The association of yeast Ku with subtelomeric core X sequences prevents recombination involving telomeric sequences.

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ABSTRACT

The yKu protein of *Saccharomyces cerevisiae* is important for genome stability by repressing recombination involving telomeric sequences. The mechanism of this repression is not known but silent heterochromatin such as *HML*, *HMR* and telomeres are compartmentalized at the nuclear periphery and yKu is proposed to interact with these regions and play a role in telomeric silencing and tethering. We have utilized ChIP on chip, QPCR and quantitative recombination assays to analyze yKu binding and its effect on genome stability in wild-type and mutant backgrounds. Our data suggests that although yKu binds to the TG1-3 repeats and other parts of the genome when needed, such as during non-homologous-end-joining, it specifically binds to core X sequences in addition to the mating type-loci, *HML* and *HMR*. Association with core X occurred in the absence of Sir-proteins and enhanced binding was observed at silenced ends compared to non-silenced ends. In contrast, binding to *HML* and *HMR* was totally dependent on Sir2-4p and partially-dependent on Sir1p with stronger association at *HML* in both MATa and MATa strains. Using yku80 separation-of-function mutants we show a direct correlation between core X binding and recombination rate. We believe our findings support our hypothesis that yKu and core X play a pivotal role in maintaining genome stability through nuclear architecture, by mediating a defensive fold-back structure at yeast chromosome ends.
INTRODUCTION

*S. cerevisiae* telomeres are protective structures at the end of linear chromosomes that consist of irregular TG-rich repeats that are maintained by telomerase (reviewed in (SMOGORZEWSKA and DE LANGE 2004). Adjacent to these repeats are conserved subtelomeric elements called core X and Y’s, which have no known function. Core X elements are found at every chromosome end, suggesting an important biological function, whereas Y’s are only found at approximately half the chromosome ends (see http://www.nottingham.ac.uk/genetics/people/louis/images/ends-lowres.jpg for map).

The yKu heterodimer is a DNA-binding protein encoded by *YKU70* and *YKU80* (FELDMANN et al. 1996; FELDMANN and WINNACKER 1993) with the ability to recognize exposed ends in a sequence-independent manner. This enables it to be utilized for DNA repair via non-homologous-end-joining (NHEJ reviewed in (AYLON and KUPIEC 2004). Paradoxically, yKu predominantly binds to chromosome termini where NHEJ must be averted to prevent deleterious end fusions, but is not unique for this as the NHEJ regulator, *NEJ1*, has been shown to prevent chromosome end fusions, albeit in telomerase-negative cells (LITI and LOUIS 2003). However, how NHEJ at chromosome termini is prevented still remains to be elucidated. Additional end-related duties for yKu include the recruitment of telomerase, preventing end degradation, tethering telomeres to the nuclear envelope (NE) and promoting subtelomeric epigenetic silencing known as the telomere position effect, or TPE (DOWNS and JACKSON 2004; THAM and ZAKIAN 2002). These additional roles are not fully understood, but may be manifested through the formation of a nucleoprotein telosome structure that protects the end of linear chromosomes.
Deletion of *YKU70* or *YKU80* results in the loss of all yKu heterodimer-associated activity, with both null-mutants displaying identical phenotypes (DOWNS and JACKSON 2004). One such phenotype is a reduction of the telomere repeats (PORTER et al. 1996) as yKu genetically interacts with a number of telomerase components including Cdc13p, Tlc1p, Est1p and Est2p (FISHER et al. 2004; GRANDIN et al. 2000; MEIER et al. 2001; PETERSON et al. 2001; STELLWAGEN et al. 2003). However, it is still unclear whether the loss of telomeric DNA in yKu mutants is primarily due to its interaction with telomerase-associated proteins, or through the loss of some additional end-protection properties.

Studies of *yku80* differentiation-of-function mutants have described alleles proficient for NHEJ and chromosome end-binding, but defective for telomeric-associated functions (BERTUCH and LUNDBLAD 2003). Furthermore, it has been proposed that telomeric and subtelomeric functions of yKu are separable activities, with the former resulting from direct binding to DNA and the latter through protein-protein interactions. (BERTUCH and LUNDBLAD 2003). Studies have shown that each yKu subunit plays a specialized functional role through α-helix 5 motifs, with yKu70p mainly responsible for NHEJ activity and yKu80p for telomeric functions. (BERTUCH and LUNDBLAD 2003; PALMBOS et al. 2005; RIBES-ZAMORA et al. 2007; STELLWAGEN et al. 2003). To explain this a ‘two faced model’ has been proposed where yKu binds to a chromosome end like it would at a double-strand-break during NHEJ (RIBES-ZAMORA et al. 2007), with the α-helix 5 of yKu70p facing outwards towards the chromosome terminus and the α-helix 5 of yKu80p facing inwards towards the heterochromatic subtelomeric DNA (RIBES-ZAMORA et al. 2007).
Epigenetic silencing is mediated by a number of proteins in *S. cerevisiae*, including yKu, Sir1p, the silent information regulator Sir2p-4p complex, Rap1p and histones H3, H4 and H2A (*COCKELL and GASER 1999; LAURENSON and RINE 1992; MILLAR et al. 2006; PATTERSON and FOX 2008; VANDRE et al. 2008*). Different mechanisms appear to control silencing, as deletion of yKu disrupts telomeric silencing but does not disrupt silencing of the mating-type (*HM*) loci (*VANDRE et al. 2008*). Two hybrid screens have shown that yKu interacts with Sir4p (*TSUKAMOTO et al. 1997*) and TPE is thought to be promoted by yKu playing an access role in the competition between Sir4p and Rif1-2p for telomeric bound Rap1p, initiating the establishment of silent heterochromatin *via* further interactions between the Sir-complex and subtelomeric histone proteins (*FEESER and WOLBERGER 2008; MISHRA and SHORE 1999*). Core X and Y’s may also play a role, as silencing assays have shown TPE is different at native ends compared to truncated ends that lack these sequences (*FOUREL et al. 1999; PRYDE and LOUIS 1999*). Interestingly, silencing at native ends is discontinuous, with maximum repression at core X and the TG1-3 repeats, with little at Y’s (*PRYDE and LOUIS 1999*).

