000 c.p.m. of *CAN1*, *ADE2* and *HIS3* probe, respectively. Samples were fractionated on 5% acrylamide (19:1 acrylamide–bisacrylamide)–8.3 M urea gels. Control experiments were performed to confirm that the S1 nuclease protection procedure can detect differences in RNA levels under the conditions used. A constant amount of labeled antisense probe was added to samples containing increasing amounts of

target RNA. Signals detected with probes to *CAN1*, *ADE2* and *HIS3* were quantitated as described below and responded linearly over a 10-fold range of input RNA encompassing the experimental 20 μg per lane, indicating sufficient probe excess for quantitative analysis.

Unexpectedly large transcripts in addition to the expected wild-type messages were detected in strains carrying the *rDNA::ADE2-CAN1* insert using probes homologous to the 5′ ends of the *CAN1* and *ADE2* genes. In contrast, both probes detected only the expected wild-type species in strains with wild-type *ADE2* and *CAN1* loci and no rDNA inserts. Subsequent Northern and S1 analyses demonstrated that the novel transcripts represent large, unpolyadenylated messages with a size expected for Pol I read-through transcripts from the nearest upstream 35S rRNA promoter. Production of the novel transcripts depended upon the presence of rDNA sequences upstream of the *ADE2-CAN1* insert since they were found in episomal constructs with an upstream rDNA unit but not in related constructs lacking rDNA sequences (unpublished data). A similar pattern of transcription was noted in another instance where markers were placed downstream of an rDNA transcription region (Stewart and Roeder, 1989).

Probes

DNA probes were gel-purified and labeled via the manufacturer's instructions with the GENIUS™ kit (Boehringer-Mannheim). The RNA probe for *CAN1* was generated by digesting p5′CAN1 with *Bsm*AI followed by *in vitro* transcription with T7 RNA polymerase in the presence of [α-^{32}P]dUTP. A 623 bp RNA probe is produced which contains 50 bp of plasmid sequence at its 5′ end, followed by 573 bp of *CAN1* sequence beginning at the *Afl*2 site in the coding region and ending at the *Bsm*AI site in the *CAN1* upstream region. The RNA probe for *ADE2* was generated by digesting pBE288 with *Dra*I and *in vitro* transcription with T3 RNA polymerase in the presence of label. A 945 bp RNA probe is produced which contains 20 bp of plasmid sequence at the 5′ end followed by 925 bp of *ADE2* spanning the *Hin*dIII site at the 5′ end of the *ADE2* coding region through the *Dra*I site in the *ADE2* upstream region. Both probes were shown to be specific by their failure to detect messages in RNA prepared from a Δ*ade2* Δ*can1* strain (CFY558, not shown). *HIS3* RNA probe has been described previously (Surosky and Esposito, 1992).

For the MNase studies, DNA probes p2, p3 and p6 were prepared directly from the rDNA inserts of plasmids pCH2, pCH3 and pCH6, respectively. *HMR**a*** and *MAT*α MNase profiles were analyzed by digesting the DNA with *Hin*dIII and hybridization with a unique DNA probe that adjoins the distal *Hin*dIII site for each locus: a *Hin*dIII–*Nru*I fragment for *MAT*α and a *Bam*HI–*Pst*I fragment for *HMR**a***. These fragments were purified by gel electrophoresis and labeled to a specific activity of 1×10^9 c.p.m./μg using the Stratagene random priming kit. In all other studies, DNA probes were labeled via the manufacturer's instructions with the GENIUS™ kit (Boehringer-Mannheim).

Image analysis and figure production

S1 gels were quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software. In other cases as indicated, autoradiograms were scanned using an Apple Scanner and AppleScan software in grayscale mode at 300 d.p.i. Scanned images were analyzed by densitometry with NIH Image v1.54. Band intensities were generated by manually identifying bands and determining the background density (BD) for each lane. The software calculated average density (AD) and area (AR) for each selected band; these were combined to yield total band density (TD) as TD = AR(AD–BD). Images of scanned autoradiograms and phosophorimagery files were imported to Adobe's Photoshop v 2.5 software. Pertinent sections of the images were selected and arranged for presentation; no image manipulations were performed and all data presented in any figure is the result of the same experimental trial. Output was generated on a Kodak 7700 dye-sublimation printer using Kodak paper.

