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The DNA End-Binding Protein Ku Regulates Silencing at the Internal *HML* and *HMR* Loci in *Saccharomyces cerevisiae*

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ABSTRACT

Heterochromatin resides near yeast telomeres and at the cryptic mating-type loci, *HML* and *HMR*, where it silences transcription of the α- and **a**-mating-type genes, respectively. Ku is a conserved DNA end-binding protein that binds telomeres and regulates silencing in yeast. The role of Ku in silencing is thought to be limited to telomeric silencing. Here, we tested whether Ku contributes to silencing at *HML* or *HMR*. Mutant analysis revealed that yKu70 and Sir1 act collectively to silence the mating-type genes at *HML* and *HMR*. In addition, loss of yKu70 function leads to expression of different reporter genes inserted at *HMR*. Quantitative chromatin-immunoprecipitation experiments revealed that yKu70 binds to *HML* and *HMR* and that binding of Ku to these internal loci is dependent on Sir4. The interaction between yKu70 and Sir4 was characterized further and found to be dependent on Sir2 but not on Sir1, Sir3, or yKu80. These observations reveal that, in addition to its ability to bind telomeric DNA ends and aid in the silencing of genes at telomeres, Ku binds to internal silent loci via protein–protein interactions and contributes to the efficient silencing of these loci.

DISTINCT regions of eukaryotic genomes are packaged into different types of chromatin, which are broadly categorized as euchromatin and heterochromatin. The type of chromatin in any particular region controls the functional potential of that region. In general, euchromatic regions of the genome are replicated early in S-phase and the genes in those regions are maintained in a state that is permissive for transcription, whereas heterochromatic regions of the genome are replicated late in S-phase and transcription of the genes in heterochromatic regions is repressed (Gilbert 2002; Schwaiger and Schèubeler 2006).

In the budding yeast *Saccharomyces cerevisiae*, heterochromatin resides in the regions adjacent to each of the telomeres and at the silent mating-type loci *HML* and *HMR* (Rusche *et al.* 2003; Millar and Grunstein 2006; Shahbazian and Grunstein 2007). Heterochromatin represses transcription of the endogenous genes in these regions and can repress transcription of genes experimentally inserted into these regions (Rusche *et al.* 2003; Pirrotta and Gross 2005; Millar and Grunstein 2006; Talbert and Henikoff 2006). Repression of transcription by heterochromatin in yeast is typically referred to as silencing (Herskowitz *et al.* 1977; Haber and George 1979; Klar and Fogel 1979; Rine *et al.* 1979). Silencing is dependent on Sir2, Sir3, and Sir4, which bind the nucleosomes in the silent regions. The complex of these Sir proteins with the histones is thought to comprise the structural unit of heterochromatin in yeast (Grunstein 1997; Rusche *et al.* 2003).

Silencing is also dependent on DNA control elements associated with each of the silent regions. Two control regions are associated with *HML*, the *HML-E* and *HML-I* silencers, and two with *HMR*, the *HMR-E* and *HMR-I* silencers (Brand *et al.* 1985; Mahoney and Broach 1989; Rivier *et al.* 1999). The telomeres, which are made up of the terminal telomeric repeats and the physical end of the chromosome, are one type of control region essential for telomeric silencing (Gottschling *et al.* 1990; Tham and Zakian 2002). In addition, some telomeres contain short tandem repeats (STR), which also contribute to telomeric silencing (Louis 1995; Mondoux and Zakian 2007). Four of these control regions—*HML-E*, *HML-I*, *HMR-E*, and the telomeres—can act autonomously to direct silencing in the absence of any other control region (Loo and Rine 1995; Rusche *et al.* 2003). The other two, *HMR-I* and the STRs, contribute to the overall efficiency of silencing at their respective loci but cannot direct silencing autonomously (Rivier *et al.* 1999; Mondoux and Zakian 2007).

Four DNA-binding proteins—Rap1, Orc (the origin recognition complex), Ku, and Abf1—bind to silencers, telomeres, and/or STRs and play a role in silencing. Three of these proteins—Rap1, Orc and Abf1—bind specific DNA sequences (Shore 1994; Loo and Rine 1995; Grunstein 1997; Rusche *et al.* 2003), whereas Ku

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binds specifically to DNA ends (Gravel *et al.* 1998; Ribes-Zamora *et al.* 2007). The binding sites for these proteins that contribute to silencing are contained within the control regions that direct silencing. Of these four proteins, the roles of Rap1, Orc, and Ku in silencing are most well understood. Rap1 is a general regulator of silencing that acts at all three silent loci, *HML*, *HMR*, and the telomeres. Binding sites for Rap1 are found in control regions associated with each of the three silent loci *HML*, *HMR*, and the terminal telomeric repeats (Shore and Nasmyth 1987; Buchman *et al.* 1988; Hofmann *et al.* 1989; Longtine *et al.* 1989; Kurtz and Shore 1991). Furthermore, mutations in *RAP1* result in silencing defects at *HML*, *HMR*, and the telomeres (Kyrion *et al.* 1993; Moretti *et al.* 1994).

In contrast, Ku appears to be a locus-specific regulator of silencing that acts in some silent regions but not in others. In particular, deletion of the gene encoding either subunit of the Ku heterodimer results in complete loss of telomeric silencing (Mishra and Shore 1999; Teo and Jackson 2001) but no detectable loss in silencing at *HML* or *HMR* (Boulton and Jackson 1998; Laroche *et al.* 1998; Nugent *et al.* 1998; Martin *et al.* 1999; Gartenberg *et al.* 2004; Roy *et al.* 2004). Also, a Ku-binding site (a DNA end) is found at the telomere but not at the internal *HML* or *HMR* loci (Gravel *et al.* 1998). Thus, Ku is thought to be a locus-specific regulator of silencing that acts at the telomeres but not at *HML* or *HMR*.

The focus of this work is to further understand the role of Ku in silencing. As described above, Ku is not thought to act at *HML* and *HMR*; however, the evidence that Ku does not act at these internal loci is limited. *HML* and *HMR* are internal loci that are not associated with DNA ends and therefore do not contain a DNA-binding site for Ku; however, some DNA-binding proteins have two modes of binding to DNA: one mode resulting from the DNA-binding activity intrinsic to the protein itself and another mode resulting from protein–protein interactions (Valenzuela *et al.* 2008). If Ku is endowed with two modes of binding DNA, then the fact that *HML* and *HMR* lack a DNA-binding site for Ku is not sufficient evidence to conclude that Ku does not bind these loci. Furthermore, although neither subunit of Ku is required for silencing *HML* or *HMR* (Boulton and Jackson 1998; Laroche *et al.* 1998; Nugent *et al.* 1998; Martin *et al.* 1999; Gartenberg *et al.* 2004; Roy *et al.* 2004), as they are for telomeric silencing (Mishra and Shore 1999; Teo and Jackson 2001), silencing at *HML* and *HMR* is more efficient than silencing at the telomeres and at least some DNA elements within silencers are known to be redundant. Therefore, it is possible that the increased efficiency and redundancy of silencing at *HML* and *HMR* has masked a possible role of Ku in silencing at these loci.