Fluorescent microscopy studies have shown that *S. cerevisiae* telomeres are compartmentalized within the nucleus, being clustered in 4-7 foci at the NE, with some chromosomes forming complete loops (*BYSRICKY et al. 2005*). This is believed to promote heterochromatic silencing (reviewed in *TADDEI and GASER 2004*). Two partially redundant pathways exist for anchoring telomeres to the NE, one dependent on yKu and the other on Sir4p (*HEDIGER et al. 2002b; TADDEI et al. 2004a*). Which pathway is utilized may depend on the specific end and is under the control of the cell cycle. Sir4p is involved in both silencing and nuclear tethering, suggesting both
properties are closely related (TADDEI and GASSER 2004; TADDEI et al. 2004b).
However, loss of silencing does not always correlate with loss of tethering, as during
interphase a silencing defective sir4 mutant can anchor some telomeres to the NE
using the yKu-dependent mechanism (HEDIGER et al. 2002b; THAM et al. 2001).
Possible components for NE anchoring include Esc1p, Mlp1 and Mlp2p and Mps3p
(ANDRULIS et al. 2002; GALY et al. 2000; HEDIGER et al. 2002a; SCHOBER et al.
2009), but it has also been suggested subtelomeric sequences play an important role
(SCHOBER et al. 2009; TADDEI and GASSER 2004). Interestingly, some ends display
differing tethering capabilities depending on the presence or absence of Y’s (HEDIGER
et al. 2002b; TADDEI and GASSER 2004; THAM et al. 2001).

We have previously proposed a fold-back model that maintains genome stability in S.
cerevisiae (PRYDE and LOUIS 1999). It has previously been shown that yKu binds to
the telomeric TG1-3 repeats and subtelomeric DNA (MARTIN et al. 1999) and similar
binding patterns have been observed for Rap1p, Rif1-2p and Sir2p-4p (LIEB et al.
2001; SMITH et al. 2003; STRAHL-BOLSINGER et al. 1997). TPE, a subtelomeric
phenomenon, can be altered or abolished by inactivating or mutating any of these
proteins, or by transcribing telomeres to interrupt Rap1p/Sir-mediated heterochromatin (DE BRUIN et al. 2000; FEESER and WOLBERGER 2008; LAROCHE et
al. 1998; MISHRA and SHORE 1999). Furthermore, transcription of a subtelomeric
gene with an enhancer placed 1-2 kb from it is activated if the reporter is placed at a
telomere, but not internally (DE BRUIN et al. 2001). These findings have led to the
hypothesis that a protective heterochromatic fold-back structure exists at S. cerevisiae
chromosome ends where the TG1-3 repeats loop back to associate with subtelomeric
Using heteroalleles embedded in Y’s, we have recently identified a direct role for yKu and core X as part of the same pathway for repressing recombination near telomeres (MARVIN et al. 2009a). This repression could be related to TPE and/or tethering to the NE. In an attempt to elucidate the mechanism for maintaining genome stability, we have performed chromatin immunoprecipitation (ChIP) using Myc-tagged alleles of yKu and analyzed the enriched DNA using microarray and QPCR analysis to fine resolution (BUCK and LIEB 2004; MOCKLER and ECKER 2005). Coupled with recombination assays in various strain backgrounds we have determined yKu binding sites and the effects of defective binding on recombination. We propose that our results provide further evidence of the existence of a protective fold-back structure at chromosome ends. This structure may be mediated by yKu binding to the TG1-3 repeats and pulling them to core X to form a silent heterochromatic region over this subtelomeric element.

MATERIALS AND METHODS

Strains, genetic manipulation and growth: Strains are shown in Table S1 and for ChIP on chip were derived from YP1 (LOUIS and HABER 1989). MEM1 contains heteroallelic leu2::URA3 constructs at two genomic locations, an internal intergenic site and a telomere proximal site. The leu2-B heteroallele contains a 2-bp frame shift mutation introduced into the BstXI site of LEU2 by site-directed mutagenesis, it is located in the Y’ element, 250 bp from the telomere on XVR. The leu2-C heteroallele has a 2-bp insertional frame shift at the Clal site of LEU2 and is inserted 88,273 bp away from the telomere on XVIL, 150 bp away from any promoters or terminators (MARVIN et al. 2009a). Strains used for study of specific chromosome ends were
derived from S288C strain FYBL1-8B (PRYDE and LOUIS 1999) and strains used for analyzing differentiation of function mutants were derived from Y55 (McCusker and Haber 1988) and harbored the \textit{lys2-Nde1-1; lys2-Nde1-2} alleles to monitor ectopic recombination rates (Marvin et al. 2009a). All were constructed using PCR-directed mutagenesis (Lontine et al. 1998) and homologous recombination at the correct genomic locus confirmed by PCR (see Table S2 for primers). Strains harboring Myc-tagged alleles of \textit{YKU70} and \textit{YKU80} were tested for wild-type yKu function by observing: growth on leucine drop-out medium to check for a hyper-recombination phenotype (Figure S1); growth on YEPD medium at 37º (Figure S2) to check for temperature sensitivity (Feldmann et al. 1996; Feldmann and Winnacker 1993); loss of telomeric DNA (Figure S3) following 12 generations of growth in YEPD by Southern hybridization of genomic DNA using a probe consisting of a XhoI-SalI fragment of Y’ and TG1-3 sequence cloned from pYP1-S1 (Louis and Haber 1992).

Transformations were carried out using the lithium acetate method (Gietz et al. 1992). All strains were grown at 30º on medium prepared according to (Sherman et al. 1986).

**ChIP on chip:** ChIPs were performed as previously described (Laloraya et al. 2000; Meluh and Koshland 1997) with the following modifications: Cultures were set up from individual colonies and grown to a cell density of $2 \times 10^7$ cells/ml and then fixed \textit{in situ} with 1% formaldehyde for 2 hrs; The fixed cells were treated with Zymolyase 20T from \textit{Arthrobacter luteus} supplied by the Seikagaku Corporation to obtain spheroplasts; which were sonicated 17 times in an ice-cold water bath with 10-sec pulses and 30-sec rests. Total genomic DNA was purified from each spheroplast preparation to be used as a reference sample for microarray hybridizations and as a
positive control for QPCR. Immunoprecipitations were performed using a monoclonal anti-Myc (mouse IgG1 isotype) antibody supplied by Sigma®. Immunoprecipitations were performed in duplicate or triplicate with the addition of anti-Myc (IP) and without the addition of the antibody (Mock IP or NoAb) as a negative control. For ChIP on chip, eight individual IPs were performed on three cultures. Sequence independent amplification of IP DNA and purified MEM4 input DNA was performed as previously described (Bohlander et al. 1992; Gerthon et al. 2000). Amino-allyl-dUTP was incorporated into both the experimental and reference samples (Hughes et al. 2001; Randolph and Waggoner 1997) followed by coupling to N-hydroxysuccinimidyl-esterified Cy5 and Cy3 dyes (Amersham Biosciences).