Nuclear isolation

Nuclei were isolated following the Percoll gradient procedure of (Ide and Saunders, 1981), with some modifications in the pretreatment, spheroplasting and lysis steps according to (Huigbregtse *et al.*, 1987): 4–8 g of cells (wet weight) grown to late log or stationary phase in YPDA (2% bactodextrose, 2% bactopeptone, 1% yeast extract, 0.001% adenine sulfate) were pretreated by washing in 28 ml/g wet weight 40 mM EDTA, pH 8.5, 90 mM β-mercaptoethanol. Spheroplasting was done in 1 M sorbitol, 1 mM EDTA, pH 8.5, 3 mM DTT with 6 mg Zymolyase 20.000T/g wet weight at 30°C until 75% of the cells appeared swollen and without buds when suspended in digestion buffer (50 mM Tris, pH 7.5, 75 mM KCl, 0.5 mM $CaCl_2$, 1 mM PMSF). Spheroplasts were gently resuspended in 5 ml/g wet weight ice-cold digestion buffer. The suspension was kept on ice for 3 min to allow lysis of the spheroplasts, and the lysates were loaded on 30 ml prespun 30% Percoll (Pharmacia) gradients. Nuclei were withdrawn from the gradient, washed with 150 ml ice-cold digestion buffer and resuspended in 2 ml of the same buffer.

MNase digestion

Aliquots of 0.6 ml nuclear suspension were digested step-wise with 0 (untreated) to 18 units of micrococcal nuclease (MNase-Worthington) for 5 min at 25°C. The reactions were stopped by the addition of 1.8 ml GuHCl solution (6 M GuHCl, 133 mM EDTA, 200 mM NaCl, 0.066% Sarkosyl, pH 8). Purification of the DNA was performed according to Holm *et al.* (1986).

For the naked DNA controls, 100 μg of total yeast DNA was resuspended in 1 ml 25 mM Tris, pH 7.5, 5 mM $CaCl_2$ and digested at 25°C with 1.8 units of MNase. Aliquots of 300 μl were removed from the reaction after 3, 5 and 8 min and brought to a final concentration of 20 mM EGTA, 0.1% SDS to stop the reaction. The DNA was incubated at 65°C for 30 min with 0.1 mg/ml Proteinase K (Boehringer-Mannheim) and then extracted thoroughly with phenol/chloroform.

Southern hybridizations

For the MNase studies, digestion of DNA with restriction enzymes was carried out according to specifications of the manufacturer (Boehringer-Mannheim). Restricted DNA was run on agarose gels and blotted overnight onto nitrocellulose or Nytran nylon membranes (Schleicher & Schuell) in 20× SSC according to (Southern, 1975). Hybridizations were done at 65°C overnight in 5× SSC, 0.5% Sarkosyl, 1× Denhardt's, 0.1 mg/ml sonicated salmon sperm DNA, 10% dextran sulfate. Membranes were washed for 2×10 min in 2× SSC, 0.1% SDS at room temperature and subsequently, when necessary, in 0.2× SSC, 0.1% SDS at 55°C for 30 min. Membranes were exposed to Kodak XAR films overnight at room temperature.

For the analysis of GATC methylation, genomic DNA was prepared from 5 ml logarithmic cultures by the method of Hoffman and Winston (1987) with the addition of a second organic extraction after RNase treatment. Approximately 10 μg DNA was digested overnight in 100 μl with a minimum of 6 units enzyme. Digests were precipitated and run on 1% agarose gels. Transfer to Magna nylon membranes (Micron Separations) was accomplished by the method of Kempter *et al.* (1991) with transfers of 45 min to 1.5 h. Filters were incubated to dryness at 80°C. Hybridization was carried out in roller bottles at 65°C in a solution of 5× SSC, 0.5% blocking reagent (Boehringer-Mannheim), 0.1% sarcosyl and 0.02% SDS for a minimum of 4 h. Filters were subsequently washed twice for 15 min at 65°C with 0.1% SDS, 0.5× SSC. Development by chemiluminescent detection proceeded as per instructions in the Boehringer-Mannheim GENIUS™ kit, with the modification that Wash #2 contains 2% blocking reagent. When needed, blots were stripped for re-probing by incubation in 0.2 M NaOH, 0.1% SDS at 37°C for 20 min.

Acknowledgements

The authors would like to thank J.Ivy, J.Rine, O.Aparicio, H.Renauld and D.Gottschling for the gift of materials used in this study and S.Gasser for her critical comments on the manuscript. This work was supported by an NRSA training grant to C.F. (GM07183) and grant NIH-G29182 to R.E.E.

