Distinct Roles for the Essential MYST Family HAT Esa1p in Transcriptional Silencing[□]

Astrid S. Clarke,^{*†} Eva Samal,^{‡§} and Lorraine Pillus^{*}

^{*}Division of Biological Sciences, UCSD Cancer Center and Center for Molecular Genetics and [‡]Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093

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Among acetyltransferases, the MYST family enzyme Esa1p is distinguished for its essential function and contribution to transcriptional activation and DNA double-stranded break repair. Here we report that Esa1p also plays a key role in silencing RNA polymerase II (Pol II)-transcribed genes at telomeres and within the ribosomal DNA (rDNA) of the nucleolus. These effects are mediated through Esa1p's HAT activity and correlate with changes within the nucleolus. Esa1p is enriched within the rDNA, as is the NAD-dependent protein deacetylase Sir2p, and the acetylation levels of key Esa1p histone targets are reduced in the rDNA in *esa1* mutants. Although mutants of both *ESA1* and *SIR2* have enhanced rates of rDNA recombination, *esa1* effects are more modest yet result in distinct structural changes of rDNA chromatin. Surprisingly, increased expression of *ESA1* can bypass the requirement for Sir2p in rDNA silencing, suggesting that these two enzymes with seemingly opposing activities both contribute to achieve optimal nucleolar chromatin structure and function.

INTRODUCTION

Histone modifications such as phosphorylation, methylation, acetylation, and deacetylation provide a mechanism by which chromatin structure is modulated to affect gene expression both positively and negatively (for reviews see Strahl and Allis, 2000; Iizuka and Smith, 2003; Kurdistani and Grunstein, 2003). The acetylation of lysine residues on histone N-terminal tails is classically linked to increases in gene expression and more directly to roles in transcriptional activation. By contrast, deacetylation of histones is most frequently correlated with transcriptionally silent chromatin. As more is learned about the enzymes catalyzing these modifications, the histone acetyltransferases (HATs) and histone deacetylases (HDACs), simple functional distinctions between acetylation and deacetylation of histones do not hold (Iizuka and Smith, 2003). For example, in several organisms, mutations in *RPD3*, a gene encoding a deacetylase, lead to increases in silencing rather than the expected decreases (DeRubertis *et al.*, 1996; Rundlett *et al.*, 1996). This brings forward the possibility that histone acetylation may play an important part in both silent and active chromatin. In addition, coactivator proteins, such as the histone acetyltransferases p300 and CBP and nuclear hormone receptors, appear to have roles in transcriptional repression through acetylation and association with particular binding partners

(for examples, see Chen and Li, 1998; Waltzer and Bienz, 1998; Baluchamy *et al.*, 2003; Girdwood *et al.*, 2003; Rajabi *et al.*, 2005). Thus, understanding of multiple roles for acetylation in controlling gene expression is expanding.

In *Saccharomyces cerevisiae*, there are three regions of the genome controlled by chromatin-mediated transcriptional silencing: telomeres, the silent mating-type loci *HMR* and *HML*, and the rDNA array. Many factors are important for transcriptional silencing at subsets of these loci including the four core histones, the repressor activator protein Rap1p, the Silent Information Regulator proteins, Sir1-4p, and other proteins with chromatin modifying activities (reviewed in Rusche *et al.*, 2003; Fox and McConnell, 2005). Sir2p, an NAD-dependent histone deacetylase (Imai *et al.*, 2000; Landry *et al.*, 2000; Smith *et al.*, 2000), is unique among the Sir proteins because it is important for silencing at all three loci (reviewed in Gottschling, 2000; Guarente, 2000; Shore, 2000; Moazed, 2001). Sir2p is part of the RENT complex and plays a role in rDNA silencing and rDNA chromatin structure that is mediated through its deacetylase activity (Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith and Boeke, 1997; Shou *et al.*, 1999; Straight *et al.*, 1999; Ghidelli *et al.*, 2001). Sir2p also plays an important role in suppressing homologous recombination within the repetitive rDNA array, highlighting its contribution to chromatin structure at this locus (Gottlieb and Esposito, 1989). Studies of chromatin modification at silenced loci in yeast have been critical for the emerging understanding of how changes in chromatin structure lead to changes in gene expression.

Esa1p is an essential HAT in *S. cerevisiae* (Smith *et al.*, 1998a; Clarke *et al.*, 1999) that is a member of the MYST family that is distantly related to the GNAT family of HATs (reviewed in Neuwald and Landsman, 1997; Utley and Côté, 2003). There are two other nonessential MYST proteins in yeast, Sas2p and Sas3p. Sas2p is necessary for silencing at telomeres and together with Sir1p plays a role in silencing at the silent mating-type loci (Reifsnnyder *et al.*, 1996; Ehrenhofer-Murray *et al.*, 1997), and it has been proposed that Sas2p and Sir2p act in opposition to one another (Kimura *et*

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Present addresses: [†] Whitehead Institute, Massachusetts Institute of Technology, 9 Cambridge Center, Cambridge, MA 02142; [§] Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, Room 430B, 1650 Owens Street, San Francisco, CA 94158.

Address correspondence to: Lorraine Pillus (lpillus@ucsd.edu).

al., 2002; Suka *et al.*, 2002). Sas3p is the catalytic HAT component of the NuA3 complex, with roles in transcriptional activation through acetylation of histone H3 (Takechi and Nakayama, 1999; John *et al.*, 2000).

Esa1p has a range of cellular functions distinct from those of other HATs. It primarily acetylates histone H4 and, to a limited extent H2A and H3, and is required for cell cycle progression (Smith *et al.*, 1998a; Clarke *et al.*, 1999). Several *esa1* temperature-sensitive mutants display a conditional G2/M arrest, which is dependent upon the RAD9 DNA damage checkpoint, and these mutants fail to segregate their chromosomes properly. Under restrictive conditions, nucleolar structure is severely disrupted in *esa1* mutants as revealed by electron microscopy and the overall level of histone H4 acetylation is reduced in the *esa1* temperature-sensitive mutants under permissive and restrictive conditions (Clarke *et al.*, 1999). Esa1p is the catalytic component of the 1.4-MDa NuA4 HAT complex that is important for transcriptional activation both in vitro and in vivo at specific target loci, including many ribosomal protein genes (reviewed in Doyon and Côté, 2004). Esa1p also functions in the highly active, smaller picNuA4 complex (Boudreault *et al.*, 2003).

The NuA4 complex itself is broadly conserved (Doyon and Côté, 2004) and the human catalytic subunit Tip60 functions in nontranscriptional components of apoptosis and signaling processes (Ikura *et al.*, 2000; Sheridan *et al.*, 2001). Tip60 has been linked to DNA repair (Ikura *et al.*, 2000) and a role for Esa1p and the Yng2p NuA4 subunit was found in replication-coupled DNA double-stranded break repair (Bird *et al.*, 2002; Choy and Kron, 2002). Indeed, several subunits of NuA4 are shared with the Swr1 chromatin remodeling complex and are linked at additional levels to chromosomal stability (Kobor *et al.*, 2004; Krogan *et al.*, 2004; Mizuguchi *et al.*, 2004).

Because Esa1p regulates global levels of acetylation in yeast and these modifications are not restricted to promoter-proximal regions (Clarke *et al.*, 1999; Vogelauer *et al.*, 2000), we considered that Esa1p may have additional roles in genomic regulation. We found that Esa1p contributes to locus-specific transcriptional silencing and appears to have a prominent role within the rDNA of the nucleolus. In particular, Esa1p is associated with the rDNA and affects its acetylation status. Our studies reveal a surprising connection between Esa1p and the NAD-dependent deacetylase Sir2p that also functions within the nucleolus. Sir2p is itself required for rDNA silencing and in all previous studies has been considered essential for this process. Yet, we find that modest increases in gene dosage of *ESA1* can bypass the rDNA silencing defects of *sir2* null mutants. This effect is reciprocal, although restricted to partially functional *esa1* mutants. Together, these results demonstrate an increasingly universal role for Esa1p in genomic structure and function. In addition, these results suggest that seemingly opposing chromatin-modifying activities can both contribute to achieve optimal function.

MATERIALS AND METHODS

Structural Image Production

The Esa1p structural representation was generated using the RasMol program with Brookhaven file 1FY7 with the generous help of Dr. R. Dutnall.

Strain Construction and Genetic Methods

Strains used are listed in Table 1 and plasmids are listed in Table 2. The W303 *esa1* mutant strains were generated by digesting pLP952 (*esa1-414/URA3*/integrating) with BstEII and digesting pLP949 (*esa1-L254P/URA3*/integrating) with SphI (New England Biolabs, Beverly, MA) and transforming these linearized plasmids into LPY5. These URA⁺ transformants were cultured on

5-FOA plates to identify cells that had excised the *URA3* marker and either the *ESA1* wild-type locus or the *esa1* mutant locus. The 5-FOA-resistant, ura⁻ colonies were tested for temperature sensitivity at 37°C to identify candidates in which the *ESA1* locus had been excised. Strains LPY4679 and LPY4680 (*esa1-414*) and LPY5001 (*esa1-L254P*) were confirmed as such integrants. Subsequently, standard genetic analysis and molecular amplification were utilized to test for appropriate integration of the *esa1* mutant alleles at the *ESA1* locus. Molecular amplification was carried out with Taq polymerase (Promega, Madison, WI) following manufacturer instructions with primers **ESAWAYUP** 5'-CTCACGTAACAGTTGGTG3' and **TAS1PST** 5'-TGCACTG-CACCGTGTGATATGTTCTCATC3'.