The goal of this work was to test directly whether Ku plays a role in silencing at *HML* and *HMR*. We show that Ku contributes to silencing at both *HML* and *HMR*, that Ku binds both these internal loci, and that binding of Ku to *HML* and *HMR* is dependent on Sir4.

MATERIALS AND METHODS

Strain construction: Strains used in this study are isogenic to W303-1a and are listed in Table 1. Strains were constructed by cross or PCR-mediated gene disruption (Baudin *et al.* 1993) and confirmed by PCR or DNA blots. The *kanMX4* gene of plasmid pFA6-*kanMX4* (Wach *et al.* 1994) and the *natMX4* gene of plasmid pFA6-*natMX4* (Goldstein and Mccusker 1999) were integrated into pBluescript to make pDR759 and pDR1848, respectively, and to avoid any growth defects associated with auxotrophic markers. The plasmids were PCR amplified by hybrid disruption primers containing homology to both a specific gene and pUC1 or pUC2 sequences, as previously described (Replogle *et al.* 1999). The gene-specific confirmation primers were used in conjunction with pUC complement sequences to confirm PCR-mediated gene disruptions. All PCR-mediated gene disruption and confirmation primers are listed in Table 2.

Strains DRY5430-5433 and DRY5438-5440, used in quantitative mating analysis, were derived from a cross of DRY5397 and DRY5401 (W303-1a; *hdf1Δ::natMX4 sir1Δ::kanMX4 lys2Δ-::hisG*). Strains DRY5406 and DRY5408, DRY5414 and DRY5416, and DRY5418 and DRY5420, used in *HMR::ADE2* color assays, were generated by PCR-mediated gene disruption in strains DRY829, DRY826, and DRY815, respectively. Strains DRY5402 and DRY5404, used in the *HMR::URA3pr-ADE2* growth assays, were generated by PCR-mediated gene disruption in DRY1667. Strains DRY5119, DRY5136, DRY5122, DRY5125, DRY5129, and DRY5137, used in two-hybrid analysis, were generated by PCR-mediated gene disruption of PJ69-4A (DRY2805) (James *et al.* 1996). These strains were transformed with the two-hybrid plasmids pDR1344 (*pGBD-KU70*) and pDR1473 (*pGAD-SIR4*, aa1205–1348) to produce strains DRY5447, DRY5456, DRY5474, DRY5483, DRY5492, and DRY5501. Strain DRY3762 (*KU70-MYC9-TRP1*), used in chromatin immunoprecipitation (ChIP), was generated by the addition of a sequence that encoded by nine copies of the Myc epitope to the C-terminal end of the native *YKU70* gene. In particular, a segment of pYM6 (Knop *et al.* 1999) was PCR amplified using primers DHR265 and DHR266 and the resting product was transformed into strain DRY3348. PCR confirmation for correct integration was as previously described (Knop *et al.* 1999). Strain DRY5539 was generated by disrupting *SIR4* in DRY5532, a derivative of DRY3762.

Media and genetic manipulations: Rich medium (YPD) and minimal medium (YM) were as described (Sherman 1991). Medium for red/white colony assays was as described (Gottschling *et al.* 1990), except no L-aspartic acid was added. The *kanMX4* genes were selected for on YPD containing 200 mg/liter G418 and *natMX4* genes were selected for on YPD containing 100 mg/liter nourseothricin. Transformation was by a modified lithium–acetate method (Gietz and Schiestl 1991).

Quantitative and patch mating analysis: Quantitative matings were performed as described previously (Xu *et al.* 1999). Strains JRY2726 and JRY2728 were used as tester strains. Values reported are the average of a minimum of three independent trials.

Two-hybrid analysis: The two-hybrid analysis was as described previously (James *et al.* 1996). An *Eco*RI/*Bam*HI *HDF1* fragment was amplified by PCR using primers DHR195 and DHR196 and cloned into plasmid pDR1406 [pGBD (c1)] to generate plasmid pDR1344. A *Bam*HI/*Pst*I *Sir4* (aa 1205–

TABLE 1

Strains used in this study

Strain	Genotype
DRY705	*MAT*α *his3-11 leu2-3,112 lys2*Δ*::hisG trp1-1 ura3-1*
DRY5430	*MAT***a** *ADE2 lys2*Δ*::hisG*
DRY5431	*MAT***a** *yku70*Δ*::natMX4 ADE2 lys2*Δ*::hisG*
DRY5432	*MAT***a** *sir1*Δ*::kanMX4 ADE2 lys2*Δ*::hisG*
DRY5433	*MAT***a** *yku70*Δ*::natMX4 sir1*Δ*::kanMX4 ADE2 lys2*Δ*::hisG*
DRY5438	*MAT*α *yku70*Δ*::natMX4 ADE2 lys2*Δ*::hisG*
DRY5439	*MAT*α *sir1*Δ*::kanMX4 ADE2 lys2*Δ*::hisG*
DRY5440	*MAT*α *yku70*Δ*::natMX4 sir1*Δ*::kanMX4 ADE2 lys2*Δ*::hisG*
DRY829	*MAT*α *HMR::ADE2 ade2::HIS3 his3-11 leu2-3,112 trp1-1, ura3-1*
DRY5406	*sir1*Δ*::kanMX4* in DRY829
DRY5408	*yku70*Δ*::natMX4* in DRY829
DRY826	*MAT*α *HMR::ADE2* Δ*I ade2::HIS3 his3-11 leu2-3,112 trp1-1, ura3-1*
DRY5414	*sir1*Δ*::kanMX4* in DRY826
DRY5416	*yku70*Δ*::natMX4* in DRY826
DRY815	*MAT*α *HMR-SS::ADE2 ade2::HIS3 his3-11 leu2-3,112 trp1-1, ura3-1*
DRY5418	*sir1*Δ*::kanMX4* in DRY815
DRY5420	*yku70*Δ*::natMX4* in DRY815
DRY640 (JRY2334)[a]	*MAT*α *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100*
DRY707	*MAT***a** *ADE2 his3-11 leu2-3,112 lys2::hisG trp1-1 ura3-1*
DRY1667	*MAT*α *HMR::URA3pr-ADE2 ade2::HIS3*
DRY1665	*MAT*α *HMR::URA3pr-ADE2* Δ*I ade2::HIS3*
DRY5404	*MAT*α *HMR::URA3pr-ADE2 yku70*Δ*::natMX4 ade2::HIS3*
DRY5402	*MAT*α *HMR::URA3pr-ADE2 sir1*Δ*::kanMX4 ade2::HIS3*
DRY3348	*MAT***a** *TELVIIL::URA3 ADE2 LYS2 ura3*Δ*0::kanMX4*
DRY3762	*MAT***a** *YKU70-MYC9-TRP1* in DRY3348
DRY5539	*MAT*α *sir4*Δ*::natMX4 YKU70-MYC9-TRP1 ADE*
DRY2805 (PJ69-4A)[b]	*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*
DRY5119	*sir1*Δ*::kanMX4* in DRY2805
DRY5122	*sir3*Δ*::kanMX4* in DRY2805
DRY5125	*sir4*Δ*::kanMX4* in DRY2805
DRY5129	*yku70*Δ*::kanMX4* in DRY2805
DRY5136	*sir2*Δ*::kanMX4* in DRY2805
DRY5137	*yku80*Δ*::kanMX4* in DRY2805
DRY5447	pDR1344 (*pGBD-YKU70*) and pDR1473 (*pGAD-Sir4*, aa 1205–1348) in DRY2805
DRY5448	pDR1473 (*pGAD-Sir4*, aa 1205–1348) and pDR1406 (*pGBD-TRP1-empty*) in DRY2805
DRY5449	pDR1344 (*pGBD-YKU70*) and pDR1405 (*pGAD-LEU2-empty*) in DRY2805
DRY5456	pDR1344 (*pGBD-YKU70*) and pDR1473 (*pGAD-Sir4*, aa 1205–1348) in DRY5129
DRY5465	pDR1344 (*pGBD-YKU70*) and pDR1473 (*pGAD-Sir4*, aa 1205–1348) in DRY5137
DRY5474	pDR1344 (*pGBD-YKU70*) and pDR1473 (*pGAD-Sir4*, aa 1205–1348) in DRY5119
DRY5483	pDR1344 (*pGBD-YKU70*) and pDR1473 (*pGAD-Sir4*, aa 1205–1348) in DRY5136
DRY5492	pDR1344 (*pGBD-YKU70*) and pDR1473 (*pGAD-Sir4*, aa 1205–1348) in DRY5122
DRY5501	pDR1344 (*pGBD-YKU70*) and pDR1473 (*pGAD-Sir4*, aa 1205–1348) in DRY5125