References

Aguilera,A. and Klein,H.L. (1990) HPR1, a novel yeast gene that prevents intrachromosomal excision recombination, shows carboxy-terminal homology to the *Saccharomyces cerevisiae* TOP1 gene. *Mol. Cell. Biol.*, **10**, 1439–1451.

Alani,E., Cao,L. and Kleckner,N. (1987) A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. *Genetics*, **116**, 541–545.

Aparicio,O.M. and Gottschling,D.E. (1994) Overcoming telomeric silencing: a *trans*-activator competes to establish gene expression in a cell cycle-dependent way. *Genes Dev.*, **8**, 1133–1146.

Aparicio,O.M., Billington,B.L. and Gottschling,D.E. (1991) Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. *Cell*, **66**, 1279–1287.

Berk,A.J. and Sharp,P.A. (1977) Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell*, **12**, 721–732.

Bonven,B.J., Gocke,E. and Westergaard,O. (1985) A high affinity Topoisomerase I binding sequence is clustered at DNase I hypersensitive sites in *Tetrahymena* R chromatin. *Cell*, **41**, 541–551.

Brachmann,C.B., Sherman,J.M., Devine,S.E., Cameron,E.E., Pillus,L. and Boeke,J.D. (1995) The SIR2 gene family, conserved from bacteria to humans, functions in silencing cell cycle progression, and chromosome stability. *Genes Dev.*, **9**, 2888–2902.

Brand,A.H., Breeden,L., Abraham,J., Sternglanz,R. and Nasmyth,K. (1985) Characterization of a 'silencer' in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. *Cell*, **41**, 41–48.

Brand,A.H., Micklem,G. and Nasmyth,K.A. (1987) A yeast silencer contains sequences that can promote autonomous plasmid replication and transcriptional activation. *Cell*, **51**, 709–719.

Braunstein,M., Rose,A.B., Holmes,S.G., Allis,C.D. and Broach,J.R. (1993) Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev.*, **7**, 592–604.

Brewer,B.J. and Fangman,W.L. (1988) A replication fork barrier at the 3′ end of yeast ribosomal RNA genes. *Cell*, **55**, 637–643.

Bryk,M., Banerjee,M., Murphy,M., Knudsen,K.E., Garfinkel,D.J. and Curcio,M.J. (1997) Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast. *Genes Dev.*, **11**, 255–269.

Buchman,A.R., Kimmerly,W.J., Rine,J. and Kornberg,R.D. (1988) Two DNA-binding factors recognize specific sequences at silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **8**, 210–225.

Chien,C.T., Buck,S., Sternglanz,R. and Shore,D. (1993) Targeting of SIR1 protein establishes transcriptional silencing at HM loci and telomeres in yeast. *Cell*, **75**, 531–541.

Christianson,T.W., Sikorski,R.S., Dante,M., Shero,J.H. and Hieter,P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene*, **110**, 119–122.

Christman,M.F., Dietrich,F.S. and Fink,G.R. (1988) Mitotic recombination in the rDNA of *S. cerevisiae* is suppressed by the combined action of DNA topoisomerases I and II. *Cell*, **55**, 413–425.

Dammann,R., Lucchini,R., Koller,T. and Sogo,J.M. (1993) Chromatin structures and transcription of rDNA in yeast *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **21**, 2331–2338.

Derbyshire,M.K., Weinstock,K.G. and Strathern,J.N. (1996) HST1, a new member of the SIR2 family of genes. *Yeast*, **12**, 631–640.

Diffley,J.F. and Stillman,B. (1988) Purification of a yeast protein that binds to origins of DNA replication and a transcriptional silencer. *Proc. Natl Acad. Sci. USA*, **85**, 2120–2124.

Dower,W.J., Miller,J.F. and Ragsdale,C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.*, **16**, 6127–6145.

Ferguson,B.M. and Fangman,W.L. (1992) A position effect on the time of replication origin activation in yeast. *Cell*, **68**, 333–339.

Fritze,C.E. (1994) Control of recombination and gene expression in the rDNA tandem repeated array of *S. cerevisiae*. PhD Thesis, The University of Chicago, Chicago, IL, USA.

Gangloff,S., McDonald,J.P., Bendixen,C., Arthur,L. and Rothstein,R. (1994) The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog: a potential eukaryotic reverse gyrase. *Mol. Cell. Biol.*, **14**, 8391–8398.