To generate strains marked at the three silenced loci in the W303 background, strains LPY4679, LPY4680, and LPY5001 were crossed with CCF101 (rDNA::ADE2-CAN1, TELVR::URA3, *hmrΔRap1::TRP1*), generating the diploids LPY4801, LPY4804, and LPY5099, respectively. LPY4801 was sporulated and dissected to yield strains LPY4819, LPY4821, LPY4909, LPY4911, LPY4913, and LPY4915. LPY4804 was sporulated and dissected to generate LPY4848, LPY5099 was sporulated and dissected to generate the strains LPY5055, LPY5059, and LPY5066. To generate *sir2Δ::HIS3* mutant combinations with the three reporter genes, LPY4821 (*esa1-414* triple-marked) was crossed with LPY11 (*sir2Δ::HIS3*) transformed with pLP1237 (*SIR2/LEU2/CEN*) to yield the diploid LPY4906. LPY4906 was sporulated and dissected to generate the strains LPY4978 and LPY4980. All *sir2Δ* strains generated no longer contain pLP1237.

Quantitative Mating and Silencing Assays

The *MATα* strains LPY3498 (*ESA1*), LPY3500 (*esa1-L254P*), LPY2991 (*ESA1*), and LPY3291 (*esa1-414*) were grown in YPD. Cultures in log phase were diluted 1:5000 in YPD, and 100 μl of each dilution was either plated on SC or mixed with 100 μl of a freshly growing *MATα* mating tester strain (LPY78) and spread onto minimal plates. Samples were plated in triplicate for each medium at 28°C. The ratio of the average number of colonies on the three minimal plates to the average number of total colonies on the three SC plates defines the mating efficiency of that culture. The wild-type mating efficiency was set to 1.00 and mutant mating efficiencies were normalized to the appropriate wild type. Mating efficiencies of all strains were measured in two independent experiments and are reported as their average.

Assays measuring telomeric silencing were carried out by incubating 2-ml YPD cultures for 4–5 d at 28°C and then plating the cultures in fivefold serial dilutions on synthetic complete medium (SC) and SC containing 5-FOA at 1 g/l. For transformants with *TRP1* plasmids, tryptophan was left out of the medium to select for the plasmids. Plates were incubated at the indicated temperatures for 2–4 d, depending on media and temperature. Assays measuring *HMR* silencing were carried out by incubating 2-ml YPD cultures for 2–4 d at 28°C and plating these cultures on SC or SC without tryptophan at the indicated temperatures for 2–4 d, depending on the temperature.

Assays measuring rDNA silencing were carried out by incubating 2-ml cultures in liquid SC without adenine and arginine for 2–4 d at 28°C. For strains that were transformed with plasmids, cultures were grown in SC without adenine, arginine, and either tryptophan or uracil to select for the plasmids. Cultures were plated in fivefold serial dilutions on SC, SC without adenine, SC without adenine and arginine, and SC without adenine and arginine (except strains with plasmids, as above) but containing canavanine (Sigma, St. Louis, MO) at concentrations ranging from 8 to 60 μg/ml. Strains were tested for general canavanine sensitivity on SC containing 60 μg/ml canavanine without arginine.

Recombination Assays

Recombination assays were carried out by incubating cultures in medium lacking adenine and arginine to select for the *ADE2* reporter gene in the rDNA array during logarithmic growth for several cell cycles (4–6 h). Cultures of different isolates were serially diluted 10-fold and then spread to 10 YPD plates with 500 cells each. Cell concentrations were estimated using the conversion of 2.0×10^7 cells per ml yields an A_{600} of 1.0. Plates were incubated at 28 or 33°C for 2 d, and colonies were counted. Approximately 3000–4000 total colonies for each isolate and experiment were counted. Plates were incubated at 4°C to allow red color development that occurs when cells become ade⁻ due to loss of the reporter cassette. The number of half-sectorized colonies was divided by the total number of colonies plated to measure the rate of recombination. This ratio provides the frequency of cells in a given population that had undergone a recombination event during the first cell division. Averages of 3–4 independent isolates for each temperature and strain are provided with standard deviations. Statistical analysis of these data (Supplementary Table S1) was performed using the Wilcoxon test (Wonnacott and Wonnacott, 1984) with the assistance of Dr. Vincent Carey (Harvard Medical School and Whitehead Institute).

Chromatin Immunoprecipitation

Immunoprecipitation of formaldehyde cross-linked chromatin was performed as described previously by Strahl-Bolsinger *et al.* (1997). Briefly, a 100-ml culture was grown to $A_{600} = 1.0$ and fixed with 0.86% formaldehyde for 30 min at room temperature. Harvested cells were washed twice with tris-buffered saline (20 mM Tris-HCl, pH 7.5, 200 mM NaCl) and lysed in 1.6 ml FA-lysis buffer (50 mM

Table 1. Strains used in this study

Strain	Genotype	Source
LPY5	<i>MATa</i>	This lab
LPY11	<i>MATa sir2Δ::HIS3</i>	This lab
AMR53	<i>MATa top1-8::LEU2</i>	R. Sternglanz
LPY78	<i>MATα his4</i>	This lab
LPY2991	<i>MATa his3Δ200 leu2-3,112 trp1Δ1 ura3-52 esa1Δ::HIS3 + pLP795</i>	This lab
LPY3291	<i>MATa his3Δ200 leu2-3,112 trp1Δ1 ura3-52 esa1Δ::HIS3 + pLP863</i>	This lab
LPY3498	<i>MATa his3Δ200 leu2-3,112 trp1Δ1 ura3-52</i>	This lab
LPY3500	<i>MATa his3Δ200 leu2-3,112 trp1Δ1 ura3-52 esa1Δ::HIS3 esa1-L254P::URA3</i>	This lab
CCF101	<i>MATα hmr::TRP1 rDNA::ADE2-CAN1 TELVR::URA3</i>	K. Runge
LPY4679	<i>MATa esa1-414</i>	This study
LPY4680	<i>MATa esa1-414</i>	This study
LPY4801	<i>MATa/α +/esa1-414 +/hmr::TRP1 +/rDNA::ADE2-CAN1 +/TELVR::URA3</i>	This study
LPY4804	<i>MATa/α +/esa1-414 +/hmr::TRP1 +/rDNA::ADE2-CAN1 +/TELVR::URA3</i>	This study
LPY4819	<i>MATα hmr::TRP1 rDNA::ADE2-CAN1 TELVR::URA3</i>	This study
LPY4821	<i>MATα esa1-414 hmr::TRP1 rDNA::ADE2-CAN1 TELVR::URA3</i>	This study
LPY4848	<i>MATα esa1-414 hmr::TRP1 rDNA::ADE2-CAN1 TELVR::URA3</i>	This study
LPY4906	<i>MATa/α +/esa1-414 +/sir2Δ::HIS3 +/hmr::TRP1 +/rDNA::ADE2-CAN1 +/TELVR::URA3</i>	This study
LPY4909	<i>MATα rDNA::ADE2-CAN1</i>	This study
LPY4911	<i>MATα esa1-414 rDNA::ADE2-CAN1</i>	This study
LPY4913	<i>MATα hmr::TRP1</i>	This study
LPY4915	<i>MATα esa1-414 hmr::TRP1</i>	This study
LPY4917	<i>MATα TELVR::URA3</i>	This study
LPY4919	<i>MATα esa1-414 TELVR::URA3</i>	This study
LPY4978	<i>MATα sir2Δ::HIS3 rDNA::ADE2-CAN1</i>	This study
LPY4980	<i>MATα sir2Δ::HIS3 hmr::TRP1</i>	This study
LPY5001	<i>MATa esa1-L254P</i>	This study
LPY5017	<i>MATa sir2Δ::TRP1 rDNA::ADE2-CAN1 TELVR::URA3</i>	This study
LPY5055	<i>MATα esa1-L254P rDNA::ADE2-CAN1</i>	This study
LPY5059	<i>MATα esa1-L254P hmr::TRP1</i>	This study
LPY5066	<i>MATα esa1-L254P hmr::TRP1 rDNA::ADE2-CAN1 TELVR::URA3</i>	This study
LPY5099	<i>MATa/α +/esa1-L254P +/hmr::TRP1 +/rDNA::ADE2-CAN1 +/TELVR::URA3</i>	This study
LPY5136	<i>MATα esa1-414 rDNA::ADE2-CAN1 + pLP1402</i>	This study
LPY5141	<i>MATα esa1-414 rDNA::ADE2-CAN1 + pLP796</i>	This study
LPY5143	<i>MATα esa1-414 rDNA::ADE2-CAN1 + pLP37</i>	This study
LPY5144	<i>MATα esa1-L254P rDNA::ADE2-CAN1 + pLP1402</i>	This study
LPY5150	<i>MATα esa1-L254P rDNA::ADE2-CAN1 + pLP796</i>	This study
LPY5151	<i>MATα esa1-L254P rDNA::ADE2-CAN1 + pLP37</i>	This study
LPY5159	<i>MATα sir2Δ::HIS3 rDNA::ADE2-CAN1 + pLP1402</i>	This study
LPY5164	<i>MATα sir2Δ::HIS3 rDNA::ADE2-CAN1 + pLP796</i>	This study
LPY5166	<i>MATα sir2Δ::HIS3 rDNA::ADE2-CAN1 + pLP37</i>	This study
LPY6181	<i>MATα TELVR::URA3 + pLP1347</i>	This study
LPY6182	<i>MATα TELVR::URA3 + pLP1348</i>	This study
LPY6184-7	<i>MATα TELVR::URA3 + pLP1349</i>	This study
LPY6189	<i>MATα esa1-414 TELVR::URA3 + pLP1347</i>	This study
LPY6190	<i>MATα esa1-414 TELVR::URA3 + pLP1348</i>	This study
LPY6192-5	<i>MATα esa1-414 TELVR::URA3 + pLP1349</i>	This study
LPY6217	<i>MATα rDNA::ADE2-CAN1 + pLP1347</i>	This study
LPY6219	<i>MATα rDNA::ADE2-CAN1 + pLP1348</i>	This study
LPY6220-3	<i>MATα rDNA::ADE2-CAN1 + pLP1349</i>	This study
LPY6225	<i>MATα esa1-414 rDNA::ADE2-CAN1 + pLP1347</i>	This study
LPY6227	<i>MATα esa1-414 rDNA::ADE2-CAN1 + pLP1348</i>	This study
LPY6228-31	<i>MATα esa1-414 rDNA::ADE2-CAN1 + pLP1349</i>	This study
LPY6244	LPY5 with 3HA-ESA1	This study