Tiling DNA microarrays were constructed using an in-house built contact printing robot. Probes consisted of unmodified overlapping PCR products that covered S. cerevisiae chromosomes III (0.3 Mb), VII (1.1 Mb) and XI (0.7 Mb) from telomere to telomere. These were chosen to be printed on our tiling array as they encompass the various types of chromosome ends found in S. cerevisiae, specifically silenced or non-silenced, X-only or X-Y’ ends and include the silent mating-type loci HML and HMR. In addition, we already had data on silencing and recombination at many of these ends, so had strains with marked telomeres for further study (Marvin et al. 2009a; Pryde and Louis 1999). Sequence specific primers for PCR amplification were selected using the Lasergene® package from DNASTAR, inc. The resolution of the generated chromosome maps from these arrays is limited by the size of the PCR products printed (0.8 to 1.2 kb), their overlap (~300 bp) and the size of the sheared immunoprecipitated DNA (200 to 600 bp), so can be estimated at approximately 1 Kb. Primer sequences used to construct the microarray probes can be obtained from
the correspondent author on request. To represent telomere DNA at the end of each chromosome, a short (~200 bp) TG_{1-3} tract was PCR-amplified using plasmid pGEM3Zf as a template (KRAMER and HABER 1993) and primers M13-FWD and M13-RVS (Invitrogen). Probes were printed as individual spots onto amine coated glass slides supplied by Genetix.

Immunoprecipitated DNA and ‘no antibody added’ counterparts were labeled with Cy5 and competitively hybridized with MEM4 total genomic DNA that had been labeled with Cy3. All hybridizations were carried out at 63° in a water bath overnight. The arrays were washed twice with 0.6× SSC; 0.03% SDS and twice with 0.06× SSC before being dried by centrifugation and scanned immediately. Prior to hybridization, the printed microarray slides were subjected to post-processing. This consisted of rehydrating the spots using 0.5× SSC at 37° and snap drying them on a hotplate set at 100°, UV cross-linking at 60 mJ.cm^{-1} and incubating them in 3× SSC; 0.2% SDS at 65° for 5 min. Free amine groups on the surface of the slide were blocked using succinic anhydride and the slides were boiled in sterilized distilled water to remove any residual solvent and denature the DNA probes in order to make them available for hybridization.

**Computational analysis and quantification:** Slide images were obtained using an Affymetrix® 428TM scanner. Spots were analyzed and data prepared using ImaGene version 5.6 and GeneSight Lite from Biodiscovery. Any spots that ran together, displayed irregular shape, high background or contamination with unknown exogenous material were omitted from analysis. Quantified Cy5 and Cy3 channel pixel intensity data was transformed using the median pixel value for each arrayed
genomic segment with default settings. The 75 percentile was used to normalize and the combined ratios of the median pixel intensity values were used for further analysis. Analysis of our ChIP on chip microarray data using the PeakFinder program was carried out using previously described filter settings (GLYNN et al. 2004) but the height in log₂ space was set at less than 1.0.

**PCR and QPCR analysis:** All primers were supplied by Invitrogen™ and DNA amplified in a DNA Engine® Tetrad™ 2 thermal cycler (MJ Research®). QPCR analysis was performed using SYBR® Green JumpStart™ Taq ReadyMix™ supplied by Sigma®. All QPCR reactions were performed in duplicate 28 µl samples using an Mx4000® Multiplex QPCR System from Stratagene® and the mean values taken. Analysis was performed on IP DNA, NoAb controls and total genomic DNA extracted from each strain. Analysis was performed on at least three individual IP’s from independent cultures. Fold enrichment was calculated by dividing the mean IP values by the corresponding mean NoAb values and then normalizing each sample to the corresponding value given by the *MAT* locus as a negative binding control. To enable quantification of each sample, a standard curve was constructed using 4-fold serial dilutions of a single known standard concentration of S288C genomic DNA. All reactions were carefully monitored to ensure acceptable efficiency and that unknown quantities fell into the linear range of amplification. The standard error of the mean is included in all data shown. QPCR amplicons were run on a 3% NuSieve® agarose gel containing 0.5 µg/ml of ethidium bromide and visualized on a Kodak Gel Logic 200 Imaging System using 1D Image Analysis Software to confirm the correct size.
Quantitative Recombination Assay: Recombination assays were performed as previously described (MARVIN et al. 2009a), except strains were plated onto synthetic medium omitting lysine in order to detect recombinant colonies and leucine in order to maintain the replication of plasmid DNA.

Plasmids containing the separation of function yku80 alleles: Two sets of plasmids were used that contained the yku80-1 through to yku80-8 mutant alleles and wild-type YKU80 alleles with and without Myc-tags (BERTUCH and LUNDBLAD 2003). Plasmids pAB109-117 were constructed by subcloning a XhoI-NotI fragment containing the YKU80 promoter and a wild-type or mutant YKU80-Myc coding sequence obtained from plasmids pVL1352, pVL1353, pVL1354, pVL1809, pVL1810, pVL1816, pVL1817, and pVL1818, and pVL1819 (BERTUCH and LUNDBLAD 2003), respectively, into pRS415 restricted with XhoI and NotI. The parent plasmids of these constructs were used as negative controls.

RESULTS

Determination of yKu70p binding sites by ChIP on chip

In order to determine yKu binding sites in S. cerevisiae, we performed ChIP on a strain harboring a Myc-tagged yKu70 allele (MEM4) and hybridized the resulting enriched immunoprecipitated (IP) DNA to tiling arrays covering chromosomes III, VII and XI from telomere to telomere (see Materials and Methods for details). The normalized data was then processed to determine areas of significant enrichment along each chromosome using two previously described methods (GLYNN et al. 2004; LIEB et al. 2001).
The first method employed involves assigning each probe a median percentile rank calculated from the median ratio intensities for each IP and NoAb hybridizations (Lieb et al. 2001). Significantly enriched DNA is then determined by plotting the number of probes falling into each percentile (1 percentile bins), with NoAb data usually showing a unimodal distribution, representing no strong selection of any particular sequences and IP data showing a bimodal distribution, representing consistent enrichment of a subset of sequences (Lieb et al. 2001). Using this method we found a small but distinct group of enriched DNA segments that overlapped and appeared above the 96 percentile in our IP hybridizations (Figure S4).