Gangloff,S., Zou,H. and Rothstein,R. (1996) Gene conversion plays the major role in controlling the stability of large tandem repeats in yeast. *EMBO J.*, **15**, 1715–1725.

Gotta,M., Strahl-Bolsinger,S., Renauld,H., Laroche,T., Kennedy,B.K., Grunstein,M. and Gasser,S.M. (1997) Localization of Sir2p: the nucleolus as a compartment for silent information regulators. *EMBO J.*, **16**, 3243–3255.

Gottlieb,S. (1989) Genetic control of recombination between repeated genes in the yeast *Saccharomyces cerevisiae*. PhD Thesis, University of Chicago, IL, USA.

Gottlieb,S. and Esposito,R.E. (1989) A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA. *Cell*, **56**, 771–776.

Gottlieb,S., Wagstaff,J. and Easton Esposito,R. (1989) Evidence for two pathways of meiotic intrachromosomal recombination in yeast. *Proc. Natl Acad. Sci. USA*, **86**, 7072–7076.

Gottschling,D.E. (1992) Telomere-proximal DNA in *Saccharomyces cerevisiae* is refractory to methyltransferase activity *in vivo*. *Proc. Natl Acad. Sci. USA*, **89**, 4062–4065.

Gottschling,D.E., Aparicio,O.M., Billington,B.L. and Zakian,V.A. (1990) Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. *Cell*, **63**, 751–762.

Grenson,M., Mousset,M., Wiame,J.M. and Bechet,J. (1966) Multiplicity of amino acid permeases in *Saccharomyces cerevisiae* IV: evidence for a specific arginine transporting system. *Biochim. Biophys. Acta*, **127**, 325–338.

Haber,J.E. and George,J.P. (1979) A mutation that permits expression of normally silent copies of mating-type information in *Saccharomyces cerevisiae*. *Genetics*, **93**, 13–35.

Hecht,A., Laroche,T., Strahl,B.S., Gasser,S.M. and Grunstein,M. (1995) Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. *Cell*, **80**, 583–592.

Hecht,A., Strahl,B.S. and Grunstein,M. (1996) Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. *Nature*, **383**, 92–96.

Henikoff,S. and Eghtedarzadeh,M.K. (1987) Conserved arrangement of nested genes at the *Drosophila* Gart locus. *Genetics*, **117**, 711–725.

Herskowitz,I. (1990) A regulatory hierarchy for cell specialization in yeast. *Nature*, **286**, 854–857.

Hoffman,C.S. and Winston,F. (1987) A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene*, **57**, 267–272.

Hoffmann,W. (1985) Molecular characterization of the CAN1 locus in *Saccharomyces cerevisiae*. A transmembrane protein without N-terminal hydrophobic signal sequence. *J. Biol. Chem.*, **260**, 11831–11837.

Holm,C., Meeks-Wagner,D., Fangman,W. and Botstein,D. (1986) A rapid efficient method for isolating DNA from yeast. *Gene*, **42**, 169–173.

Huang,G.S. and Keil,R.L. (1995) Requirements for activity of the yeast mitotic recombination hotspot HOT1: RNA polymerase I and multiple *cis*-acting sequences. *Genetics*, **141**, 845–855.

Huigbregtse,J.M., Evans,C.F. and Engelke,D.R. (1987) Comparison of tRNA gene transcription complexes formed *in vitro* and in nuclei. *Mol. Cell. Biol.*, **7**, 3212–3220.

Ide,G. and Saunders,C. (1981) Isolation of nuclei from *Saccharomyces cerevisiae*. *Curr. Genet.*, **4**, 85–90.

Ivy,J.M., Klar,A.J. and Hicks,J.B. (1986) Cloning and characterization of four SIR genes of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **6**, 688–702.

Kayne,P.S., Kim,V.J., Han,M., Mullen,J.R., Yoshizaki,F. and Grunstein,M. (1988) Extremely conserved histone H4 N terminus is dispensable for growth but is essential for repressing the silent mating loci in yeast. *Cell*, **55**, 27–39.

Keil,R.L. and Lin,Y.-H. (1991) Mutations affecting RNA polymerase I-stimulated exchange and rDNA recombination in yeast. *Genetics*, **127**, 31–38.

Keil,R.L. and McWilliams,A.D. (1993) A gene with specific and global effects on recombination of sequences from tandemly repeated genes in *Saccharomyces cerevisiae*. *Genetics*, **135**, 711–718.