All strains are in the W303 background with the genotype *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*, and additional pertinent genotype information is noted. Exceptions are the mating tester strain LPY78 and the S288c background strains LPY2991, LPY3291, LPY3498, and LPY3500.

HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% sodium deoxycholate, and the protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 5 μg/ml TPCK, 2 μg/ml leupeptin and pepstatin) at 4°C by the glass bead method. The extract was sonicated to shear DNA and clarified by centrifugation at 16,000 × g for 5 min at 4°C. The clarified supernatant was incubated overnight with either α-HA (12CA5, BabCo), αH4AcLys5, αH4AcLys12, αH4AcLys16, or αH3Ac (Serotech) at 4°C. Immune complexes were immunoprecipitated with protein A Sepharose (Pharmacia Biotech, Piscataway, NJ). Precipitates were washed successively with 1 ml FA-lysis buffer, FA-lysis buffer with 0.5 M NaCl, LiCl solution (10 mM Tris-HCl, pH 8, 0.25 M

LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA) and TE (10 mM Tris, pH 7.5, 1 mM EDTA) followed by RNase A and proteinase K treatment. After reversal of cross-linking, DNA was purified by phenol-chloroform extraction and recovered by ethanol precipitation. Precipitated DNA was analyzed by molecular amplification using the 25S-1, 25S-2, 5S, and NTS primers described in Gotta *et al.* (1997) and the *POL1* primers described in Papamichos-Chronakis *et al.* (2002).

To generate the triple-HA-tagged *ESA1* construct (*ESA1-3HA*), sequence immediately 3' to the *ESA1* ORF was amplified using Deep-Vent polymerase (New England Biolabs) and oligos T3 and D1-*ESA1* (5'-CGCGTTTAAACGCG-

Table 2. Plasmids used in this study

Plasmid	Description	Source
pLP37	<i>SIR2/URA3/2-micron</i>	This lab
pLP61	<i>TRP1/CEN</i> vector	This lab
pLP690	<i>ESA1/TRP1/CEN</i>	This lab
pLP796	<i>ESA1/URA3/2-micron</i>	This lab
pLP948	<i>ESA1/URA3/integrating</i>	This lab
pLP949	<i>esa1-L254P/URA3/integrating</i>	This lab
pLP952	<i>esa1-414/URA3/integrating</i>	This lab
pLP1070	rDNA 5'-BglII fragment/PKC7	S. Gasser
pLP1071	rDNA 3'-BglII fragment/PKC7	S. Gasser
pLP1237	<i>SIR2/LEU2/CEN</i>	This lab
pLP1309	<i>PPR1/pUC8</i>	This lab
pLP1347	<i>TRP1/2-micron</i> vector	S. Berger
pLP1348	<i>ESA1/TRP1/2-micron</i>	S. Berger
pLP1402	<i>URA3/2-micron</i> vector	This lab

TATGTAGTTTCCTAAAC-3'). The amplification product was inserted into pFA6a-3HA-kanMX6 (Longtine *et al.*, 1998) using PmeI and EcoRV (New England Biolabs) to construct pLP1452. The entire *ESA1* coding region was amplified using oligos TAS-1059 (5'-AACATATCACACGAGGATG3') and C-Bam-*ESA1* (5'-CTCGAGGATCCACCAGCAAGCGTAAC-3'). A PvuII-PacI-digested fragment from pLP1455 was used to insert *ESA1* in frame with 3HA in pLP1452 to generate pLP1453. To generate the *ESA1-3HA* strain, a SacII-SnaBI fragment of pLP1453 was transformed into LPY4819. Integrants that were G418 resistant were isolated and integration at the *ESA1* locus was verified with oligos kanMX6-sense (5'-GATGACGAGCGTAATGCTG-3') and TAS1-2132 (5'-GGCTGTATATCTTAAGTAAG-3') to generate LPY6934. Expression of *Esa1-3HA* from its endogenous promoter was verified by SDS-PAGE and Western blotting analyses of whole-cell extracts.

Pulsed-Field Gel Electrophoresis and Southern Blotting

Sample preparation for pulsed-field gel electrophoresis was carried out, in part, as described (Birren and Lai, 1993). Briefly, the strains AMR53, LPY4909, and LPY4911 were grown in log phase in YPD for 24 h at 28 and 37°C and for 4 h at 37°C. Before sample preparation, portions of the cultures were reserved for flow cytometric analysis, viability determination, and standard genomic DNA preparation. The remainder was washed twice in 10 ml of CSE (20 mM citric acid, pH 5.6, 20 mM Na₂HPO₄, 50 mM EDTA, pH 8, 1.2 M sorbitol), resuspended in 10 ml CSE with 0.43 mg/ml zymolyase 70T (ICN), and incubated in a 37°C water bath for 1 h. Cells were pelleted and resuspended in TSE (10 mM Tris, pH 7.5, 45 mM EDTA, pH 8, 0.9 M sorbitol) at a concentration of 6×10^8 cells per ml. Cells were then prewarmed briefly at 37°C and mixed 1:1 with warmed (55°C) liquefied 1% Incert agarose (FMC) in TSE. Multiple 80- μ l plugs were poured into a mold, and plugs were incubated in 5 ml of 0.25 M EDTA, pH 8, 50 mM Tris, pH 7.5, 1% SDS in sealed 50-ml conical tubes that were immersed in a 55°C water bath for 2 h. Plugs were transferred to fresh conical tubes and incubated in 5 ml of 1% lauryl sarcosine, 0.5 M EDTA, in a 55°C water bath for 72 h total. Fresh solutions of 5 ml of 1% lauryl sarcosine, 0.5 M EDTA, 1 mg/ml proteinase K were replaced every 24 h. Plugs were transferred to fresh 50-ml conical tubes and stored at 4°C in 5 ml of 1% lauryl sarcosine, 0.5 M EDTA.

Before electrophoresis, plugs were washed four times in TE over a 1-h period with gentle agitation. Gels containing 1% PFGE Seakem Gold agarose (FMC) in 0.5 $\times$ TBE (89 mM Tris-borate, pH 8.3, 25 mM EDTA) were cast, and plugs were sealed in the wells with residual agarose. Gels were electrophoresed in chilled (4°C) 0.5 $\times$ TBE for 72 h at 50 Volts (2 V/cm) with 30-min fixed pulse times. Gels were stained with ethidium bromide at a final concentration of 0.5 μ g/ml for 30 min to visualize chromosomes.

To digest whole chromosomes with NotI, plugs were dialyzed in 50 ml TEN (10 mM Tris, pH 7.4, 50 mM NaCl) overnight with gentle agitation at room temperature. A single plug was then incubated in 250- μ l final volume reactions with either no enzyme (mock) or 5 units of NotI (New England Biolabs) overnight at 37°C. After 8 h of digestion, 5 additional units of NotI were added to the reactions. BSA (bovine serum albumin) was added to NotI digests at a final concentration of 1 μ g/ml.

To determine the relative number of rDNA repeats in a given sample, genomic DNA corresponding to a PFGE experiment was digested with HindIII or BglII, both of which liberate satellite genomic bands that were resolved on a standard 1% agarose gel stained with ethidium bromide. Satellite bands, corresponding to rDNA unit fragments, were normalized to nonsatellite resolved DNA and quantified using the Alpha Digital Imaging System 2000 software. This analysis yielded an estimate of the relative number of repeats between samples. In the HindIII-digested samples, the *esa1-414* mutant possessed 98% of the number of repeats present in wild type, and in the BglII-

digested samples the *esa1-414* mutant possessed 84% of the number of repeats present in wild type, averaged together as 91% of the repeats in wild type. This small difference in repeat numbers cannot fully explain the mobility difference observed for the rDNA array in the *esa1-414* mutant. For example, if there were 200 rDNA repeating units at 9.1 kb, the array size would be 1820 kb in wild-type cells and 1656 kb in *esa1-414* cells, the difference of which is 164 kb. This size difference is not enough to explain the mobility differences observed for the entire chromosome XII nor for the NotI fragment containing the array between wild-type and *esa1-414* mutants.