All strains are isogenic to W303-1a.
[a] From J. Rine.
[b] From E. Craig.

1348) fragment was amplified by PCR using primers DHR239 and DHR240 and cloned into plasmid pDR1405 [pGAD (c1)] to generate plasmid pDR1473. The pGBD and pGAD plasmids were sequentially transformed into the prepared two-hybrid deletion strains.

Chromatin immunoprecipitation: Chromatin immunoprecipitations of strains DRY3762 and DRY5539 were performed as previously described (Meluh and Broach 1999). Crosslinking was performed at a final concentration of 1% formaldehyde for 15 min. Genomic DNA was sonicated to fragments in a 200- to 400-bp range using both a Diagenode Bioruptor (20; 30-sec pulses with 1-min intervals on ice) and a Branson Sonifier 450 (sonicate 5 min, 20 sec on high, stopping to change the ice bath every 80 sec). A monoclonal mouse 9E11 Myc antibody from Abcam (ab56) was used to precipitate the DNA. Bound DNA was eluted from a Calibiochem Protein G Plus/Protein A Agarose suspension using a 10% BioRad Chelex-100 molecular grade resin solution.

Real-time PCR and data analysis: Quantitative ChIP analysis was performed on a Corbett Research Rotor-Gene RG3000A instrument using Invitrogen Platinum SYBR Green qPCR SuperMix-UDG as the detection dye. The primers used in the real-time PCR are listed in Table 2 and were screened prior to use for similar melting temperatures and amplification efficiencies. Real-time PCR was carried out as follows: step 1—50° for 2 min; step 2—95° for 2 min; step 3—95° for 15 sec; step 4—53° for

TABLE 2

Primers used in this study

Region	Primer
	Chromatin immunoprecipitation
HMR-E	1. gttttaacattacgtatcttgtaccc
	2. tgcaaatgtggaggaaaagaaatgcg
HMR-I	1. gaagagacttatgatcaacataattttgc
	2. catatacgaaaatgttggtgacatgtaatc
HML-E	1. taaagttttcggcacggacttatttgg
	2. atgtgcgctagatataaaaatcttattgtg
HML-I	1. cgaaatttctctaatgccagctgagt
	2. tgaaaataatcgggtgaaaaagagga
GIT1	1. ctaggttagctatggtaacgag
	2. gggaatggaaatatatggtatagcg
0.5 TEL	1. gacaaataaaaattcagctttttcaag
	2. gttcgaatccttaagtaaaacacattc
7.5 TEL	1. gtggaaagtatcgagttatgtgtacct
	2. gtcattcaaatacagtgggaagtctac
	PCR-mediated gene disruption
KU70 disruption	1. tcaacagtaaagctatgatttgttaagtgactctaagcctgattttaaaacgggaatatt-
	2. ataccctaccctaccaaatattgtatgtaacgttatagatatgaaggatttcaatcgtct-
KU70 confirmation	1. ccgactgaatgcggccgcctcaatttcatgtattaggg
	2. ccgactgaatgcggccgcaggttttttgagaatgccgc
KU80 disruption	1. aaaacctaattaacgagagtgcaggacatatgcacaaataatatatctcacaccataata-
	2. ttttttttctctttaactgtggtgacgaaaacataactcaaaggatgttagacctttt-
KU80 confirmation	1. ccgactgaatgcggccgcgctgcatacataattctc
	2. ccgactgaatgcggccgcgcagtcatccagattctg
SIR1 disruption	1. aaagtttgtcgcgagaatttgggcacatgtgacccggaatgtatattgagtaatataaga-
	2. tgaaatgagacatcacccgcttatatgttggtatccataactgataatcttaccaactat-
SIR1 confirmation	1. ccgactgaatgcggccgcactaagaagccggacctagg
	2. ccgactgaatgcggccgcccacccacgcattattgtcgg
SIR2 disruption	1. aggcatcgcttcggtagacacattcaaaccatttttccctcatcggcacattaaagctgg-
	2. tgccatactatgtaaattgatattaatttggcacttttaaattattaaattgccttctac-
SIR2 confirmation	1. ccgactgaatgcggccgcggtccaggacagccaggacc
	2. ccgactgaatgcggccgcgctgttccacctgcccttc
SIR3 disruption	1. atcaccttccttacaggggtttaagaaagttgttttgttctaacaattggattagctaaa-
	2. gaagagactgcatgtgtacataggcatatctatggcggaagtgaaaatgaatgttggtgg-
SIR3 confirmation	1. ccgactgaatgcggccgccaggggaacaaagtattcggg
	2. ccgactgaatgcggccgcgagtcctggaatttccagcgg
SIR4 disruption	1. gggataaaaaaaaaaaaggaagcttcaacccacaataccaaaaaagcgaagaaaacagcca-
	2. aaaacgacaaagaaaaacagggtacacttcgttactggtcttttgtagaatgataaaaag-
SIR4 confirmation	1. ccgactgaatgcggccgccgtccttaaacatgtgcac
	2. ccgactgaatgcggccgcggcaaggtcggtttggatgac
pUC1[a]	-ccggctcgtatgttgtgtgg (attach to 3′-end disruption primer 1)
pUC2 [a]	-cgacgttgtaaaacgacggcc (attach to 3′-end disruption primer 2)
Complement to pUC1	ccacacaacatacgagccgg
Complement to pUC2	ggccgtcgttttacaacgtcg
	Yeast two-hybrid construction
DHR195	gcccggaattcatgcgctcagtcactaatgc
DHR196	ctcgcggatccttatatattgaatttcggc
DHR239	gcccggatccgatcgtcgagtgaaacaactc
DHR240	gcccgctgcagtcaatacggttttatctcc
	Myc epitope tag for YKU70
DHR265	gataacatttcgataaaagaagaaaagaagccctttgataaaaagccgaaattcaatatacgtacgctgcaggtcgac
DHR266	ataccctaccctaccaaatattgtatgtaacgttatagatatgaaggatttcaatcgtctatcgatgaattcgagctcg

All primers are described in the 5′ to 3′ direction.
[a] From D. Rivier.