Keil,R.L. and Roeder,G.S. (1984) *Cis*-acting, recombination-stimulating activity in a fragment of the ribosomal DNA of *S. cerevisiae*. *Cell*, **39**, 377–386.

Kempter,B., Luppa,P. and Neumeier,D. (1991) A short procedure for Southern blotting on neutral and anionic membranes. *Trends Genet.*, **7**, 109–110.

Kennedy,B.K., Gotta,M., Sinclair,D.A., Mills,K., McNabb,D.S., Murthy,M., Pak,S.M., Laroche,T., Gasser,S.M. and Guarente,L. (1997) Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in *S.cerevisiae*. *Cell*, **89**, 381–391.

Klar,A.J.S., Fogel,S. and MacLeod,K. (1979) *MAR1* – a regulator of HM**a** and HMα loci in *Saccharomyces cerevisiae*. *Genetics*, **93**, 37–50.

Laurenson,P. and Rine,J. (1992) Silencers, silencing, and heritable transcriptional states. (Review). *Microbiol. Rev.*, **56**, 543–560.

Lewin,B.E. (1990) *Genes IV.* Cell Press, Cambridge MA.

Lin,Y.H. and Keil,R.L. (1991) Mutations affecting RNA polymerase I-stimulated exchange and rDNA recombination in yeast. *Genetics*, **127**, 31–38.

Linskens,M.H. and Huberman,J.A. (1988) Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **8**, 4927–4935.

Lucchini,R. and Sogo,J.M. (1994) Chromatin structure and transcriptional activity around the replication forks arrested at the 3′ end of the yeast rRNA genes. *Mol. Cell. Biol.*, **14**, 318–326.

Mahoney,D.J., Marquardt,R., Shei,G.J., Rose,A.B. and Broach,J.R. (1991) Mutations in the HML E silencer of *Saccharomyces cerevisiae* yield metastable inheritance of transcriptional repression. *Genes Dev.*, **5**, 605–615.

McNally,F.J. and Rine,J. (1991) A synthetic silencer mediates SIR-dependent functions in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **11**, 5648–5659.

Miller,A.M., Sternglanz,R. and Nasmyth,K.A. (1984) The role of DNA replication in the repression of the yeast mating-type silent loci. *Cold Spring Harbor Symp. Quant. Biol.*, **49**, 105–113.

Moretti,P., Freeman,K., Coodly,L. and Shore,D. (1994) Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. *Genes Dev.*, **8**, 2257–2269.

Nasmyth,K. (1982) The regulation of yeast mating type chromatin structure by *SIR*: an action at a distance affecting both transcription and transposition. *Cell*, **30**, 567–578.

Ohta,K., Shibata,T. and Nicolas,A. (1994) Changes in chromatin structure at recombination initiation sites during yeast meiosis. *EMBO J.*, **13**, 5754–5763.

Ozenberger,B.A. and Roeder,G.S. (1991) A unique pathway of double-strand break repair operates in tandemly repeated genes. *Mol. Cell. Biol.*, **11**, 1222–1231.

Petes,T.D. (1979) Yeast ribosomal DNA genes are located on chromosome XII. *Proc. Natl Acad. Sci. USA*, **76**, 410–414.

Petes,T.D. and Botstein,D. (1977) Simple Mendelian inheritance of the reiterated ribosomal DNA of yeast. *Proc. Natl Acad. Sci. USA*, **74**, 5091–5095.

Pillus,L. and Rine,J. (1989) Epigenetic inheritance of transcriptional states in *S. cerevisiae*. *Cell*, **59**, 637–647.

Planta,R.J. and Raue,H.A. (1988) The control of ribosome biogenesis. *Trends Genet. Sci.*, **4**, 64–68.

Renauld,H., Aparicio,O.M., Zierath,P.D., Billington,B.L., Chhablani,S.K. and Gottschling,D.E. (1993) Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. *Genes Dev.*, **7**, 1133–1145.

Rine,J. and Herskowitz,I. (1987) Four genes responsible for a position effect on expression from HML and HMR in *Saccharomyces cerevisiae*. *Genetics*, **116**, 9–22.

Roman,H. (1956) Studies of gene mutation in *Saccharomyces*. *Cold Spring Harbor Symp. Quant. Biol.*, **21**, 175–185.

Saffer,L.D. and Miller,O.J. (1986) Electron microscopic study of *Saccharomyces cerevisiae* rDNA chromatin replication. *Mol. Cell. Biol.*, **6**, 1148–1157.