Pulsed-field gels were prepared for Southern blotting, in part, as described (Birren and Lai, 1993). Briefly, after gels were stained with ethidium bromide and photographed, each side of the gels was UV-irradiated in a Stratilinker apparatus (Stratagene, La Jolla, CA) at maximum output for 60 s. Gels were soaked in 0.4 M NaOH, 1.5 M NaCl for 15 min and transferred to Zetabind membrane (Cuno Laboratory Products) in 0.4 M NaOH, 1.5 M NaCl for 24–36 h. Blots were rinsed in 0.5 M Tris, pH 7.5, for 5 min, rinsed briefly in 2 $\times$ SSC (333 mM NaCl, 30 mM sodium citrate), and dried, and each side of the blots were UV-irradiated in a Stratilinker (Stratagene) at 100 μ J. An rDNA/chromosome XII-specific probe for hybridization was prepared from pLP1070 by digestion with BglII to liberate a 4.5-kb fragment, which contains part of the rDNA repeat. pLP1309 was digested with BamHI and Sall to liberate a 2-kb fragment for use as a *PPR1*-specific probe for hybridization.

Indirect Immunofluorescence

Indirect immunofluorescence was carried out as described (Gotta *et al.*, 1997; Stone *et al.*, 2000). Briefly, cells were logarithmically grown in YPD for 4 h at either 28 or 37°C and fixed by adding paraformaldehyde to a final concentration of 3.3% at 30°C for 10 min. Cells were washed twice in YPD, resuspended at 1 ml per 0.1 g of cells in 0.1 M EDTA, KOH, pH 8.0, 10 mM DTT, and incubated at 30°C for 10 min with gentle agitation. Cells were resuspended at 1 ml per 0.1 g of cells in YPD containing 1.2 M sorbitol. Lyticase (ICN) was added to a 3000 U/ml final concentration, and samples were incubated at 30°C with gentle agitation for 30 min to form spheroplasts. Samples were diluted with 5 volumes of YPD, washed twice in 20 ml YPD containing 1.2 M sorbitol, resuspended in YPD at 3 ml per 0.1 g cells, and incubated on appropriate slides for 2 min. Slides were subsequently fixed in 100% methanol at –20°C for 6 min and in 100% acetone at –20°C for 1 min and blocked in PBST (phosphate-buffered saline [PBS], 0.1% Triton X-100) with 1% ovalbumin (Sigma) for 1 h. Slides were incubated with both the affinity-purified anti-Sir2p antibody (rabbit 2916, bleed 8, polyclonal; Smith *et al.*, 1998b; Ersfeld and Stone, 2000) at 1:1 dilution and the anti-Nop1p antibody (mouse D77, monoclonal; Aris and Blobel, 1988) at 1:100 in PBST overnight at 37°C. Slides were washed five times in PBST. The secondary antibodies, FITC-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA) were diluted 1:50 in PBST and preincubated at 4°C for 30 min with cells from all samples, and the resulting supernatant was incubated on slides for 1 h at 37°C. Slides were then washed three times in PBST, stained with 0.25 mg/ml DAPI for 5 min, washed again, and mounted in 24 mg/ml DAPCO in PBS, 87% glycerol, pH 7.5. Staining was visualized with an Applied Precision Deltavision optical sectioning deconvolution microscope, Olympus IX70, with six deconvolved 0.2- μ m sections. Deltavision version 2.10 software and a Princeton Instruments MicroMax camera were utilized to capture images.

RESULTS

A Structural Basis for *esa1* Mutant Defects

To begin to investigate the function of Esa1p in transcriptional regulation, we focused on two temperature-sensitive *esa1* mutants, *esa1-L254P* and *esa1-414* (Clarke *et al.*, 1999). From the crystal structure of Esa1p (Yan *et al.*, 2000), the lesions in the two temperature-sensitive mutants are found to reside very near the highly conserved HAT domain (Figure 1B, purple). The leucine residue 254 (orange) is buried within helix α 2, just four positions away from two residues that contact coenzyme-A (CoA). The *esa1-414* lesion is a frameshift mutation resulting in 10 changed residues (amino acids 414–423, in blue, starting in helix α 7) and the remaining C-terminal 22 residues deleted from 424 to 445 (black). The arginine residue 421 (blue spacefill), which is changed to glutamic acid in the *esa1-414* mutant, normally contacts CoA. The highlighted glutamic acid residue 338 (red spacefill), between strand β 10 and helix α 4 is also an essential residue for catalysis (Yan *et al.*, 2000) that by both structure and sequence is conserved among many HATs. A dominant mutant harboring a glutamine substitution at this position, *ESA1-E338Q*, interferes with growth in wild-type cells when overexpressed, mimicking the cell cycle arrest observed in

Figure 1. Mutants of *ESA1* contain lesions in residues near and within the catalytic HAT domain. (A) A schematic box diagram of *Esa1p* highlights lesions (asterisks) residing near and within the highly conserved GNAT family HAT domain (purple) in three distinct *esa1* mutants: *esa1-L254P*, *esa1-E338Q*, and *esa1-414*. The *esa1-L254P* and *esa1-414* mutants are temperature-sensitive for growth above 34°C (Clarke *et al.*, 1999) and the *esa1-E338Q* mutant is inviable and displays dominant negative properties (Yan *et al.*, 2000). The chromodomain (aqua) is not part of the solved crystal structure. (B) The solved crystal structure of *Esa1p* (Yan *et al.*, 2000) from residues 165-435 illustrates the location of *esa1* mutant lesions with respect to CoA (green) and the HAT domain (purple) corresponding to motifs A and D (Yan *et al.*, 2000). The serine residue 254 (orange spacefill), mutated in *esa1-L254P*, is located in helix α_2 , which precedes strand β_7 of motif D. The *esa1-414* frameshift mutation changes 10 amino acids (blue) beginning in helix α_7 and deletes the remaining C-terminal 22 amino acids (black), 10 of which were not included in the original solved structure. The arginine residue 421 (blue spacefill), which contacts CoA, is replaced with glutamic acid in *esa1-414*. The critical catalytic glutamic acid residue 338 (red spacefill), changed to glutamine in the *esa1-E338Q* mutant, is within the pocket comprising the A motif where the acetyl group in acetyl-CoA would reside.

esa1 mutants (Yan *et al.*, 2000). Effects on transcriptional control, in either positive or negative directions, were investigated for the *esa1-L254P* and *esa1-414* recessive temperature-sensitive mutants (below) and the *ESA1-E338Q* dominant HAT mutant (see Supplementary Figure S2).

esa1 Mutants Are Defective in Transcriptional Silencing at Telomeres and the rDNA

Because *Esa1p* is an essential histone acetyltransferase with connections to transcriptional activation (Allard *et al.*, 1999; Galarneau *et al.*, 2000; Reid *et al.*, 2000; Eisen *et al.*, 2001), it might be expected that the *esa1* temperature-sensitive mutants that have total decreases in histone H4 acetylation would have collateral increases in transcriptional silencing. To investigate whether *Esa1p* may function in both transcriptional activation and silencing, we asked if *esa1* mutants displayed changes in silencing at three well-characterized loci. Expression of reporter genes placed at the silent mating-type locus *HMR*, within the rDNA array and at telomeres (reviewed in van Leeuwen and Gottschling, 2002) was examined in the two *esa1* temperature-sensitive mutants to test for effects on transcriptional silencing.

Figure 2. Temperature-sensitive *esa1* mutants display transcriptional silencing defects within the rDNA array and at telomeres but only modest silencing defects at *HMR*. (A) The *esa1-414* mutant (LPY4915) displayed no defect in silencing at *HMR*, whereas the *esa1-L254P* mutant (LPY5059) had a modest defect compared with wild-type (LPY4913) and to a *sir2Δ* mutant (LPY4980), which was completely disrupted for silencing at *HMR*. Silencing at *HMR* was assessed by measuring growth on plates lacking tryptophan (right) compared with a synthetic complete (SC) growth control plate (left) of strains containing the *TRP1* reporter gene within the *HMR* locus. (B) The *esa1-414* (LPY4821) mutant had an intermediate telomeric silencing defect compared with wild-type (LPY4819), yet not as severe as a *sir2Δ* mutant (LPY5017). Telomeric silencing was assessed for strains containing the telomere V-proximal *URA3* reporter gene by measuring growth on plates containing 5-FOA (right) compared with an SC growth control plate (left). (C) The *esa1-414* (LPY4821 and LPY4848) mutant displayed strong defects in rDNA silencing compared with wild-type (LPY4819), even more so than that of a *sir2Δ* mutant (LPY5017). Silencing in the rDNA array was assessed by measuring growth on 8 mg/ml canavanine plates lacking adenine (right) of strains containing the *ADE2-CAN1* rDNA reporter cassette compared with a growth control plate (left). Note that the concentration of canavanine used in the rDNA silencing assay was eightfold lower than in earlier studies (Roy and Runge, 2000) to highlight the severity of the *esa1* defect relative to that of *sir2Δ*. Plates in A were incubated at 28°C, those in B and C at 33°C.