We grouped these overlapping fragments into 17 foci for further investigation (File S2). Seven of these foci contained DNA segments that also appeared above the 96 percentile in the NoAb data and these should have been omitted from further analysis using the described methodology (Lieb et al. 2001). However, probes representing the TG1-3 repeats were also positioned above the 96 percentile in both the IP and NoAb data, yet yKu is known to bind to the chromosome termini (Martin et al. 1999). Closer examination of the remaining six foci revealed that their high ranking in both sets of data most likely resulted from a bias in hybridization as these segments also contained repetitive genomic elements such as transposons, LTRs, ARSs and tRNAs. However, each of these segments had neighboring overlapping partners that did not appear above the 96 percentile in the NoAb data. Therefore, these foci, like the TG1-3 repeats could still represent real interactions with yKu and so were kept for further analysis.
Determination of yKu70p binding sites using PeakFinder

The second method of analysis utilized the PeakFinder program previously utilized to identify cohesin binding sites (GLYNN et al. 2004). We took the median of the median ratio intensities for each genomic segment and used these to plot chromosome maps (Figure S5). A total of 30 peaks were identified using the parameters described (Materials and Methods). PeakFinder is unable to identify single-sided peaks such as those that would result from yKu70p binding to the ends of the telomeres (GLYNN et al. 2004), therefore these were included in our data as manually identified (M/C) peaks (Table 1).

Although we cannot unambiguously determine whether or not all our probes are completely specific to each of the chromosome ends we analyzed (LIEB et al. 2001) as a result of subtelomeric sequence duplication leading to cross-hybridization in some regions (see http://www.nottingham.ac.uk/genetics/people/louis/images/clusters-fixed-lowres.jpg for a map of the duplicated ends found in S. cerevisiae), our PeakFinder outputs produced several interesting observations (Figure S5). Twin peaks were observed on both ends of chromosome III and chromosome VII. Closer examination of the median of median ratio values for these ends (Figure 1) showed that yKu binding on chromosome III occurred both at the telomeres and HML and HMR and on the right arm of chromosome VII (VIIR), binding was observed at both the telomere and the subtelomeric core X element. In addition, we observed a spread of enriched DNA segments between the twin peaks (Figure 1A and 1D) on the left arm of chromosome III (IIIL) and the right arm of chromosome VII. Interestingly, on VIIR, one peak is centered over core X and the other at the TG1-3 repeats but hybridization is alleviated along the Y’ element (Figure 1D). This illustrated a direct
correlation with silencing assays previously performed in our laboratory (PRYDE and LOUIS 1999) that had shown maximal repression at core X and the TG\textsubscript{1,3} repeats, but alleviated repression at Y’ elements and more centromere proximal regions.

Our data also suggested that association with core X was weaker at IIIR and VIIL (Figure 1 and S5), which correlates with the absence of Sir2p-4p at these non-silenced ends (LIEB et al. 2001). This suggested that the Sir complex may facilitate stronger binding of yKu at core X. We also saw a spread of yKu70p enriched fragments that declined towards the centromere (Figure 1A, D, E and F), which correlated with similar studies on Rap1p and Sir-proteins (LIEB et al. 2001). In addition, Sir-proteins bind to the \textit{HM} loci and telomeres, which were also enriched in our data (COCKELL and GASSER 1999; GRUNSTEIN 1997; LAURENSON and RINE 1992). This could suggest that DNA centromere proximal to core X was enriched via yKu70p interacting with Sir-proteins. Alternatively, this could be due to spatial association of yKu70p binding targets at the nuclear periphery (COCKELL and GASSER 1999; GRUNSTEIN 1997; LAURENSON and RINE 1992).

**yKu70p preferentially binds to subtelomeric core X in addition to \textit{HML} and \textit{HMR}**

To confirm our microarray results the enriched genomic segments were analyzed by QPCR. As targets, we chose those deemed significant by both types of analysis (Table 1A-C). We also included transposable elements, as yKu has been implicated in retro-transposition and Sir-proteins have been implicated in controlling recombination rates, particularly of Ty\textsubscript{1} elements (RADFORD et al. 2004).
QPCR analysis revealed that yKu70p bound primarily to core X sequences and the HM loci with enriched DNA being maximal at HML, followed by HMR (Figure 2A). No enrichment was seen at MAT, demonstrating that cross-hybridization with the HM loci was responsible for its high ranking in our microarray screen. No other sequences selected from our data showed enrichment, including transposons (with exception to the Ty5 element positioned close to core X on IIIL) tRNA sites, the centromere of XI and all the ORFs tested (see Table S3 for all the primers used, Figure 3 for subtelomeric specific primer binding sites). The only Ty5 element in S. cerevisiae and the Ty5-LTRs (remnants of Ty5 retrotransposition) showed approximately 10-fold enrichment and Y’ sequences showed approximately 4-fold enrichment in the chromatin IP’s. The enrichment observed at Ty5 and Y’ sequences, is likely to result from their close proximity to core X and TG1-3 sequences. To determine whether or not enrichment was specific for Ty5, we targeted three additional loci with similar relative positions to core X, but on different chromosome ends (primers targeted AAD3 and AAD15 on IIIR and XVL simultaneously and 1240 bp upstream of COS12 on VIIL, see Figure 2A). Both displayed comparable levels of binding to that observed for Ty5, demonstrating that enrichment most likely resulted from close proximity to core X and was not specific to the Ty5 transposon itself.

To determine if the high level of enrichment at HML compared to HMR was related to mating-type, enrichment of yKu70p at specific loci was examined in an isogenic strain of the opposite mating-type, (MATa, Figure 2A). We found no significant difference between MATa and MATα strains, with yKu70p still associating more strongly with HML than with HMR (Figure 2A). Furthermore, in agreement with a recent finding (Bystricky et al. 2009), we did not see a significant amount of
enrichment at the recombination enhancer (RE) element in either mating-type (data not shown).