15 sec; step 5—68° for 20 sec (40 cycles: steps 3–5); step 6—melt curve from 68° to 95° and then hold at 4°. The annealing temperature in step 4 was increased to 58° for the *HML* primers in comparison to the *HMR* primers to decrease the number of primer dimers. The same amount of DNA, 2.5 μl of each sample and 2.5 μl of 1/1000 dilution of input, was analyzed in duplicate

TABLE 3

Mating efficiencies of strains with different alleles of *SIR1* and *YKU70*

Relevant genotype	Mating efficiencies	
	HMR	*HML*
Wild type	1.0	1.0
*yku70*Δ*::natMX4*	1.05 ± 0.11	0.81 ± 0.14
*sir1*Δ*::kanMX4*	0.72 ± 0.13	0.65 ± 0.10
*yku70*Δ*::natMX4 sir1*Δ*::kanMX4*	$3.6 \times 10^{-2} \pm 4.0 \times 10^{-3}$	0.26 ± 0.14

All strains were isogenic to W303. At least three independent mating assays were performed on all strains and the averages and standard error are reported. *MAT*α strains were used to determine mating efficiencies for *HMR*, and *MAT***a** strains were used to determine mating efficiencies for *HML*. Strains tested were DRY705 (wild type), DRY5438 (y*ku70*Δ), DRY5439 (*sir1*Δ), and DRY5440 (y*ku70*Δ *sir1*Δ) at *HMR* and DRY5430 (wild type), DRY5431 (y*ku70*Δ), DRY5432 (*sir1*Δ), and DRY5433 (y*ku70*Δ *sir1*Δ) at *HML*.

for each primer set to determine a relative increase in DNA from the immunoprecipitation (IP) compared to the input DNA. The C_T values were between 20 and 30 cycles, which indicated that the values were within the linear range. The fold increase was then normalized to the *7.5 TEL* primer set. The standard error for a minimum of three crosslinks and five IPs was calculated for each strain and primer set.

RESULTS

Ku contributes to silencing at the internal *HML* and *HMR* loci: As an initial test of whether Ku contributes to silencing at *HML* or *HMR*, we performed quantitative mating-type assays. Wild-type *MAT*α cells can mate with *MAT***a** cells; however, disruption of silencing in *MAT*α cells results in the nonmating phenotype due to the simultaneous expression of the *MAT*α genes and the *HMR***a** genes. Similarly, disruption of silencing in *MAT***a** cells results in transcription of the *MAT***a** genes and the *HML*α genes, also resulting in the nonmating phenotype. To test whether Ku contributes to silencing at *HMR*, we constructed a *MAT*α strain in which the entire coding region of *YKU70* was deleted (*yku70*Δ) and performed quantitative mating analysis (Table 3). This *yku70*Δ strain had the same mating efficiency as an isogenic wild-type strain, confirming previous observations that Ku is not required for silencing at *HMR*. In contrast, an isogenic *sir1*Δ strain had a slightly reduced mating efficiency of 0.72 relative to the wild-type strain, consistent with previous results (Pillus and Rine 1989). To test whether Ku contributes to the overall efficiency of silencing at *HMR*, we compared the mating efficiency of a strain lacking both *YKU70* and *SIR1* (*yku70*Δ *sir1*Δ) to the isogenic wild-type strain and to the two isogenic *yku70*Δ and *sir1*Δ strains. The mating efficiency of the *yku70*Δ *sir1*Δ strain relative to the wild-type strain was 0.036, a 28-fold reduction in silencing relative to the wild-type strain and a 20-fold reduction relative to the *sir1*Δ strain. These results suggest that yKu70 normally contributes to silencing at the wild-type *HMR***a** locus and, furthermore, that Sir1 and Ku are collectively required for efficient silencing at *HMR*.

To test for a role of Ku in silencing *HML*, quantitative mating analysis was performed on an isogenic set of *MAT***a** strains: wild-type, *yku70*Δ, *sir1*Δ, and *yku70*Δ *sir1*Δ (Table 3). The *yku70*Δ strain mated with an efficiency of 0.81 relative to the wild-type strain, suggesting that loss of yKu70 function alone is sufficient to result in a slight silencing defect at *HML*. The mating efficiency of the *yku70*Δ *sir1*Δ strain was 0.26 relative to the wild-type strain and was less than either the *yku70*Δ strain or the *sir1*Δ strain, further suggesting that yKu70 contributes to silencing at the wild-type *HML*α locus.

Silencing is a general mechanism of repression that can inhibit transcription directed by a variety of different promoters. In principle, the results presented above could be due to a role for Ku in specifically acting on the mating-type gene promoters, rather than due to a role for Ku in silencing *per se* at *HML* and *HMR*. As a second test for a role of Ku in silencing at an internal locus, we determined whether loss of yKu70 function resulted in an increase in expression of a reporter gene. The yeast *ADE2* gene can serve as a reporter gene that sensitively detects a reduction in the efficiency of silencing when it is inserted into *HMR* (*HMR::ADE2*) (Gottschling *et al.* 1990; Sussel *et al.* 1993; Maillet *et al.* 2001). Yeast cells that do not transcribe *ADE2* form red colonies on media containing adenine in contrast to wild-type cells that form white colonies. *HMR::ADE2* cells form red colonies on media containing adenine, whereas *HMR::ADE2* strains in which silencing is disrupted form white colonies, or pink colonies if silencing is partially disrupted (Sussel *et al.* 1993; Rivier *et al.* 1999). In addition, we previously determined that this reporter assay is more sensitive to silencing defects than quantitative mating assays (Rivier *et al.* 1999). As observed previously, an *HMR::ADE2* strain displays the red color phenotype, whereas an isogenic strain in which the *SIR1* coding region was deleted (*sir1*Δ *HMR::ADE2*) displays a white phenotype (Figure 1). In contrast, an isogenic strain lacking the entire *YKU70* coding region displayed a pink color phenotype, indicating that Ku contributes to silencing at *HMR* and that Ku's contribution to the overall efficiency of silencing at *HMR* is less than that of Sir1.