Expression of a *TRP1* reporter gene placed at a sensitized *HMR* locus was measured in the *esa1-L254P* and *esa1-414* mutants by assessing growth on medium lacking tryptophan (Figure 2A). If *HMR* silencing is intact, as in wild-type cells, *TRP1* expression is low and the strain will grow poorly on medium lacking tryptophan. However, if *HMR* silencing is disrupted, as in the *sir2Δ* control strain, *TRP1* expression will be adequate to promote full growth. A modest defect in silencing at *HMR* was observed for the *esa1-L254P* mutant compared with a *sir2Δ* mutant at 28°C, and this defect was not influenced by temperature (unpublished data). No defect was detected for the *esa1-414* mutant. We also tested silencing at *HML* by quantitative mating analysis, where only modest changes were observed (Table 3) by comparison with those of original *sir* mutants, (for example, see Rine and Herskowitz, 1987). In these assays, no significant increases in silencing were detected at either *HMR* or *HML* in the *esa1* mutants, as might be expected for mutants with an overall loss in histone H4 acetylation. Instead, only slight disruptions of *HMR* and *HML* silencing were detected

Table 3. The *esa1* mutants silence *HML*

MATa strain	Mating efficiency
<i>ESA1</i> (LPY2991)	1.00
<i>esa1-414</i> (LPY3291)	0.42
<i>ESA1</i> (LPY3498)	1.00
<i>esa1-L254P</i> (LPY3500)	1.15

Mating efficiencies were measured in two independent experiments at 28°C and are reported here as their average. See *Materials and Methods* for details.

for either mutant. Therefore, it appears that *ESA1* is neither essential for nor antagonizes transcriptional silencing of the *HM* loci.

In a second series of assays, telomeric silencing, also known as telomeric position effect (Gottschling *et al.*, 1990) was evaluated. Here, expression of a *URA3* reporter gene placed proximal to telomere V was measured by assessing growth on 5-FOA, a suicide substrate for the *URA3* gene product. Surprisingly both *esa1-414* (Figure 2B) and *esa1-L254P* (unpublished data) mutants had defects in telomeric silencing, as demonstrated by decreased growth on 5-FOA. This silencing defect is most severe at 33°C in the *esa1* mutants but is also detectable at 28°C, both temperatures at which growth and viability are completely normal. Note that although the *esa1* mutants are defective in telomeric silencing, and readily distinguishable from wild-type cells, their defect is not as severe as in a *sir2Δ* strain (Figure 2B).

Finally, the expression of an *ADE2-CAN1* reporter cassette placed in the rDNA array within the 25S transcription unit (Fritze *et al.*, 1997) was measured in the *esa1* mutants by assessing growth on medium containing canavanine. Expression of *CAN1* leads to production of the arginine permease that transports canavanine, a toxic arginine analog, into cells at the cell membrane. Thus, strains will grow on medium containing canavanine only if the *CAN1* gene is silenced within the rDNA array. Both *esa1-414* (Figure 2C) and *esa1-L254P* (unpublished data) mutants displayed severe rDNA silencing defects, as evidenced by lack of growth on the canavanine plate. At 33°C, this *esa1* rDNA-silencing defect was more severe than that of a *sir2Δ* mutant (Figure 2C) and at 28°C was roughly equal to a *sir2Δ* (unpublished data). Viability was measured at 100% and importantly, cell growth rates are equivalent for wild-type and the *esa1* temperature-sensitive mutants at both 28 and 33°C, so the defects observed were not due to nonspecific effects on growth. In addition, *esa1* mutants are not generally sensitive to canavanine; therefore, the effects observed were due to aberrantly increased expression of the reporter gene.

ESA1 Function within the rDNA

The pronounced effects of loss of *ESA1* function on rDNA silencing could be mediated directly or indirectly. For example, if *SIR2* were a transcriptional target of *ESA1*, its expression might be down-regulated in the *esa1* mutants, leading to defects at this Sir2p-dosage-sensitive locus (Smith *et al.*, 1998b). This does not appear to be the case however because *SIR2* is not a transcriptional target of *ESA1* as assessed by either chromatin immunoprecipitation (ChIP) or expression-based microarray studies (Reid *et al.*, 2000 and our unpublished observations). Further, the steady state levels of Sir2p appear normal in *esa1* mutants (Supplementary Figure S1) and there are no gross defects in the Sir2p-dependent processes of mating (see above).

To test the alternative possibility that Esa1p might be acting directly within the rDNA, we took several independent approaches. In the first, we asked whether Esa1p was itself found within the rDNA. In previous ChIP experiments to evaluate promoter occupancy, Esa1p was found at many ribosomal protein gene promoters (Reid *et al.*, 2000). It is also known that *ESA1* influences total levels of H4 acetylation (Clarke *et al.*, 1999) and that much of this global acetylation is not restricted to promoter regions (Vogelauer *et al.*, 2000). We performed ChIP experiments using a fully functional Esa1-HA epitope-tagged strain and compared recovery of rDNA sequences to a control untagged strain (Figure 3, A and B). We observed Esa1-HA-dependent recovery of rDNA sequences from the 25S, 5S, and NTS regions of the repetitive rDNA array from chromosome XII compared with the untagged strain. That enrichment at these sites was specific was established by comparison to the control *POL1* locus, where no differences were observed between tagged and untagged strains. Recovery of Esa1p from these sites in the rDNA thus demonstrates that Esa1p maps to the site of its effects on silencing, as observed for targeting of Sir2p (Huang and Moazed, 2003).

Because *esa1* mutants affect global levels of histone H4 acetylation (Clarke *et al.*, 1999), we tested the effect of *esa1* mutants on the levels of acetylation in the rDNA at specific lysine residues on histone tails. We performed ChIP analysis using modification specific antisera at the 25S, 5S, and NTS regions within the rDNA, comparing *ESA1* and *esa1-414* strains at 33°C where both strains have complete viability (Figure 3C). We observed multiple *ESA1*-dependent changes of acetylation within the rDNA that varied depending on both the location and the isoform examined. The overall acetylation level of H4 Lys5 was reduced to the greatest extent, varying from 25 to 42% to that of wild type. This correlates well with H4 Lys5 being the primary target of Esa1p in vitro and in in vivo analysis of *esa1* total histones (Clarke *et al.*, 1999). Acetylation of H4 Lys12 and 16 was also reduced. To a lesser extent, H3 was affected, correlating with minor targets of Esa1p. These results demonstrate that Esa1p both localizes to and functions directly within the rDNA.

In addition to being found directly in the nucleolus and rDNA (Gotta *et al.*, 1997; Huang and Moazed, 2003), Sir2p functions to suppress recombination within the highly repetitive rDNA array (Gottlieb and Esposito, 1989). Indeed, many rDNA silencing defective mutants are correlated with increases in rDNA recombination (Smith *et al.*, 1999). We tested this function for *ESA1* at both 28 and 33°C using a sectoring assay based on colony color associated with the *ADE2-CAN1* reporter. Recombination rates were measured as the number of events that excise the reporter cassette per cell division (Figure 4, A and B). In these experiments, both wild-type and *sir2Δ* mutant strains were included as controls. We observed that in the *esa1-414* mutant, the mitotic rDNA recombination rate was slightly elevated relative to wild-type cells. In contrast, the *sir2Δ* strain had substantially elevated mitotic recombination, as previously reported. When the values for recombination were evaluated with the Wilcoxon ranking test (Wonnacott and Wonnacott, 1984; Supplementary Table S1), wild-type and *esa1-414* mutant rates appeared different at 28°C, and both *esa1-414* and wild type were significantly different from *sir2Δ* mutants. Thus, the *esa1* mutant was modestly defective in suppressing mitotic recombination within the rDNA array under conditions in which Pol II silencing was disrupted, but viability was intact. Together, these effects are distinct from loss of Sir2p function.

Figure 3. Esa1p functions within the rDNA. (A) A diagram of a single rDNA transcription unit highlighting the features of each unit and orientations of transcription is shown. Dotted lines above show the position of primer sets. Replication fork barrier (RFB) and ARS1 (Abf1p binding site) are also indicated as black boxes within the nontranscribed spacer (NTS) regions. (B) Esa1p is enriched within the rDNA array. Formaldehyde cross-linked chromatin of untagged control *ESA1* (LPY5) or *3HA-ESA1* (LPY6244) strains was immunoprecipitated using the α -HA antibody. Molecular amplification was carried out on total (Input) and immunoprecipitated chromatin (IP α -HA) using two primer sets specific to the 25S region (25S1 and 25S2), one set near the 5S region, an NTS primer set, and a control primer set for the *POL1* locus. Two dilutions within the linear range of amplification are shown for each. The fold-enrichment values indicated are the ratio of IP/Input for tagged/untagged strains after normalization with *POL1*. In all cases 3HA-ESA1p is enriched within the rDNA array compared with the untagged strain. (C) Acetylation of Esa1p targets is reduced in the rDNA. Wild-type and *esa1-414* cells were grown at a permissive temperature of 33°C with full viability. Chromatin was immunoprecipitated with antibodies against acetylated lysines H4 Lys5, H4 Lys12, H4 Lys16, and total Ach3. Levels of acetylation were compared in wild-type and *esa1-414* strains in the rDNA using primer sets 25S1, 25S2, 5S, and NTS. The % wild-type value shown below each *esa1* IP (gray boxes) is calculated by *esa1-414*/wild-type signals after input normalization. Acetylation of H4 Lys5 is reduced to the greatest extent throughout the rDNA at all sites tested, which correlates with the primary acetylation target of Esa1p. In addition, acetylation of H4 Lys12 and 16 are also somewhat reduced, as is total acetylated H3, again correlating with other minor acetylation targets of Esa1p.