**Binding of yKu70p to HML and HMR is Sir-dependent but binding to core X is Sir-independent**

In order to establish whether or not yKu binding to HML, HMR and core X was dependent upon interaction with Sir-proteins, we performed ChIP on Myc-tagged yKu70p in a sir1, sir2, sir3 or sir4-null mutant background. The resulting enriched IP and NoAb DNA was then used for QPCR analysis (Figure 2B). Our results showed that deletion of any one of the genes encoding Sir2p-4p abolishes binding of yKu70p to HML and drastically reduces binding to HMR, indicating that association with these sequences is dependent on the Sir-complex. Binding of yKu70p in a sir1-null mutant also resulted in reduced association with HML and HMR to approximately half when compared to wild-type. In contrast, binding of yKu70p to core X sequences was not abolished in any of the sir-mutants, demonstrating that yKu can bind to core X independently of all Sir-proteins. In a sir1 mutant, binding to core X was slightly enhanced and in a sir3, or sir4 mutant background, the strength of binding was slightly reduced. This suggests that the Sir2p-4p complex plays a role in enhancing binding at core X, probably through further protein interactions to establish the formation of core heterochromatin over this region.

**Binding of yKu70p to core X occurs at both silenced and non-silenced ends and in the presence and absence of Y’ elements**

Our microarray data had shown that yKu binding at subtelomeric sequences is increased at silenced chromosome ends. However, our microarray probes and QPCR
primers were not specific for a single chromosome end, but were generic for many ends (see Figure S6 for mismatches). In order to investigate yKu70p binding at specific chromosome ends, we employed strains from a previous study that carried URA3 inserts immediately centromere proximal to core X (Pryde and Louis 1999). By introducing the Myc-tagged allele of yKu70p and performing ChIP and QPCR, we were able to target individual ends, using primers specific to the URA3 marker and immediately adjacent flanking sequences (Figure 3). Three types of end were studied: XVR is non-silenced and is an X-Y’ end; XIL is a silenced X-only end; IIIR is a non-silenced X-only end (Pryde and Louis 1999).

Our results (Figure 2C) showed that for all ends, binding of yKu70p is stronger at core X sequences telomere-proximal to the URA3 insert, compared to the corresponding subtelomeric centromere-proximal sequences. Binding of yKu70p to core X was observed for all the ends tested, but binding was considerably weaker at IIIR and XVR compared to the XIL. This is consistent with yKu binding more strongly with core X at silenced ends than non-silenced ends. The same was observed for the subtelomeric centromere-proximal sequences, with the silenced end showing stronger binding of yKu. Interestingly, over the URA3 ORF at the silenced end, binding was weaker than that at sequences centromere proximal to this position. Binding at core X also differed among the non-silenced ends, with the IIIR showing stronger binding at core X (TEL-Prox) than XVR.

These observations and the microarray data both support the hypothesis that the Sir complex affects both the extent and strength of yKu binding. The stronger binding observed at non-silenced IIIR compared to XVR is probably due to additional co-
association with Sir-proteins present at the *HMR* locus. Alternatively, it may simply be down to the physical distance of core X from its associate TG<sub>1-3</sub> repeats, as XVR contains a Y' element and displays weaker binding than IIIR.

**Differentiation-of-function *yku80* mutants display hyper recombination phenotypes and defective binding to core X.**

Previously, it has been shown that specific mutations in *YKU70* and *YKU80* lead to defective NHEJ and telomere maintenance, respectively (*BERTUCH* and *LUNDBLAD* 2003; *PALMBOS* *et al.* 2005; *RIBES-ZAMORA* *et al.* 2007; *STELLWAGEN* *et al.* 2003). Furthermore, we have recently found that deletion of either gene and/or core X results in increased recombination involving telomeric sequences (*MARVIN* *et al.* 2009a). Therefore, we decided to ascertain whether or not telomeric defects, previously associated with differentiation-of-function mutants of *YKU80*, also displayed a hyper-recombination phenotype and defective yKu-binding.

We obtained three mutant genes; *yku80-1*, *yku80-4* and *yku80-8*, which had been Myc-tagged and cloned into the low copy number plasmid pRS415 (Materials and Methods). Each mutant allele had previously been shown to display differing degrees of telomeric defects, but each was proficient for NHEJ (*BERTUCH* and *LUNDBLAD* 2003). According to the severity of each telomeric defect previously observed, we categorized them into highly defective (*yku80-1*), medium defective (*yku80-4*) and slightly defective (*yku80-8*) mutations of *YKU80*. We then transformed a *yku80*-null strain with each allele or the wild-type *YKU80* allele and performed ChIP, followed by QPCR analysis of the known yKu binding sites. The results (Figure 4) showed that
yKu80p binds in a similar manner to yKu70p, which is consistent with heterodimer activity. Binding was greatly reduced in strains expressing the \textit{yku80-1} and \textit{yku80-4} alleles, but not the \textit{yku80-8} allele which only displayed reduced binding at HML (Figure 4A). Interestingly, strains harboring the \textit{yku80-1} allele had previously been shown to have more defective silencing and end-protection properties than those carrying the \textit{yku80-4} allele (BERTUCH and LUNDBLAD 2003). However, in our study a strain harboring the \textit{yku80-1} allele displayed slightly stronger binding to core X than its \textit{yku80-4} counterpart (Figure 4C).

Following the ChIP analysis we decided to perform a quantitative recombination assay (MARVIN et al. 2009a) to observe ectopic recombination between telomeric and interstitial sites in strains harboring all the mutant alleles, \textit{yku80-1} through to \textit{yku80-8} (Figure 5). This enabled us to study the genome-stabilizing properties of each mutant allele that had previously been studied for NHEJ and telomere maintenance properties (BERTUCH and LUNDBLAD 2003). With exception to the \textit{yku80-1} allele, our results showed that there was a correlation between defective TPE and end-protection properties and genome instability. Importantly, there was an inverse relationship observed between the strength of yKu binding at core X and recombination rate in \textit{yku80-1}, \textit{yku80-4} and \textit{yku80-8} mutants, suggesting that increased binding at core X decreased genomic instability [see Table 2 for a summary of phenotypes from Bertuch and Lundblad (2003) and this study].

**DISCUSSION**

All chromosome ends may contain a yKu-core X mediated fold-back structure
The yKu heterodimer has been implicated in many telomeric functions (Downs and Jackson 2004) functionally separate from NHEJ (Bertuch and Lundblad 2003). The paradox of having a NHEJ protein at chromosome ends may be explained by nuclear architecture providing a telomere capping mechanism by sequestering them from the rest of the genome. One proposed hypothesis, is the presence of a protective fold-back structure forming part of the telosome (De Bruin et al. 2000; De Bruin et al. 2001; Pryde and Louis 1999). We have now demonstrated that yKu specifically binds to core X at silenced, non-silenced, X-only and X-Y’ ends. We propose that this association is via telomeric bound yKu and is further evidence for a protective fold-back structure at S. cerevisiae chromosome ends (see Figure 6 for our model). Our results show that the yKu-core X association prevents recombination involving telomeric sequences and correlates with maximal silencing at core X (Pryde and Louis 1999). In support of this model, it has been found that a transplaced core X can enhance repression of already silenced regions of the genome, suggesting a role in promoting silencing (Lebrun et al. 2001). Furthermore The core X sequence on its own is unlikely to facilitate the binding of yKu, as increased dosage of this sequence on a multicopy plasmid has no effect on telomeric silencing and translocation to an internal locus does not result in it being silenced (Pryde and Louis 1999).

