THE LEFT TELOMERE ON CHROMOSOME III IN SACCHAROMYCES CEREVISIAE: ISOLATION AND CHARACTERIZATION

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Telomeres, the molecular ends of eukaryotic chromosomes, are essential for chromosome stability and complete replication of the termini. A yeast *Saccharomyces cerevisiae* telomeric region was isolated by chromosome walking from *HMLα*, the most distal known gene on the left end of chromosome III. The terminal SalI restriction fragment on IIL (chromosome III left end) mapped 8.6 kb distal to *HMLα* and was cloned in a circular vector to generate a probe for the IIL telomere. This SalI fragment on the end of chromosome IIL was heterodisperse in length, having an average size of 3.3 kb and a length distribution of +/- 0.2 kb. Southern blot and DNA sequence analyses indicated that the IIL end conforms to the T-X class, rather than the T-Y'-X class, of yeast telomeres. The terminal (T) region on IIL consists of 0.6 kb (+/- 0.2 kb) of 5'-C1-3A-3' tandem repeat sequence, adjacent to the 1.2 kb type X *ARS* region. There is no homology to the type Y' *ARS* region between *HMLα* and the IIL telomere. The telomeric probe from IIL hybridized to multiple genomic restriction fragments; both heterodisperse and defined length bands were observed in Southern hybridization analyses and these mapped to chromosome ends. Heterogeneity of the length of the terminal fragment on a given chromosome end was observed among various yeast strains and this length variation was localized to the T region.

The cloned fragment from the IIL telomere lacked the terminus due to the cloning method used to isolate it, however this T-X fragment functioned as a telomere on linear plasmids in yeast. Plasmids constructed in vitro with IIL ends were maintained as linear molecules in yeast, and were more stable mitotically than linear plasmids constructed with *Tetrahymena* rDNA termini. Plasmids constructed with IIL end fragments that had the entire T region deleted were replicated as circular molecules in yeast. Addition of Y' regions did not occur on any of the linear plasmids constructed with fragments from the IIL telomere during replication in yeast, but T region was presumably added to the equivalent extent as that observed on the natural chromosome IIL end [0.6 kb (+/- 0.2 kb)]. A rationale is discussed for the existence of two telomeric classes in yeast, T-X and T-Y'-X, and for the addition of T region to chromosome ends in replication.
The region adjacent to *HMLx* on III, which extends 8.6 kb toward the telomere, is homologous to an alternate telomeric region in the genome of haploid yeast strains. Southern hybridization analyses with *HMLx* distal probes revealed that at least the terminal 6.5 kb of this region is retained in some circular chromosome III strains that were expected to have the III L and III R telomeric regions deleted. Northern hybridization indicated that an unidentified RNA transcript is homologous with a fragment from the *HMLx* distal region.
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<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>ATP</td>
<td>riboadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>DEP</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyriboadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ddATP</td>
<td>dideoxyriboadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxyribocytosine 5'-triphosphate</td>
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<tr>
<td>dTTP</td>
<td>deoxyribothymidine 5'-triphosphate</td>
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<td>ddTTP</td>
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</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>ddNTP</td>
<td>dideoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>ds</td>
<td>double-stranded</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetate</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
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<td>ethanol</td>
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<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>IIIIL</td>
<td>yeast chromosome III left arm</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthiogalactoside</td>
</tr>
<tr>
<td>KAac</td>
<td>potassium acetate</td>
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kb  kilobase pair (s)
kilodalton (s)
Luria-Bertani
milliamperes
minute (s)
nucleotides
polyethylene glycol, carbowax
plaque-forming units
restriction enzyme (s)
replicative form
ribonucleic acid
revolutions per minute
room temperature (23°C)
sodium dodecyl sulfate
second (s)
single-stranded
N,N,N',N'-tetramethylene aminomethane
tris (hydroxymethyl) amino methane
unit (s)
ultraviolet
volts
vanadyl ribonucleoside complex
watts
5-dibromo 4-chloro 3-indolygalactoside
To Mum and To Dave:

If only you were here...
INTRODUCTION

The replication and stability of eukaryotic chromosomes is dependent on at least three types of elements: centromeres, the attachment sites for mitotic and meiotic spindle fibers (25), ARS elements, the presumptive origins of replication (210), and telomeres, the molecular ends of the chromosomal DNA molecules (25). Such yeast chromosomal elements have been cloned and characterized using artificial yeast chromosomes (29, 56, 140, 141).