Alteration of the rDNA Array in *esa1* Mutants

In *S. cerevisiae*, the rDNA resides as a repetitive array at a single locus on the right arm of chromosome XII (Figures 4A and 5A). The rDNA array consists of 100–200 repeating units on average, with each 9.1-kb unit containing the genes for the 35S and 5S transcription units. We observed previously that *esa1* mutants have striking structural aberrations in the nucleolus, the site of the rDNA, when evaluated by electron microscopy (Clarke *et al.*, 1999). Whereas the normal yeast

Figure 4. Mitotic recombination in the rDNA array. (A) Mitotic recombination within the rDNA array was evaluated using a marker loss assay. In the table, the average recombination rate of four independent isolates is shown with indicated standard deviations, except for the *sir2Δ* strain where the range of five (28°C) or two (33°C) isolates is presented. (B) Graphed are values for recombination assays performed at 28°C. Wilcoxon ranking tests indicated that *esa1* and wild-type rates of loss are significantly different from *sir2Δ* rates and that *esa1* rates are elevated relative to wild type (see Supplementary Table S1).

nucleolus is a coalesced, hemispherical mass of electron-dense material in close apposition to the nuclear membrane, in *esa1* mutants, this structure was not observed at elevated temperatures. Instead, there was very little electron-dense material and what was present appeared dispersed and scattered throughout the nucleus. These observations raised the possibility that chromatin structure itself was altered within the nucleolus. The strong rDNA silencing defects in the *esa1* mutants also support the idea that chromatin structure might be changed within the rDNA array.

To determine if the chromosomes of *esa1* mutants were substantially different from wild-type, we examined them by pulsed-field gel electrophoresis (PFGE). Under conditions that maximized resolution of the entire chromosomal complement, we observed that wild-type and *esa1* strains had similar chromosomal migration patterns, demonstrating that the *esa1* mutants did not have massive, genome-wide defects (unpublished data). However, there appeared to be clear and reproducible differences in chromosome XII, the most slowly migrating chromosome in these gels. It has previously been observed that the electrophoretic mobility of chromosome XII and of the rDNA array can be greatly affected by their structure. In particular, mutations in the topoisomerase I gene (*TOP1*) lead to extreme chromatin alterations that prevent either chromosome XII or the rDNA array from entering pulsed-field gels and that influence chromosomal compaction (Christman *et al.*, 1993; Castaño *et al.*, 1996). In fact, *top1* mutants are defective in the suppression of rDNA recombination as well as rDNA silencing (Christman *et al.*, 1988; Smith *et al.*, 1999). To investigate the electrophoretic mobility of chromosome XII in the *esa1-414* mutant, whole chromosomes were prepared and analyzed by PFGE and Southern blotting, using wild-type, *sir2Δ*, and *top1* mutants for comparison (Figure 5B).

We observed that the electrophoretic mobility of chromosome XII in the *esa1-414* mutant was distinct from that of wild-type, irrespective of temperature (Figure 5B). This mobility difference was also clearly distinct from the behavior of either the *sir2Δ* or *top1* strains. Chromosome XII entered the gel in the *esa1-414* mutant, rather than being trapped in

Figure 5. The rDNA array is affected by *esa1* mutants. (A) The 2.2-Mb rDNA array as diagrammed resides on the right arm of chromosome XII, the largest *S. cerevisiae* chromosome (not drawn to scale). NotI restriction enzyme sites (N) flank the array and the region containing the *PPR1* gene (gray box). A chromosomal NotI digest liberates a 2.2-Mb fragment containing the rDNA array and a 1.4-Mb fragment containing the *PPR1* gene. (B) The *esa1-414* mutant exhibited altered chromatin structure of chromosome XII as evidenced by pulsed-field gel electrophoretic mobility. Whole chromosome preparations from *ESA1* (LPY4909), *esa1-414* (LPY4911), *sir2Δ* (LPY4978), and *top1-8* (AMR53) strains incubated at the temperatures indicated (28, 33, and 37°C) were subjected to pulsed-field gel electrophoresis and analyzed by ethidium bromide staining (top) and Southern blotting (bottom) with a *PPR1* probe, as diagrammed in A. In the *esa1-414* mutant, chromosome XII (as identified in the Southern blot) displayed an altered electrophoretic mobility at all temperatures compared with wild-type (left arrows), which was distinct from the *sir2Δ* and *top1-8* strains, where chromosome XII failed to enter the gel. (C) The altered electrophoretic mobility of chromosome XII in the *esa1-414* mutant is due to the rDNA array. Whole chromosome preparations as in B were digested with NotI, subjected to pulsed-field gel electrophoresis, and analyzed by ethidium bromide staining (top) and Southern blotting (bottom) with the *PPR1* probe. The rDNA array has an altered electrophoretic mobility in the *esa1-414* mutant compared with wild-type, as evidenced by ethidium bromide staining (top arrows). However, the *PPR1* NotI fragment (bottom arrow, Southern) migrated equivalently in all strains, including in the *sir2Δ* and *top1-8* strains. In both B and C relevant marker sizes are indicated to the right. Asterisks indicate the marker chromosome XII and the wells are indicated. In B and C, the wells are detected by the *PPR1* probe, because not all chromosomal material enters the gels.

the wells as previously characterized for *top1* mutants, or as shown here for *sir2Δ* mutants.

Because the rDNA array migrated more rapidly in the *esa1* mutants compared with wild type, we considered the possibility that these strains had fewer repeat units, even though the recombination rate was significantly lower than that in *sir2Δ* or *top1* mutants (Figure 4B and Christman *et al.*, 1988; Gottlieb and Esposito, 1989). We quantified the rDNA repeats for *ESA1* and *esa1-414* strains during the experiment shown in Figure 4 and found that they had essentially equivalent numbers of repeats and that the small difference in number is unlikely to account for the PFGE migration differences (range 84-98%; see *Materials and Methods*). In addition, we independently measured the relative rDNA repeat number in the *ESA1*, *esa1-414*, and *top1Δ* strains by quantitative genomic dot blotting. The relative rDNA repeat hybridization signal was compared with a single copy control locus *YBR071w* on chromosome II for *ESA1*, *esa1-414*, and *top1Δ* strains under normal growth conditions. The results of this quantification also indicated that the *esa1-414* strain had similar (although in this assay slightly more) amounts of rDNA as wild-type and *top1Δ* strains (Supple-

mentary Table S2). Thus, data from two independent assays suggest that *esa1-414* chromosome XII is not migrating faster in PFGE analysis due to fewer repeats.

To determine if the mobility difference observed in the *esa1* mutant was due to the rDNA specifically or due to a more general property affecting all of chromosome XII, we took advantage of the fact that NotI sites fall outside, but not within, the rDNA repeats. A schematic representation of chromosome XII with relevant NotI sites surrounding the rDNA array (Figure 5A) shows that when chromosome XII is digested with NotI a ~2.2-Mb fragment containing the rDNA array is liberated. A 1.4-Mb fragment containing the *PPR1* gene is also liberated (Figure 5A), along with other additional fragments not indicated. Chromosomes from the same strains examined above were digested with NotI and analyzed by PFGE and Southern blotting. We observed that, again, the rDNA array migrated differently for the wild-type, *esa1-414*, *sir2*, and *top1-8* strains (Figure 5C, top). However, the centromere-proximal 1.4-Mb fragment detected by the *PPR1* probe migrated equivalently for all three strains (Figure 5C, bottom). Therefore, the electrophoretic mobility differences observed for chromosome XII in the *esa1-414*

appear due primarily to properties of the rDNA array. Because gross changes in rDNA repeat numbers are unlikely to explain the difference in mobility we observe, we hypothesize that the changes are due to alterations in chromatin structure that are sensitive to PFGE separation but that are distinct from severe recombination phenotypes such as those occurring in *sir2* and *top1* mutants.

Altered Localization of Two Nucleolar Proteins in *esa1* Mutants

By both chromatin immunoprecipitation and fluorescence localization studies, Esa1p has been defined for its general nuclear roles and distribution (Galarneau *et al.*, 2000; Reid *et al.*, 2000; Vogelauer *et al.*, 2000; Huh *et al.*, 2003). Viewed collectively, the previously observed defects in nucleolar structure (Clarke *et al.*, 1999), in addition to the rDNA silencing, recombination and PFGE analyses reported here all suggested that *ESA1* also contributes to normal nucleolar structure and function. Many proteins are known to function in the nucleolus, so to investigate further the function of *ESA1*, we evaluated the effects of an *esa1* mutation on two of these nucleolar markers. We used indirect-immunofluorescence to compare localization of the yeast fibrillarin homolog, Nop1p (Aris and Blobel, 1988), and Sir2p in wild-type and *esa1* cells under permissive and restrictive conditions. Nop1p staining in wild-type cells at 28°C appeared as a characteristic discrete crescent shape apart from the bulk of the DAPI-staining chromatin (Figure 6A, top). Most of Sir2p colocalized with the Nop1p staining in the nucleolar crescent and some Sir2p localized to telomeric foci with the bulk of the chromatin, consistent with previous results (Gotta *et al.*, 1997).