HMR::ADE2 strains that also have a mutant version of the *HMR* silencers also provide a sensitive background for monitoring silencing. One such strain lacks the *HMR-I* silencer (*HMR::ADE2*Δ*I*) and another contains a reduced function allele of the *HMR-E*, known as the synthetic silencer (*HMR-SS::ADE2*), which has been studied extensively (McNally and Rine 1991). Both the *HMR::ADE2* Δ*I* strain and the *HMR-SS::ADE2* strain display a lighter color phenotype than wild type, indicating that silencing is partially disrupted in each case. However, both strains can display a red color phenotype

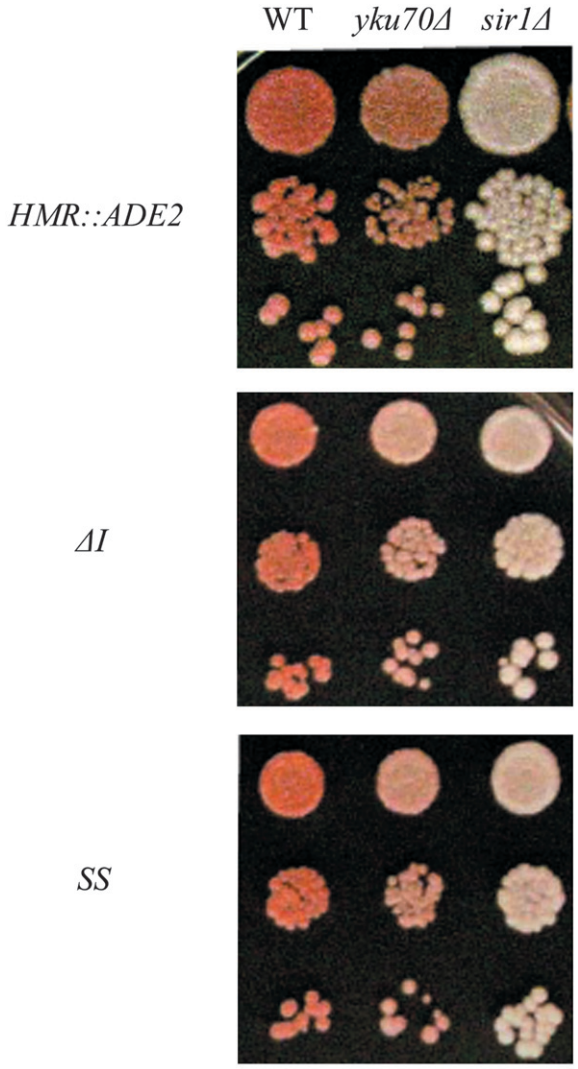

Figure 1.—yKu70 contributes to silencing of the *HMR::ADE2* reporter gene in three genetic contexts: in the presence of the wild-type (WT) *HMR* silencers (top), in the absence of the *HMR-I* silencer (middle), and when *HMR* is replaced by the synthetic silencer (bottom). (Top) A 10-fold dilution series of strains DRY829 (*HMR::ADE2*), DRY5408 (*yku70*Δ *HMR::ADE2*), and DRY5406 (*sir1*Δ *HMR::ADE2*). (Middle) A 10-fold dilution series of strains DRY826 (*HMR::ADE2* Δ*I*), DRY5416 (*yku70*Δ *HMR::ADE2* Δ*I*), and DRY5414 (*sir1*Δ *HMR::ADE2* Δ*I*). (Bottom) A 10-fold dilution series of strains DRY815 (*HMR-SS::ADE2*), DRY5420 (*yku70*Δ *HMR-SS::ADE2*), and DRY5418 (*sir1*Δ *HMR-SS::ADE2*).

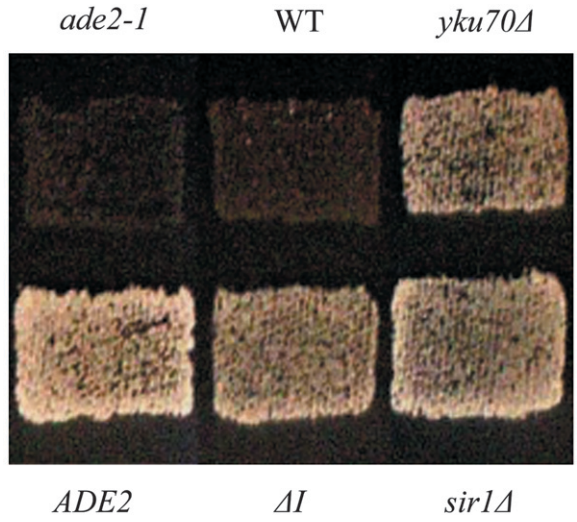

Figure 2.—Loss of yKu70 function results in expression of the *HMR::URA3pr-ADE2* reporter gene as revealed by growth on media lacking adenine. Strains shown are DRY640 (*HMR***a** *ade2-1*), DRY707 (*HMR***a** *ADE2*), DRY1667 (*HMR::URA3pr-ADE2*), DRY1665 (*HMR::URA3pr-ADE2* Δ*I*), DRY5404 (*HMR::URA3pr-ADE2 yku70*Δ), and DRY5402 (*HMR::URA3pr-ADE2 sir1*Δ).

if given sufficient time (Figure 1). Deletion of *YKU70* from the *HMR::ADE2* Δ*I* strain resulted in a lighter color phenotype than the isogenic wild-type strain but not as light a color phenotype as an isogenic strain in which *SIR1* was deleted (Figure 1). Deletion of *YKU70* from the *HMR-SS::ADE2* strain resulted in a lighter color phenotype than the isogenic wild-type strain but not as light a color phenotype as an isogenic strain in which *SIR1* was deleted (Figure 1). Collectively, these three related assays indicate that Ku contributes to silencing at *HMR*, that Ku is not required for silencing at *HMR*, and that the contribution of Ku to the overall efficiency of silencing at *HMR* is less than the contribution of Sir1.

The *URA3pr-ADE2* chimeric gene provides an assay for silencing that complements those described above (Rivier *et al.* 1999). In the case of this chimeric gene, transcription of the *ADE2* coding region is driven by the promoter region of the *URA3* gene; hence this gene provides the opportunity to study silencing of another type of promoter that is distinct from the promoters of the **a**-genes, the α-genes, and the *ADE2* gene described above. Previously, we determined that the *URA3pr-ADE2* reporter gene is silenced when inserted into *HMR* and that it provides a more sensitive metric of silencing than quantitative mating-type assays (Rivier *et al.* 1999). Wild-type yeast cells can grow on minimal media that lacks adenine whereas cells that do not express *ADE2* cannot. *HMR::URA3pr-ADE2* cells cannot grow on media lacking adenine because the *HMR::URA3pr-ADE2* gene is silenced. Disruption of silencing results in transcription of *HMR::URA3pr-ADE2* and the ability to grow on media lacking adenine. Thus, *HMR::URA3pr-ADE2* provides a gain-of-function phenotype for disruption of silencing. As a third test of whether Ku contributes to silencing at *HMR*, the entire *YKU70* coding region was deleted from an *HMR::URA3pr-ADE2* strain (*yku70*Δ *HMR::URA3pr-ADE2*). As can be seen in Figure 2, the *yku70*Δ *HMR::URA3pr-ADE2* strain is capable of growth on media lacking adenine, whereas the wild-type *HMR::URA3pr-ADE2* strain is not, providing a third line of evidence that Ku contributes to silencing at *HMR*.