Candidates for recruiting yKu to core X include ORC and Abf1p, as both influence its protosilencer properties (Lebrun et al. 2001). Binding sites for ORC are found at all core X’s and for Abf1p at all but one (Lebrun et al. 2001; Pryde and Louis 1999) and binding sites for both are found at HML and HMR (Feeser and Wolberger 2008). However, preliminary ChIP studies in our laboratory on strains with deleted ORC and Abf1p binding sites showed that yKu still bound to core X (Esther Loney,
pHD Thesis 2005) even though TPE is disrupted (PRYDE and LOUIS 1999). As TPE is abolished in yKu mutants, ORC and Abf1p may recruit the silencing machinery and yKu may target and maintain a heterochromatin state at core X.

**Binding to core X is independent of the Sir-complex per se, but is enhanced at silenced ends**

Binding of yKu at native telomeres correlates with maximal silencing at core X and the TG_{1,3} repeats (PRYDE and LOUIS 1999). Association of yKu with subtelomeric sequences has been shown to be dependent on Sir4p (MARTIN et al. 1999). We found that yKu binding was enhanced at silenced ends compared to non-silenced ends that do not contain Sir-proteins (LIEB et al. 2001). However, association of yKu with core X was always detected irrespective of silencing, suggesting that binding is independent of silencing per se.

A fold-back structure may allow additional protein-protein interactions involving yKu, telomeric capping proteins and histones to strengthen nuclear architecture. At silenced ends, this may provide a stable platform for the spread of Sir-proteins to extend and maintain TPE at more centromere proximal regions (HECHT et al. 1995; LIEB et al. 2001; MORETTI and SHORE 2001; SMITH et al. 2003; STRAHL-BOLSINGER et al. 1997; TSUKAMOTO et al. 1997). In support of this was our observation of yKu-enriched sites spreading inwards at silenced chromosome ends. Therefore, a yKu-mediated fold-back at non-silenced ends may be transient, resulting from an absence of additional interactions between yKu, Sir4p and participating partners (TSUKAMOTO et al. 1997). A combination of extrinsic factors may also affect these interactions including: the position of sequences within the chromosome, already shown to effect
silencing of HMR (THOMPSON et al. 1994); the presence or absence of Y’ elements; the variation in physical distance between TG1-3 repeats and core X sequences (HEDIGER et al. 2002b; TADDEI and GASSER 2004; THAM et al. 2001); sequence variations within or flanking core X. In addition, the phenomenon of telomere clustering and chromatin remodeling may provide an opportunity for additional interactions between proteins to occur or alter the requirement of yKu for maintaining nuclear architecture at different stages of the cell cycle (FISHER et al. 2004; SCOBER et al. 2009; SMITH et al. 2003).

**yKu binding to HML and HMR**

Binding of yKu to HML and HMR, is consistent with two hybrid interactions found between yKu and Sir4p (TSUKAMOTO et al. 1997) and binding to the E and I silencers of both mating-type loci (PATTERSON and FOX 2008; VANDRE et al. 2008). Deletion of SIR1 resulted in reduced association of yKu to HML and HMR, and slightly increased binding to core X. This is consistent with recent findings showing that Sir1p and yKu have redundant roles in establishing silencing at the mating type loci (PATTERSON and FOX 2008; VANDRE et al. 2008). In agreement with Patterson and Fox (2008), we identified stronger binding at HML than HMR. In addition, we found binding is identical in both mating types. HML is adjacent to a silenced chromosome end, whereas HMR is not, suggesting that proximity to Sir-proteins present at the telomere may be the reason for stronger binding (LIEB et al. 2001). This is particularly true for Sir4p due to its known interaction with yKu and its effects on silencing (ANDRULIS et al. 1998; Patterson and Fox 2008; ROY et al. 2004; VANDRE et al. 2008). Alternatively, this bias in binding strength may reflect differences in chromatin structure between the HM loci, or the physical distance of HML and HMR from their
corresponding telomeres. However, binding of yKu to both $HM$ loci was eliminated in $sir2$, $sir3$ and $sir4$-null mutants, whereas association with core X was only slightly affected. This suggests that $in$ $vivo$ binding of yKu with the silent mating-type loci in a wild-type strain relies on a complete Sir-complex silencing the $HM$ loci, rather than Sir4p alone (Feeser and Wolberger 2008) or by direct binding to the DNA. It also suggests that stronger binding at $HML$ is due to a more abundant presence of Sir-proteins at that locus and the adjacent silenced IIIL telomere, (Lieb et al. 2001).

Stronger binding in the presence of Sir-proteins at silenced ends suggests that the initiation of silencing by yKu at the $HM$ loci in a $sir1$-null strain (Patterson and Fox 2008; Vandre et al. 2008) is achieved via a yKu-Sir-complex interaction (Feeser and Wolberger 2008). This may be explained by the mating-type loci folding back to associate with yKu bound at core X in a similar manner to the proposed telomere fold-back (Figure 6), rather than yKu directly binding to the $HM$ loci to initiate the assembly of the silencing machinery (Feeser and Wolberger 2008; Patterson and Fox 2008; Vandre et al. 2008). A ‘double fold-back’ would promote silencing of the mating-type loci by bringing them into close proximity to a plentiful supply of silencing proteins at the telomeres. Association of $HML$ with a nearby telomere (Feeser and Wolberger 2008; Lebrun et al. 2003) and the fact that both ends of chromosome III also appear to be in close proximity (Dekker et al. 2002) promotes this hypothesis as Sir-proteins would be readily available to both $HML$ and $HMR$. The double fold-back may then be anchored at the nuclear envelope via yKu’s interaction with additional proteins that promote peripheral positioning (Andrulis et al. 2002; Galy et al. 2000; Hediger et al. 2002a; Schober et al. 2009). This may also explain the phenomenon of telomere clustering, with many yKu-associated proteins
interacting and pulling chromosome ends together. However, in the absence of yKu there is a Sir4p-mediated pathway for attachment to the nuclear periphery (HEDIGER et al. 2002b; TADDEI et al. 2004a) and silencing of HMR can be maintained in the absence of Esc1p and yKu (GARTENBERG et al. 2004; TADDEI et al. 2004a) suggesting this phenomenon is via many complex interactions and not exclusive to yKu activity alone.