In contrast, at 28°C in the *esa1-414* mutant, the anti-Nop1p staining was more punctate, although usually still found in a crescent shape (Figure 6A, bottom, asterisk) and the staining appeared to occupy a larger portion of the nucleus than in wild-type. However, in some cells, no crescent was evident but Nop1p-staining instead appeared as a small focus or was dispersed throughout the nucleus (Figure 6A, bottom, arrowheads). Sir2p staining in the *esa1* mutant was also aberrant at 28°C with more punctate nucleolar foci than in wild type. Often the Sir2p crescent shape was not visible and the staining in general appeared dispersed or diminished. Nonetheless, the Sir2p staining that was visible coincided with Nop1p and telomeric foci were visible indicating at least some Sir2p was localized properly to the nucleolus and telomeres.

After a 4-h shift to 37°C, the Nop1p and Sir2p staining in the *esa1-414* mutant (Figure 6B, bottom) were again distinct from wild type (Figure 6B, top). Many cells displayed Nop1p staining that was either bilobed (arrowheads) or in very small foci (asterisk). Sir2p staining appeared even more abnormal and localized as small condensed foci that coincided with Nop1p staining. Together these results underscore that the substructure of the nucleolus is altered in *esa1* mutants and that the effect is more severe at nonpermissive temperatures. The fact that at least part of the pool of Sir2p appears to still be localized to the nucleolus is consistent with the observation that some of its functions, such as suppression of recombination, remain at least partially intact.

Increased Gene Dosage of *ESA1* and *SIR2* Reciprocally Suppresses rDNA Silencing Defects

The data above and earlier observations establish that there are intersecting yet distinct functions for both Esa1p and Sir2p within the nucleolus. To explore this relationship fur-

Figure 6. Nop1p and Sir2p localization are affected in the *esa1-414* mutant under both permissive and restrictive conditions. (A) At 28°C, Nop1p and Sir2p localization in the *esa1-414* mutant was distinct from wild type by indirect-immunofluorescence. Nop1p staining in the *esa1-414* mutant (bottom) was more punctate, although often still in a crescent shape (asterisk) though generally occupying a larger portion of the nucleus than in wild type. In some *esa1-414* cells, the punctate staining was not crescent-shaped and was quite small or dispersed throughout the nucleus (arrowheads). Sir2p staining was also reduced to very small punctate foci that coincided with the Nop1p staining (merge), yet often these foci were not crescent-shaped. Some telomeric Sir2p foci were still visible in the *esa1-414* mutant. (B) After a 4-h shift to 37°C, the *esa1-414* mutant displayed aberrant Nop1p and Sir2p localization by indirect-immunofluorescence. Nop1p staining in the *esa1-414* mutant was distinct from wild-type with many cells displaying bilobed fragmented staining (arrowheads) or only a very small focus of staining (asterisk). Sir2p staining in the nucleolus was even more reduced and punctate in the *esa1-414* mutant at 37 than at 28°C, with very few crescent shapes, although there was still partial overlap with the Nop1p staining (merge). In addition, Sir2p telomeric foci were difficult to identify in the *esa1-414* mutant at 37°C.

ther, we asked if changes in dosage of either gene affected the rDNA silencing mutant phenotype of the other. As an initial approach, we constructed the *esa1-414 sir2Δ* double mutant through standard genetic crosses. When rDNA silencing was assayed in this strain and compared with wild-type and the single mutant strains, we observed that silencing remained as defective as in an *esa1* strain (unpublished data). Thus, these acetyltransferase and deacetylase mutants do not mutually suppress each other's transcriptional defects, as has been observed previously for other HAT/HDAC mutant pairs (Pérez-Martín and Johnson, 1998).

In addition to evaluating decreased expression with the double mutants, we also looked at effects of increased expression of the two genes. It had been noted earlier that silencing within the rDNA array is quite sensitive to the gene dosage of *SIR2*. For example, overexpression of *SIR2* or deletion of *SIR4*, which presumably redirects a pool of Sir2p from the telomeres to the rDNA, strengthens rDNA silencing (Smith *et al.*, 1998b). We first asked if increasing *SIR2*

Figure 7. Increasing the gene dosage of *SIR2* and *ESA1* reciprocally suppresses the rDNA silencing defects associated with *esa1-414* and *sir2Δ* mutants. (A) Increased gene dosage of *SIR2* from a 2-micron plasmid suppressed the *esa1-414* rDNA silencing defect. The *esa1-414* strain transformed with *ESA1* (LPY5141) or *SIR2* (LPY5143) from *URA3*-marked 2-micron plasmids grew equivalently on plates assaying for rDNA silencing (right) compared with vector transformants (LPY5136) at 33°C. (B) Similarly, increasing the dosage of *ESA1* suppressed the *sir2Δ* rDNA silencing defect. The *sir2Δ* strain transformed with *SIR2* (LPY5166) or *ESA1* (LPY5164) grew equivalently on plates assaying for rDNA silencing compared with vector transformants (LPY5159) at 28°C. The level of suppression was somewhat variable between isolates, perhaps reflecting a difference in expression levels of either *ESA1* or *SIR2* due to fluctuations in 2-micron plasmid copy number per isolate (Armstrong *et al.*, 1989). The rDNA silencing assays were performed with 32 mg/ml canavanine in order to compare silencing effects in the *sir2Δ* and *esa1* mutants directly.

gene dosage affected rDNA silencing in *esa1* mutants. The *esa1-414* strain was transformed with 2- μ m plasmids to provide moderately increased copy number (Broach, 1983) of the *URA3* vector alone, the *ESA1* gene, or *SIR2* (Figure 7A). As expected, *ESA1* fully complemented the defect evident in the vector controls. The *SIR2* transformants also had fully restored silencing, indicating that the *esa1* defect could be suppressed by increasing *SIR2*. In companion experiments, a *sir2Δ* strain was transformed with the same plasmids (Figure 7B). Again, the wild-type *SIR2* gene complemented the *sir2Δ* silencing defect. Strikingly, increased *ESA1* gene dosage also restored silencing, indicating that *ESA1* can fully bypass the requirement for Sir2p in rDNA silencing.

Interestingly, the cross-suppression between *ESA1* and *SIR2* has restricted locus specificity because increased *SIR2* could not bypass the essential requirements for *ESA1* (unpublished data). Likewise, increased *ESA1* did not restore mating to a *sir2Δ* strain (unpublished data), consistent with the lack of a central role for *ESA1* at the silent mating-type loci (Figure 2 and Table 3). Furthermore, neither gene could suppress the telomeric silencing defect of the other mutant (unpublished data). Thus, whereas *ESA1* and *SIR2* clearly have independent roles within the genome, they have the previously unsuspected capacity to compensate for each other's defects in rDNA silencing of a Pol II reporter gene.

DISCUSSION

Starting from initial links to transcriptional activation of specific target genes, HATs have now been implicated in

processes as diverse as DNA replication and double-stranded break repair and recombination. Acetylation is a readily reversible modification and long-standing correlations have associated HATs with activation and deacetylases with repression of transcription. In some cases, paired, opposing activities at a single promoter have been shown to either positively or negatively regulate transcription of the locus (reviewed in Carrozza *et al.*, 2003).

We have found that *ESA1*'s transcriptional functions are not limited to activation and repair. Instead, *Esa1p* also functions in transcriptional silencing, potentially through direct effects on chromatin structure, most evident within the rDNA and nucleolus. Perhaps even more surprisingly, we have uncovered a link between *ESA1* and *SIR2*, a prominent nucleolar NAD-dependent HDAC. These two enzymes with seemingly opposing modifying activities both have the capacity to function in silencing within the rDNA.

Expanded Roles for an Essential MYST Family HAT

The MYST family of acetyltransferases was originally defined by the human MOZ leukemia translocation partner, the two yeast Sas proteins, and the human HIV-Tat interacting protein Tip60 (Borrow *et al.*, 1996; Kamine *et al.*, 1996; Reifsnnyder *et al.*, 1996). Family members have subsequently been characterized with diverse roles in DNA replication, apoptosis, sex dosage compensation, phospholipid signaling, development, neurogenesis, and androgen receptor function (reviewed in Utley and Côté, 2003). Because multiple MYST family proteins may be expressed simultaneously in the same cells and tissues, understanding the specificity of their function has been a key goal.