Collectively, our results indicate that Ku contributes to silencing at *HML* and *HMR*. In conjunction with previous observations that Ku is required for telomeric

silencing, these results also reveal that Ku makes a contribution to silencing at *HML* and *HMR* different from the one it does at the telomeres. In particular, Ku is required for telomeric silencing, whereas Ku contributes to the overall efficiency of silencing at *HML* and *HMR* but is not absolutely required for silencing of these internal loci.

Ku binds *HML* and *HMR*: In principle, Ku could contribute to silencing at *HML* and *HMR* by acting directly as a component of the silencing machinery. Alternatively, since Ku regulates multiple chromosomal processes, it is possible that loss of Ku function disrupts the overall physiology of the cell in such a way that silencing is compromised indirectly (Bertuch and Lundblad 2003; Daley *et al.* 2005; Fisher and Zakian 2005). These two possibilities make distinct predictions. If Ku acts directly in silencing at *HML* and *HMR*, it is expected that Ku would bind these loci. In contrast, if loss of Ku function disrupts silencing at *HML* and *HMR* indirectly, it is predicted that Ku would exert its effect on silencing by acting at some other chromosomal locus or independently of DNA. In this case, Ku is not expected to bind to *HML* or *HMR*.

To determine whether Ku binds to *HML* or *HMR*, we performed quantitative ChIP experiments. The endogenous copy of the *YKU70* gene was modified so that the yKu70 protein produced would contain nine copies of the myc epitope at the C terminus (Knop *et al.* 1999). The resulting strain (*YKU70-MYC9*) displayed no growth defects and a telomeric reporter gene was silenced to the same extent in the *YKU70-MYC9* strain as in an isogenic wild-type strain, indicating that the epitope tag did not compromise Ku function (data not shown). To determine whether Ku bound to *HMR*, we performed quantitative ChIP analysis of binding of yKu70-9myc to *HMR* relative to *GIT1*, a gene that is adjacent to *HMR* but is outside the silenced region and is not bound by the silencing machinery. Each value reported was the average of a minimum of three independent crosslinking reactions and five immunoprecipitations. Using a set of primers specific for *HMR-E*, we found that the *HMR-E* DNA was enriched 3.2-fold (±0.56) in the immunoprecipitated DNA relative to *GIT1* (Figure 3). That *HMR-E* DNA is enriched to a statistically significant extent relative to *GIT1* reveals that yKu70 binds specifically to *HMR* in the vicinity of the *HMR-E* silencer. However, the absolute magnitude of the enrichment, 3.2-fold in this case, which is modest relative to quantitative enrichment values for some DNA-binding proteins, has no unique interpretation. For example, modest enrichment values could result from transient association of a protein with a specific region or could be the result of the protein being tethered to a region via protein–protein interactions rather than by directly contacting the DNA. Alternatively, the epitope could be partially masked when the protein is bound to DNA, resulting in inefficient immunoprecipitation. Therefore, while the data presented here reveal

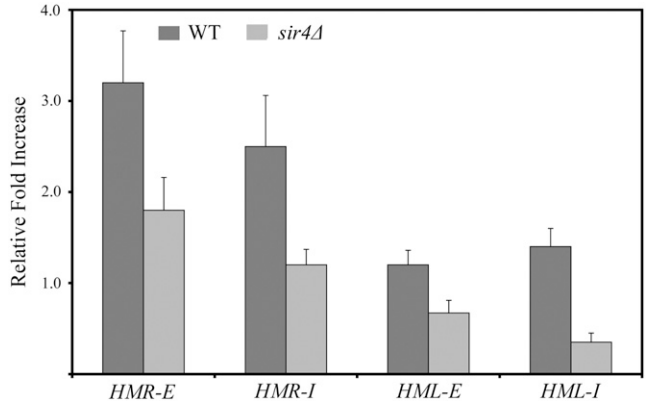

Figure 3.—yKu70 is recruited to *HMR* and *HMR* silencer regions in a Sir4-dependent manner as revealed by chromatin immunoprecipitation and real-time quantitative PCR. Fold enrichment values are normalized to the 3′ *GIT1* gene adjacent to *HMR* for the wild-type strain DRY3762 (darkly shaded bar) and the *sir4*Δ strain DRY5539, (lightly shaded bar).

that Ku binds *HMR* in the vicinity of *HMR-E*, they do not provide a detailed insight into the nature or dynamics of binding. Using the set of primers specific for *HMR-I*, we found that *HMR-I* DNA was enriched 2.5-fold (±0.56) relative to *GIT1* (Figure 3). This observation indicates that the enrichment of *HMR* DNA observed in the immunoprecipitated DNA is independent of the set of primers used and provides further evidence that Ku binds to *HMR*. As described above, we selected *GIT1* as a control site because *GIT1* is not silenced. However, since Ku has been implicated in chromosomal processes other than silencing, it is not formally known that Ku does not associate with *GIT1*. We therefore repeated each of the quantitative ChIP experiments described above using primers to a second control site that is 7.5 kb from the chromosome 6R telomere, which does not bind Ku or other silencing proteins (Martin *et al.* 1999). In the case of both *HMR-E* and *HMR-I*, we detected a modest but statistically significant enrichment of *HMR* DNA in the immunoprecipitate relative to the chromosome 6R control site (Figure 4). The observation that enrichment *HMR* DNA in the immunoprecipitate relative to both a *GIT1* and the chromosome 6R site indicates that our results are independent of the control site used and provide another line of evidence that Ku binds to *HMR*.

To determine whether Ku binds *HML in vivo*, we also used two sets of primers, one specific for *HML-E* and the other specific for *HML-I*. These two sets of primers were individually used in quantitative PCR on the same DNA samples used for *HMR* above and were run in parallel to the reactions described above. For both *HML-E* and *HML-I*, the ratio of the immunoprecipitated DNA relative to the input DNA was calculated and normalized independently to the two control sites described above. We found that *HML-E* DNA was enriched in the immunoprecipitated DNA to a statistically significant extent relative to both control sites, revealing that yKu70 binds

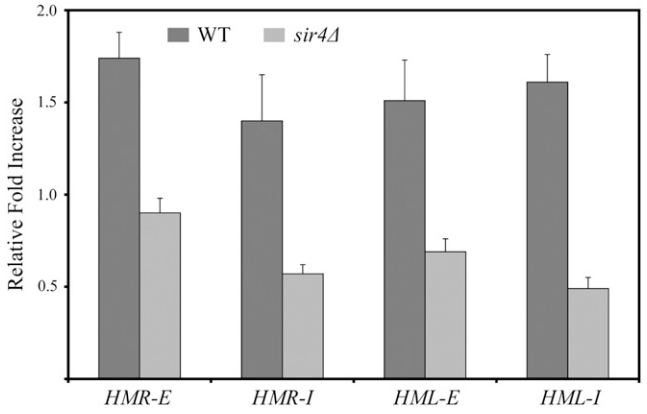

Figure 4.—yKu70 is recruited to *HMR* and *HML* silencer regions in a Sir4-dependent manner as revealed by chromatin immunoprecipitation and real-time quantitative PCR. Fold enrichment values are normalized to the *7.5 TEL* on chromosome 6R for the wild-type strain DRY3762 (darkly shaded bar) and the *sir4*Δ strain DRY5539 (lightly shaded bar).

specifically to *HML* in the vicinity of the *HML-E* silencer (Figure 3; Figure 4). Similarly, *HML-I* DNA was enriched in the immunoprecipitated DNA to a statistically significant extent relative to both control sites, providing another line of evidence that yKu70 binds specifically to *HML* (Figures 3 and 4). Collectively, these results reveal that yKu70, and presumably the Ku heterodimer, bind specifically to the two internal silent loci *HML* and *HMR*.