There is a correlation between core X-binding, telomere maintenance, TPE and genome instability.

The study of yku80 differentiation-of-function mutants suggests that there is an inverse correlation between recombination rate and binding efficiency at core X and this in turn coincides with defective TPE and a reduction in end-protection (Table 2, (BERTUCH and LUNDBLAD 2003). If a protective fold-back structure exists at the ends of chromosomes, these findings suggest that these defects are caused by a conformational change in the subtelomeric heterochromatin. Although the yku80-1 allele provided an exception to this correlation, it is worth noting that silencing was completely abolished in this mutant, whereas in all the other mutants tested it was merely impaired (BERTUCH and LUNDBLAD 2003). This difference may explain the anomaly in the correlation, as the complete breakdown of core heterochromatin may induce alternative genome stabilizing mechanisms to come into play and binding may be transient as we have proposed for non-silenced ends. Alternative mechanisms may still involve yKu binding to core X, may be linked to the sir-dependent pathway for tethering to the nuclear periphery, or to the fact that yKu-deficient strains can re-establish silencing by knocking out Rif1p or Rif2p, albeit at a truncated ends
Why would yKu mediate a fold-back structure?

A yKu mediated fold-back structure may sequester the chromosome ends from deleterious events such as exonuclease attack, end-to-end fusions and gross chromosomal rearrangements. Interestingly, Rap1p has been shown to play a role in preventing end-to-end fusions by suppressing NHEJ at telomeres (Pardo and Marcand 2005), which may or may not be related. A fold-back conformation may also play a role in controlling telomere length by preventing recombination between the TG1-3 repeats and regulating telomerase activity via chromatin remodeling (Nugent et al. 1998; Polotnianka et al. 1998). The fact that some differentiation-of-function and yku-null mutants are hyper-recombinant and subtelomeric elements such as core X, Y’s and STR’s effect anchoring, raises the possibility that tethering to the nuclear envelope also requires a fold-back structure (Hediger et al. 2006). A fold-back structure may also be required to maintain a heterochromatic state at silenced ends, correlating with maximal silencing at core X (Pryde and Louis 1999). However, we believe that TPE is merely a secondary effect to the primary function of yKu to promote genome stability. Our data also suggests that a fold-back conformation may also help to mediate compartmentalization of the HM loci at the nuclear periphery.

It still remains to be elucidated why yKu targets core X. Further insights into the roles of yKu may be acquired by further study of differentiation-of-function mutants, as this will help to determine what protein-protein interactions are taking place. We have
provided further evidence that a fold-back structure exists at the end of *S. cerevisiae* chromosomes that is dependent upon both yKu and core X. We believe this fold-back is part of the nuclear architecture of *S. cerevisiae* that promotes genome stability and explains the paradox of having a NHEJ protein at the chromosome termini. We believe such a structure may protect telomeres from unwanted recombination events and appears to target and maintain TPE at core X.
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interactions differ in core and extended telomeric heterochromatin in yeast.


TABLE 1
Genomic loci identified using median percentile rank and PeakFinder analyses

| A Chromosome III |  |
|-----------------|-----------------|-----------------|
| Loci            | Co-ordinates    | PeakFinder Output | Genomic Features |
| a               | TG1-3           | M/C Termini      | Telomere/ARS |
| b               | 463-2967        | M/C              | M/C Telomere III: X element core X ARS300 |
| c               | 12414-14878     | 1/2              | 13666-14878/89103-90381 |
| d               | 108607-109981   | 3/2              | 108607-109981/89103-90381 |
| e               | 199566-200988   | 5/10             | 199566-200988/306515-307931 |
| f               | 293200-295866   | 9/10             | 293200-294507/306515-307931 |
| g               | 306515-307931   | 10/10            | 306515-307931/306515-307931 |
| h               | 314437-316032   | M/C              | M/C Telomere III: X element core X ARS319 |
| a               | TG1-3           | M/C Termini      | Telomere/ARS |
| b               | 463-2967        | M/C              | M/C Telomere III: X element core X ARS300 |
| c               | 12414-14878     | 1/2              | 13666-14878/89103-90381 |
| d               | 108607-109981   | 3/2              | 108607-109981/89103-90381 |
| e               | 199566-200988   | 5/10             | 199566-200988/306515-307931 |
| f               | 293200-295866   | 9/10             | 293200-294507/306515-307931 |
| g               | 306515-307931   | 10/10            | 306515-307931/306515-307931 |
| h               | 314437-316032   | M/C              | M/C Telomere III: X element core X ARS319 |
| a               | TG1-3           | M/C Termini      | Telomere/ARS |
| b               | 463-2967        | M/C              | M/C Telomere III: X element core X ARS300 |
| c               | 12414-14878     | 1/2              | 13666-14878/89103-90381 |
| d               | 108607-109981   | 3/2              | 108607-109981/89103-90381 |
| e               | 199566-200988   | 5/10             | 199566-200988/306515-307931 |
| f               | 293200-295866   | 9/10             | 293200-294507/306515-307931 |
| g               | 306515-307931   | 10/10            | 306515-307931/306515-307931 |
| h               | 314437-316032   | M/C              | M/C Telomere III: X element core X ARS319 |

B Chromosome VII

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<td>Telomere/ARS</td>
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Transposons ORFs

YCL076W YCL075W YCL074W

HMLa1 YCL065W

YCL005W PGS1

MATa2

HMRa2

YCR102W-A

AAD3
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<td>1082739-1090910</td>
<td>PAU12 YGR239C</td>
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<tr>
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<td>1083361-1084797</td>
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<td>YKL225W YKL224C YKL223W</td>
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<tr>
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C Chromosome XI

> 96% Cut-off

PeakFinder Output

Genomic Features
### TABLE 2

Summary of defective phenotypes associated with *yku80*-differentiation of function mutants