ESA1 encodes the essential HAT activity that is primarily directed toward histones H4 and H2A (Smith *et al.*, 1998a; Clarke *et al.*, 1999). Analysis of conditional *esa1* mutants reveals that loss of activity results in changes in nuclear substructure and overall decreases in genomic H4 acetylation (Clarke *et al.*, 1999). Biochemical studies demonstrate that *Esa1p* is the catalytic subunit of the NuA4 complex that includes other essential subunits such as Tra1p and Act3p and other key subunits with connections to growth control and epigenetic elements of transcriptional activation (Allard *et al.*, 1999; Galarneau *et al.*, 2000). Indeed, a number of distinct transcriptional targets have been identified for *Esa1p*, along with genome-wide validation of its role as a global H4 HAT (Galarneau *et al.*, 2000; Reid *et al.*, 2000; Vogelauer *et al.*, 2000; Nourani *et al.*, 2001). More extensive expression analysis suggests nearly equal numbers of positively and negatively affected transcriptional targets for *Esa1p* (Mnaimneh *et al.*, 2004). Although some of these may be indirect, the data raise the strong possibility that *Esa1p* participates in either gene-specific or more general chromatin-mediated forms of repression.

Here, we report previously unanticipated roles for *ESA1* in transcriptional silencing and genomic structure and function. In independent support of a role for *Esa1p* HAT activity in silencing, an *ESA1-E338Q* catalytically inactive dominant mutant (Yan *et al.*, 2000) interferes with wild-type silencing at the rDNA and telomeres (Supplementary Figure S2). Nucleolar structure is also disrupted in *esa1* mutants, as assessed by mislocalization of resident nucleolar proteins and alterations in PFGE and ultrastructural profiles (Clarke *et al.*, 1999). The *esa1* silencing defects are not globally communicated through all silenced loci, because regulation of mating type control at the *HM* loci is only modestly affected.

Esa1p maps to the rDNA locus by ChIP, and mutational analyses suggest a significant contribution to this region of the genome. Acetylation levels of key *Esa1p* targets are

significantly reduced in mutants in the rDNA, supporting the idea that Esa1p functions there directly through its HAT activity. These data expand the map of genomic targets for Esa1p beyond previously identified Pol II-regulated sites (Vogelauer *et al.*, 2000; Reid *et al.*, 2000) to the territory ordinarily controlled by Pol I and Pol III.

A key question raised by the silencing phenotypes of *esa1* mutants is whether these defects are mediated through loss of NuA4 activity, previously associated with activation. A second possibility is that Esa1p-silencing functions are mediated through multiple, functionally distinct complexes. Evidence for the latter possibility comes from the observation that the canonical yeast HAT Gcn5p works through alternative complexes with different target genes (Stern and Berger, 2000) and the discovery of the Esa1p-containing Piccolo complex, picNuA4 (Boudreault *et al.*, 2003). Considering alternative complexes, one might imagine discrete subunits that mediate activating versus silencing outputs to fine-tune locus-specific functions of Esa1p. In parallel, some Esa1p-interacting partners would function generically in both activating and silencing contexts. In this regard, it is noteworthy that like *ESA1*, mutants of *EPL1*, a component of both NuA4 and picNuA4 have defects in transcriptional activation and telomeric silencing (Boudreault *et al.*, 2003). In contrast, other NuA4 subunits are themselves implicated in transcriptional activation but not silencing (Galarneau *et al.*, 2000; Nourani *et al.*, 2001). Future identification of Esa1p's interacting partners in nucleolar and telomeric silencing should refine understanding of its role in NuA4, picNuA4, or other complexes, but the results presented here demonstrate a clearly expanded role for Esa1p in genomic regulation.

Esa1p Is a Distinct rDNA Silencing Protein Important for Nucleolar Structure

A long-standing observation is that only about half of the ribosomal repeats in the rDNA cluster are transcriptionally activated under normal growth conditions. Many questions remain about the nature of the regulation of the transcriptional silencing of the remainder of the repeats (reviewed in Grummt and Pikaard, 2003). When it was discovered that Pol II-regulated genes that are engineered into the rDNA array are silenced in an epigenetic manner that is dependent on *SIR2* (Bryk *et al.*, 1997; Fritze *et al.*, 1997; Smith and Boeke, 1997), the appealing possibility was raised that Pol I transcription itself might be at least partially regulated through heterochromatin-like *SIR*-dependent mechanisms. Indeed, it had previously been observed that a Pol III reporter gene could be silenced in a *SIR*-dependent manner (Schnell and Rine, 1986). However, in contrast to early indications (Smith and Boeke, 1997), subsequent more sensitive analysis indicates that *SIR2* does not affect rDNA transcription (Sandmeier *et al.*, 2002), but may instead affect firing of origins of rDNA replication (Pasero *et al.*, 2002).

Because the discovery of nucleolar silencing of Pol II reporter genes by Sir2p, a growing number of factors have been shown to participate, including other chromatin modifying enzymes such as the Rpd3 deacetylase, the Set1 methyltransferase, and topoisomerase I (Smith *et al.*, 1999; Bryk *et al.*, 2002). Nucleolar proteins with no known catalytic activities, such as Net1p also influence rDNA silencing (Straight *et al.*, 1999). In other mutants, such as those of the Set1p methyltransferase or the Swi/Snf remodeling complex, silencing defects and effects on chromatin modification are pronounced, yet no defects have been reported for Pol I transcription and rDNA recombination may be either unaffected or suppressed (Bryk *et al.*, 2002; Dror and Winston, 2004). By comparison, Net1p directly binds Pol I and has the

capacity to promote transcription (Shou *et al.*, 2001), with no apparent effects on recombination. Intriguingly, transcription within the rDNA by Pol I itself is required for silencing and for the spread of silencing toward the centromere (Buck *et al.*, 2002). In contrast, *RPD3* is required for down-regulating rDNA transcription as cells enter the stationary phase (Sandmeier *et al.*, 2002).

We have observed that *esa1* mutants have extreme defects in rDNA silencing, but are distinguished from *sir2Δ* mutants because they have only modestly increased recombination rates between the homologous DNA repeats. The *esa1* mutants are also distinct from *sir2* mutants in that they have disrupted nucleolar structure as observed by electron microscopy (Clarke *et al.*, 1999), by the position of nucleolar marker proteins and by PFGE. Among mutants with rDNA silencing defects, *ESA1* is the only gene yet reported to also influence ribosomal protein gene transcription (Reid *et al.*, 2000). Thus, Pol I transcription defects may be part of coordinate mechanisms to shut down ribosome production when either or both rRNA or ribosomal protein synthesis are defective. How these defects would be communicated is not yet fully known, but having at least one shared regulatory molecule or mechanism provides an attractive point for coordination.

HATs and HDACs Are Both Required for Functional Optimization

HATs function in concert with other types of chromatin-modifying enzymes, such as kinases, methyltransferases, and ubiquitin ligases. A key challenge is to understand how these activities and their substrates are specified to yield optimal biological regulation (reviewed in Strahl and Allis, 2000). In some cases, pairs of enzymes with distinct activities may function sequentially to potentiate function. Such is the case for the Snf1 histone kinase and the HAT Gcn5p (Lo *et al.*, 2000, 2001). In other cases, two HATs with different substrate specificities may act on the same gene to establish a characteristic pattern of modification, as has been observed for Gcn5p and Esa1p (Vogelauer *et al.*, 2000).

In contrast, opposing modifying activities have been observed as counterbalances to one another. In these situations, mutant phenotypes resulting from the loss of one activity, such as acetylation, are effectively suppressed by loss of a paired deacetylase. Such is the case for *gcn5Δ* and *rpd3Δ* deacetylase mutants when evaluating *HO* expression (Krebs *et al.*, 1999). Whether such pairing is a general feature of chromatin modification is not yet known, but in some cases, direct interactions between HDACs and HATs have been observed (for example, see Yamagoe *et al.*, 2003).

Our observation of the mutual suppression of *sir2Δ* or *esa1* mutant rDNA silencing defects by overexpression of an opposing activity suggests the possibility for novel regulatory mechanisms. In this case, the *ESA1*-encoded HAT can actually bypass the requirement for Sir2p, which previously was a defining feature of rDNA silencing. Importantly, the suppression observed is locus-specific. That is, *ESA1* cannot fully substitute for all of *SIR2*'s functions, nor can *SIR2* bypass the essential requirements for *ESA1*. Such specificity of suppression is intriguing and suggests that in discrete regions of the genome these two enzymes contribute to achieve optimal chromosomal structure and function. We cannot yet exclude the possibility that Esa1p and Sir2p function independently within the rDNA. For example, Sir2p may affect origin firing within the rDNA and Esa1p may affect transcriptional regulation, yet both functions may be required for appropriate chromatin structure. Likewise, it is known that some regions of the rDNA array are more sen-

sitive to SIR2-silencing than others (Smith and Boeke, 1997; Buck *et al.*, 2002). It should prove interesting to map regional effects of *ESA1* control by comparison. Further investigation of the exact molecular consequences of Sir2p and Esa1p function in the rDNA is necessary to understand how these two enzymes with opposing activities contribute to silencing.

Mechanistic clues come from recent studies in which a cycle of acetylation and deacetylation was triggered at the site of an induced dsDNA lesion. The HATs Esa1p and Gcn5p were first recruited, followed by the Sir2p, Hst1p, and Rpd3p deacetylases. Histone acetylation and deacetylation patterns correlated with these ordered recruitments (Tamburini and Tyler, 2005). Deeper understanding of the *in vivo* substrates and dynamic activities of chromatin modifying enzymes such as Esa1p and Sir2p both within the rDNA and throughout the genome will establish how general the requirement for combinations of chromatin modifications at specific loci may be.

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