Binding of yKu70 to *HML* and *HMR* is dependent on Sir4: In principle, there are two mechanisms by which Ku could bind *HML* and *HMR*. Ku could bind DNA directly or, alternatively, Ku could bind *HML* and *HMR* as a result of binding to a protein or protein complex that directly binds DNA at *HML* and *HMR*. If Ku binds the internal silent loci via protein–protein interactions, it is likely that Sir4 plays a central role in that process since Ku interacts with Sir4 in two-hybrid assays. To test this possibility, we performed quantitative ChIP experiments on the *YKU70-MYC9* strain described above and on an isogenic strain in which the entire *SIR4* coding region was deleted (*YKU70-MYC9 sir4*Δ). In analyzing *HMR*, we found that *HMR-E* DNA was enriched 3.2-fold (±0.56) in the immunoprecipitate from the wild-type *YKU70-MYC9* strain relative to *GIT1* but had an ~2-fold reduced enrichment in the *YKU70-MYC9 sir4*Δ strain (1.85-fold ± 0.36) relative to *GIT1*, revealing that the binding of Ku to *HMR* is dependent to a large extent on Sir4 (Figure 3). Furthermore, *HMR-I* DNA was enriched 2.5-fold (±0.56) in the immunoprecipitate from the wild-type *YKU70-MYC9* strain but was not enriched in the *YKU70-MYC9 sir4*Δ strain (1.16-fold ± 0.17) relative to *GIT1*, providing further evidence that binding of Ku to *HMR* is dependent on Sir4 (Figure 3). Similarly, *HMR-E* and *HMR-I* DNA was enriched in the immunoprecipitate from the wild-type *YKU70-MYC9* strain but was not enriched in the *YKU70-MYC9 sir4*Δ strain when the chromosome 6R site was used as the negative control (Figure 4).

To determine whether binding of Ku to *HML* is dependent on Sir4 as it is at *HMR*, we performed quantitative PCR using *HML* primers on this same set of immunoprecipitated DNA samples. Again, we compared the fold enrichment of each of the *HML* silencers relative to both the *GIT1* and the telomere 6R control sites in the wild-type *YKU70-MYC9* strain and the *YKU70-MYC9 sir4*Δ strain. As can be seen in Figures 3 and 4, the enrichment of both *HML-E* and *HML-I* DNA in the immunoprecipitate from the *sir4*Δ strain is reduced to a statistically significant extent relative to the wild-type strain when either *GIT1* (Figure 3) or the chromosome 6R site (Figure 4) is used as the control. Collectively, analysis of Ku binding to each of the four silencers relative to two control sites in the wild-type and *sir4*Δ strains provide eight lines of evidence that Ku binds to *HML* and *HMR* and that binding of Ku to these loci is dependent on Sir4. In these experiments a value of 1.0 for the ratio of enrichment in the wild-type strain relative to the *sir4*Δ strain is expected if Sir4 is required for Ku binding to *HML* or *HMR*, whereas a ratio that is statistically >1.0 but less than the enrichment in the wild-type strain would indicate that Sir4 contributes to the overall efficiency of Ku binding to *HML* or *HMR* but is not absolutely required for Ku binding. All eight experimental observations support the conclusion that binding of Ku to *HML* and *HMR* is more efficient in the presence of Sir4 than in its absence, and seven of the eight observations support the model that Ku does not bind *HML* or *HMR* in the absence of Sir4. Thus, the preponderance of evidence presented here suggests that Sir4 is required for binding of Ku to *HML* and *HMR*; however, we cannot rule out the possibility that there is some residual binding of Ku to *HML* and *HMR* that is independent of Sir4.

The two-hybrid interaction between yKu70 and Sir4 is dependent on Sir2 but not on Sir1, Sir3, or yKu80: Our discovery that Ku plays a role in silencing of *HML* and *HMR*, that Ku binds *HML* and *HMR*, and that binding of Ku to these internal loci is dependent on Sir4 provides additional evidence that the two-hybrid interaction between Sir4 and Ku described previously (Tsukamoto *et al.* 1997; Roy *et al.* 2004) reflects *bona fide* protein–protein interactions that occur *in vivo*. To gain further insight into the nature of the Sir4–yKu70 interaction, we tested whether any of the other Sir proteins influence the interaction between yKu70 and Sir4. We constructed one plasmid that contained *YKU70* fused to the coding region of the Gal4 DNA-binding domain (*pGBD-YKU70*) and a second plasmid in which *SIR4* was fused to the *GAL4* activation domain (*pGAD-SIR4*). Introduction of both *pGBD-YKU70* and *pGAD-SIR4* into a yeast two-hybrid reporter strain resulted in expression of an *ADE2* two-hybrid reporter gene as indicated by adenine prototrophy (Figure 5). The *ADE2* reporter gene was not expressed

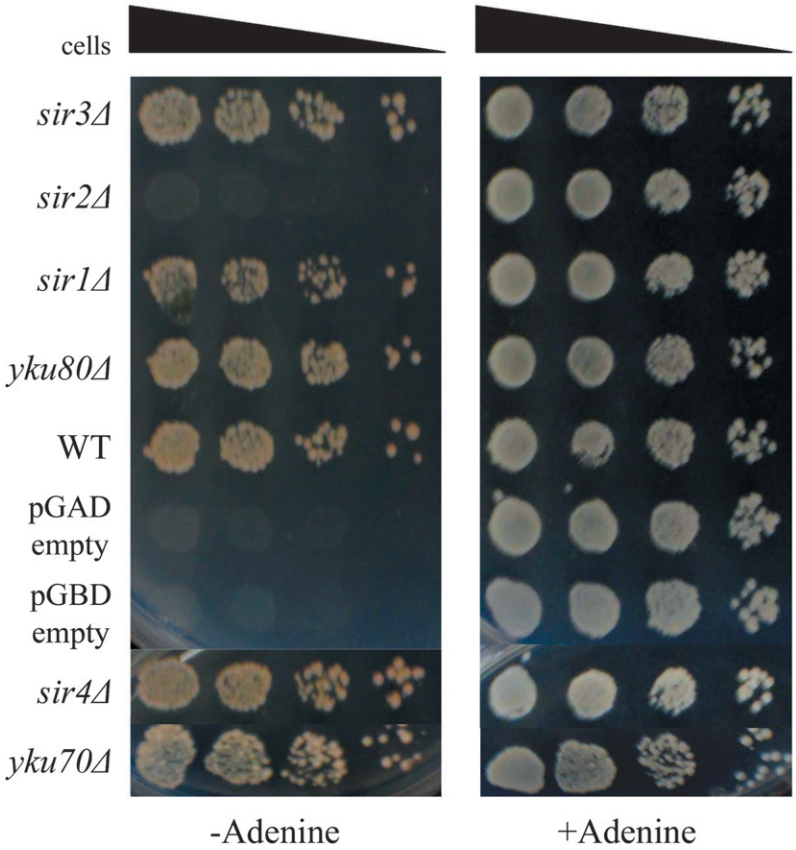

FIGURE 5.—The two-hybrid interaction of yKu70 and Sir4 is dependent on Sir2, but not on Sir1, Sir3, yKu80, or the endogenous copies of Sir4 or yKu70. Strains shown are DRY5492 (*sir3*Δ), DRY5483 (*sir2*Δ), DRY5474 (*sir1*Δ), DRY5465 (*yku80*Δ), DRY5447 (wild-type *pGBD-HDF1* and *pGAD-Sir4*, aa 1205–1348), DRY5449 (*pGAD*-empty), DRY5448 (*pGBD*-empty), DRY5492 (*sir4*Δ), and DRY5456 (*yku70*Δ).