Schiestl,R.H. and Gietz,R.D. (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.*, **16**, 339–346.

Sherman,F., Fink,G.R. and Lawrence,C.W. (1974) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shore,D., Stillman,D., Brand,A. and Nasmyth,K. (1987) Identification of silencer binding proteins from yeast: possible roles in *SIR* control and DNA replication. *EMBO J.*, **6**, 461–467.

Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.

Singh,J. and Klar,A.J. (1992) Active genes in budding yeast display enhanced *in vivo* accessibility to foreign DNA methylases: a novel *in vivo* probe for chromatin structure of yeast. *Genes Dev.*, **6**, 186–196.

Skryabin,K.G. *et al*. (1984) Structure and function of the non transcribed spacer regions of yeast rDNA. *Nucleic Acids Res.*, **12**, 2955–2968.

Smith,J.S. and Boeke,J.D. (1997) An unusual form of transcriptional silencing in yeast ribosomal DNA. *Genes Dev.*, **11**, 241–254.

Southern,E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, **98**, 503–517.

Stewart,S.E. and Roeder,G.S. (1989) Transcription by RNA polymerase I stimulates mitotic recombination in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **9**, 3464–3472.

Stone,E.M., Swanson,M.J., Romeo,A.M., Hicks,J.B. and Sternglanz,R. (1991) The *SIR1* gene of *Saccharomyces cerevisiae* and its role as an extragenic suppressor of several mating-defective mutants. *Mol. Cell. Biol.*, **11**, 2253–2262.

Strahl,B.S., Hecht,A., Luo,K. and Grunstein,M. (1997) SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. *Genes Dev.*, **11**, 83–93.

Surosky,R.T. and Esposito,R.E. (1992) Early meiotic transcripts are highly unstable in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **12**, 3948–3958.

Sussel,L. and Shore,D. (1991) Separation of transcriptional activation and silencing functions of the RAP1-encoded repressor/activator protein 1: isolation of viable mutants affecting both silencing and telomere length. *Proc. Natl Acad. Sci. USA*, **88**, 7749–7753.

Sussel,L., Vannier,D. and Shore,D. (1993) Epigenetic switching of transcriptional states: cis- and trans-acting factors affecting establishment of silencing at the HMR locus in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **13**, 3919–3928.

Thompson,J.S., Johnson,L.M. and Grunstein,M. (1994a) Specific repression of the yeast silent mating locus HMR by an adjacent telomere. *Mol. Cell. Biol.*, **14**, 446–455.

Thompson,J.S., Ling,X. and Grunstein,M. (1994b) Histone H3 amino terminus is required for telomeric and silent mating locus repression in yeast. *Nature*, **369**, 245–247.

Voelkel-Meiman,K., Keil,R.L. and Roeder,S.G. (1987) Recombination stimulating sequences in yeast ribosomal DNA correspond to sequences regulating transcription by RNA polymerase I. *Cell*, **48**, 1071–1079.

Wallis,J.W., Ghrebet,G., Brodsky,G., Rolfe,M. and Rothstein,R. (1989) A hyper-recombination mutation in *S. cerevisiae* identifies a novel eukaryotic topoisomerase. *Cell*, **58**, 409–419.

Walmsley,R.M., Johnston,L.H., Williamson,D.H. and Oliver,S.G. (1984) Replicon size of yeast ribosomal DNA. *Mol. Gen. Genet.*, **195**, 260–266.

Woodcock,D.M., Crowther,P.J., Doherty,J., Jefferson,S., DeCruz,E., Noyer,W.M., Smith,S.S., Michael,M.Z. and Graham,M.W. (1989) Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res.*, **17**, 3469–3478.

Wu,C. (1980) The 5′ ends of *Drosophila* heat shock genes in chromatin are hypersensitive to DNase I. *Nature*, **286**, 854–860.

Wu,T.C. and Lichten,M. (1994) Meiosis-induced double-strand break sites determined by yeast chromatin structure. *Science*, **263**, 515–518.

Yanisch,P.C., Vieira,J. and Messing,J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, **33**, 103–119.

Zamb,T.J. and Petes,T.D. (1982) Analysis of the junction between ribosomal RNA genes and single copy chromosomal sequences in the yeast *Saccharomyces cerevisiae*. *Cell*, **28**, 355–364.

Received on May 20, 1997; revised on August 14, 1997