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<th>Phenotype tested</th>
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<td>‡‡‡‡‡</td>
<td>‡‡‡‡‡</td>
<td>‡‡‡‡‡</td>
<td>‡‡‡‡‡</td>
<td>‡‡‡‡‡</td>
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<td>‡‡‡‡‡</td>
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<tr>
<td>TG1-3 length</td>
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<td>‡‡‡‡+</td>
<td>‡‡‡‡+</td>
<td>‡‡‡‡+</td>
<td>‡‡‡‡+</td>
<td>‡‡‡‡+</td>
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<td>I</td>
<td>I</td>
<td>I</td>
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<td>I</td>
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<td>N/A</td>
<td>N/A</td>
<td>‡‡‡‡‡</td>
<td>‡‡‡‡‡</td>
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</tbody>
</table>

\\(‡‡‡‡‡ = Fully proficient phenotype\\)
\(– = Fully defective for phenotype\\)
\(I = Impaired for phenotype\\)
\(No Shading = BERTUCH and LUNDBLAD (2003)\\)
\(Grey Shading = MARVIN et al., (2009b)\\)

Each ‘plus symbol’ indicates the level of defect and a ‘minus symbol’ indicates completely abolished activity. Where a phenotypic defect was not measurable by assay an ‘I’ symbol is used to indicate ‘impaired’. Actual values can be obtained from Bertuch and Lundblad (2003) and in this study (Figure 4 and 5).
FIGURE LEGENDS

FIGURE 1.– Collated median ratio data for each chromosome end. Each histogram above displays the median of the median log$_2$ ratio intensities for 26 arrayed genomic segments from each chromosome end on our array (1-26 = telomere proximal to centromere proximal). Some of the chosen features that were analyzed by QPCR are shown and arrows denote the number of DNA segments that span each chosen feature. Maximal peaks found at the TG$_{1-3}$ repeats (D) core X sequences (A-D) and HML and HMR are shown as a circled P. A bimodal distribution of hybridization intensities can be observed at IIII and VIIR. For more information on the features lying within each arrayed segment see File S1.

FIGURE 2.– QPCR analysis of Myc-tagged yKu70p enriched genomic segments. The relative-fold enrichment of IP DNA over the equivalent NoAb control for each genomic locus is shown. All datasets representing core X, Y’ elements and Ty5 LTRs were generated using primers that were generic for these sequences (see Figure S6 for primer mismatches at core X sequences). All values were normalized using the MAT locus as a negative binding control. (A) Shows data for isogenic MATa and MATa wild-type strains MEM4 (red) and MEM5 (blue), respectively. Please note that only HML, HMR, core X and MAT were tested for MEM5 (B) Shows data for yKu70p binding in a sir1, sir2, sir3 and sir4-null mutant background. (C) Shows data for core X binding at specific chromosome ends in three strains carrying URA3 inserts at position 1, which is
immediately adjacent to core X (PRYDE and LOUIS 1999). Standard errors are shown for all data. All primer binding sites are shown in Figure 3.

FIGURE 3.— Primer binding sites at subtelomeric loci. Primers used for QPCR analysis of subtelomeric loci are shown (see Table S3 for all primers used). Arrows indicate orientation and relative position (+ = downstream and – = upstream) to the beginning nucleotide of each genomic target. (A) Shows the primers used for targeting specific chromosome ends (Figure 2C) via a URA3 insert positioned at position 1 (P1), immediately adjacent to core X (PRYDE and LOUIS 1999); (B) shows primers targeting all chromosome ends (Figure 2 and 4), although it should be noted that the RDS1, Ty5 and COS12 primers are specific for single genomic loci; (C) shows the silencing properties of an X-Y’ end relative to the primer binding sites (PRYDE and LOUIS 1999).

FIGURE 4.— Enrichment of yKu-specific binding sites in yku80 differentiation-of-function mutants. (A-E) Show the relative-fold enrichment of IP DNA over the equivalent NoAb control for each set of primers used. All values were normalized using the MAT locus as a negative binding control. All datasets representing core X and Y’ were generated using primers that were generic for these sequences (Figure S6).
FIGURE 5.– Ectopic recombination in yku80 differentiation-of-function mutants. Recombination assays and calculations were performed as previously described (Marvin et al. 2009a). Each strain was analyzed for the production of papillae on synthetic medium without leucine and lysine. Recombination rates per $10^8$ cells are shown and the fold increase in recombination rate over the YKU80 parental strain is above each block. The rank ordered chi-squared values comparing each individual strain with all others are shown in the accompanying table. Pairs that show no significant difference in recombination rate are shaded in grey.

FIGURE 6.– Proposed model of nuclear architecture at telomeres in S. cerevisiae. It has been shown that Rap1p initiates the formation of heterochromatin by recruiting Sir2p-4p (Hecht et al. 1996; Moretti et al. 1994) and that yKu interacts with Sir4p (Tsukamoto et al. 1997). Furthermore, the efficiency of Sir4p binding to the TG1-3 repeats is reduced by inactivating yKu (Luo et al. 2002), which has been proposed to play an access role between Sir-proteins and Rif-proteins binding to Rap1p (Feeser and Wolberger 2008; Mishra and Shore 1999). This suggests yKu may promote association between Rap1p and the Sir-complex, which in turn associates with the N-terminal tails of subtelomeric situated histones H3 and H4. These set of interactions may be strengthened by further interaction between H2A and yKu (Wyatt et al. 2003). The ends of chromosome III, which contain the silent mating-type loci HML and HMR are shown. The telomere of each chromosome is folded back due to direct or indirect association.
between the yKu heterodimer and subtelomeric core X sequences. (A) This association may be stronger at ends such as IIIL that possess Sir2p-4p, possibly from additional protein-protein interactions resulting in core heterochromatin formation and epigenetic silencing (FEESER and WOLBERGER 2008). (B) Association between yKu and core X at non-silenced ends, such as IIIR, may be more transient due to the lack of these additional interactions. Both HML (A) and HMR (B) may also associate with core X due to their interaction with yKu and Rap1p via interaction with the Sir-complex (FEESER and WOLBERGER 2008).
FIGURE 1

A

CHRIIL

B

CHRIIR

Arrayed Genomic Segment

C

CHRVIIL

D

CHRVIIR

Arrayed Genomic Segment

E

CHRXLIL

F

CHRXLIR

Arrayed Genomic Segment
FIGURE 4

A

B

C

D

E
FIGURE 6

(A) Core X

yKu binding to core X

strengthened by Sir complex

TG1-3

HML

Centromere

Unsilenced region X-only or X-Y' end

(B) Core X

TG1-3

HMR

Centromere

Rap1p

Rap1p/Rif1p/Rif2p

Histones

Orc

Sir1p

Abf1p

Tbf1p

Yku70/80p

Sir4p

Sir3p

Sir2p