if the *pGBD-YKU70* plasmid and the pGAD plasmid lacking *SIR4* was introduced into the reporter strain, nor was the reporter gene expressed if the pGBD plasmid lacking the *YKU70* fusion was introduced into the reporter strain along with the *pGAD-SIR4* plasmid, indicating that expression of the reporter gene is dependent on a specific interaction between yKu70 and Sir4. These results confirm the previously observed two-hybrid interaction between yKu70 and Sir4, and since the plasmids and strains used here differ from those used previously, it indicates that the yKu70–Sir4 two-hybrid interaction is not dependent on any particular strain, any particular set of plasmids, or any particular reporter gene.

To determine whether any of the Sir proteins were required for the interaction of yKu70 with Sir4, we created a series of isogenic two-hybrid strains, each lacking one of the *SIR* genes (*sir*Δ strains), and determined whether the *pGBD-YKU70* and *pGAD-SIR4* plasmids were capable of driving expression of the reporter gene in those strains. As shown in Figure 5, the reporter gene was expressed in *sir1*Δ and *sir3*Δ strains at the same level as in the wild-type strain, indicating that neither Sir1 nor Sir3 contributed significantly to the two-hybrid interaction between yKu70 and Sir4. In contrast, expression of the two-hybrid reporter gene was abolished in the *sir2*Δ strain. This observation revealed that Sir2 is required for the *in vivo* two-hybrid interaction between yKu70 and Sir4.

To determine whether Ku80 was required for the interaction of Ku70 with Sir4, we created an isogenic two-hybrid reporter strain that lacked the entire coding region of *YKU80* (*yku80*Δ). Introduction of the *pGBD-YKU70* and *pGAD-SIR4* plasmids into the *yku80*Δ strain resulted in expression of the two-hybrid reporter gene at a level similar to that of the wild-type strain; hence, yKu70 can interact with Sir4 independently of yKu80 (Figure 5). Since yKu80 is required for telomeric silencing and can contribute to nucleation when tethered to a defective silencer (MARTIN *et al.* 1999; MISHRA and SHORE 1999), the simplest interpretation of these observations is that yKu70 and yKu80 form the classical Ku heterodimer, which plays a role in silencing in wild-type cells and that each of the yKu70 and yKu80 subunits of the dimer provide sufficient protein–protein contacts to interact with Sir4 in the two-hybrid assay in the absence of the other subunit.

We also reasoned that if the interaction between yKu70 and Sir4 involved multimers of either yKu70 or Sir4, it might be possible that the endogenous yKu70 or Sir4 proteins influence the interaction of GBD-yKu70 with GAD-Sir4. To test this possibility, we created an isogenic set of two-hybrid strains that lacked either *YKU70* (*yku70*Δ) or *SIR4* (*sir4*Δ). As shown in Figure 5,

introduction of the *pGBD-YKU70* and *pGAD-SIR4* into either the *yku70*Δ strain or the *sir4*Δ strain resulted in the same level of expression of the reporter gene as in wild-type cells, suggesting that either yKu70 and Sir4 function as monomers or the GBD–yKu70 and GAD–Sir4 fusion proteins are capable of efficient multimerization.

DISCUSSION

The key discoveries presented here are that the DNA end-binding protein Ku contributes to silencing at *HML* and *HMR*, that Ku binds these internal loci, and that binding of Ku to *HML* and *HMR* is dependent on Sir4. The observation that Ku binds *HML* and *HMR* suggests that Ku plays a direct role in silencing at both of these loci as it does at the telomeres. While these conclusions were drawn from our analysis of yKu70, recent evidence indicates that yKu80 also contributes to silencing of *HML* and *HMR* and physically associates with both of these loci (Patterson and Fox 2008). We therefore propose that the Ku heterodimer is a general regulator of silencing that acts directly at each of the known silent loci in yeast rather than as a locus-specific regulator that acts only at the telomeres. It remains to be determined exactly what role Ku plays in silencing at *HML* and *HMR*. Perhaps the simplest model is that Ku is a subunit of the protein complexes that bind the silencers and plays a role in the nucleation of silencing at *HML* and *HMR* as it does at the telomeres; however, we cannot rule out the possibility that Ku is a structural component of silent chromatin at *HML* and *HMR*.

Our observation that binding of Ku to *HML* and *HMR* is dependent on Sir4 suggests that Ku is tethered to these loci via protein–protein interactions rather than by contacting the DNA directly as a result of any sequence-specific or structure-specific binding property intrinsic to Ku itself. Taken together with previous observations, our results suggest that Ku is endowed with two modes of binding to silent regions of DNA: it can bind telomeric regions directly via its DNA end-binding activity and it can bind *HML* and *HMR* as a result of protein–protein interactions. Previous observations have also implicated Ku in binding to internal chromosomal loci and suggest that Ku may play a role in the activation of transcription and possibly in initiation of replication (Barnes and Rio 1997; Ruiz *et al.* 1999; Novac *et al.* 2001; Walker *et al.* 2001; Schild-Poulter *et al.* 2003; Sibani *et al.* 2005; Grote *et al.* 2006; Shi *et al.* 2007; Rampakakis *et al.* 2008). The data presented here support and extend the data indicating that Ku binds to internal chromosomal loci and broaden our knowledge of the number of processes in which Ku plays a role at internal loci to include silencing.

Our observation that Ku binds *HML* and *HMR* also provides a plausible resolution to a paradox associated with the internal silent loci. Each of the three silent regions in yeast—*HML*, *HMR*, and the telomeres—localize to the nuclear periphery, as do regions of heterochromatin in other eukaryotes. Ku plays a role in the nuclear localization of *HML*, *HMR*, and the telomeres (Gartenberg *et al.* 2004; Taddei and Gasser 2004; Taddei *et al.* 2004). Since Ku binds telomeres, it is thought that Ku plays a direct and central role in nuclear localization of the telomeres. However, given the previous idea that Ku does not bind *HML* or *HMR*, it was not clear how Ku could mediate nuclear localization of these loci. Our discovery that Ku binds *HML* and *HMR* suggests that Ku directly participates in localization of these loci to the nuclear periphery.

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