A. Yeast Chromosome Structure

Yeast *Saccharomyces cerevisiae* provides a choice system for eukaryotic chromosome structure and replication studies. Most details of genome organization and function are conserved in yeast and higher eukaryotic organisms (69) and the relatively simple yeast genome has been extensively characterized. Yeast chromosomes consist of linear, double-stranded DNA packaged with histones into nucleosomes (69, 155). Haploid yeast strains contain seventeen chromosomes or linkage groups which range in length from 150 kb to 2500 kb, as shown by genetic analyses (135, 136) and by electron microscopic observation of synaptonemal complexes in meiotic cells (35). The yeast genome consists of $9 \times 10^9$ daltons or $1.4 \times 10^4$ kb of DNA (118). About 80 - 85% of yeast cellular DNA is contained in the nuclear chromosomes, and the other 15 - 20% is 2μm plasmid and mitochondrial DNA (155). Only about 5% of the yeast genome is repetitive DNA, as determined by renaturation kinetics (32, 118), and this 5% consists largely of yeast rRNA and tRNA genes (69). Each yeast chromosome contains a single DNA molecule ($10^2 - 10^3$ kb), as determined by sucrose gradient sedimentation analysis (27, 152), electron microscopic analysis of DNA contour lengths (154), viscoelastic measurement (103, 118) and by karyotype analysis using orthogonal-field-alternation-gel electrophoresis, (OFAGE), (36, 37, 173). The DNA molecule is continuous through the centromere on the chromosome since circular derivatives of yeast chromosome III (109, 186), and yeast centromeric regions (25, 51, 53) have been isolated. The ends of the chromosomal DNA molecules isolated from yeast behave as free ends as determined by their sensitivity to digestion with the double-stranded exonuclease Bal31 or the single-stranded S1 nuclease (175, 193, 206).
B. The Function of Telomeres on Chromosomes

1. Telomeres and Chromosome Stability

The importance of telomeres in providing stability to a chromosome was recognized through studying chromosomes that were broken by irradiation (138) or by dicentric chromosome breakage during mitosis (120, 121, 122). Whereas broken chromosome ends undergo fusion, degradation, and recombination events, telomeres provide stability to chromosome ends. In 1938 Muller coined the term "telomere" for any monopolar chromosome end in reference to the stable polarity of genes and chromosomes in the cell. Drosophila telomeres were regarded as immutable structures due to the low frequency of healing events in irradiated chromosomes (138, 165). Dicentric chromosomes are broken due to the force exerted by pulling the two centromeres in opposing directions on the mitotic spindle during the cell cycle. McClintock described the breakage-fusion-bridge cycle in maize (120, 122), in which the unstable ends of the broken dicentric chromosome fuse, producing another dicentric chromosome which is broken during the next mitotic cycle. However, broken maize chromosomes were sometimes stabilized or "healed" during the breakage-fusion-bridge cycle, presumably due to the addition of telomeres to the broken ends. Dicentric yeast chromosomes were constructed by selecting for recombination events between circular and linear chromosomes in yeast (82, 83), or by the addition of a second centromere to a monocentric yeast chromosome (191). Resolution of dicentric chromosomes in yeast results in a novel genome organization, indicating the instability of ends broken during mitosis or meiosis, however the breakage-fusion-bridge cycle has yet to be demonstrated in yeast. The reactivity of broken ends, resulting in recombination, is evident in site specific substitutive yeast transformation. With the introduction of linear DNA fragments lacking telomeric regions in yeast, the "broken" ends readily recombine with genomic DNA at a region homologous to the ends of the introduced DNA fragment (149, 150, 196). Alternatively plasmids which contain telomeric DNA at the ends and a yeast origin of replication, i.e. an autonomously replicating segment (ARS region) (33, 47, 48, 49, 70, 184, 210), replicate autonomously as linear molecules or minichromosomes when introduced into yeast (56, 65, 140, 156, 193).
2. Telomeres and DNA Replication

The unique telomeric structure at chromosome ends must provide a mechanism for the complete replication of the ends of a linear DNA molecule (208). All DNA polymerases that have been characterized require a polynucleotide primer with a 3' hydroxyl group (3' OH) to initiate DNA synthesis and the polymerases elongate exclusively in the 5' to 3' orientation. De novo synthesized RNA fragments usually provide the priming function (114), but these RNA fragments are later replaced by DNA which is synthesized by DNA polymerase from a 5'-upstream primer. Mechanistic problems arise with linear DNA molecules because the removal of the 5'-terminal RNA primer leaves a 5'-gap, hence an incompletely replicated daughter strand on the progeny chromosomes (Fig. 1a). Circular DNA genomes do not require terminal gap filling mechanisms since primer removal, followed by strand elongation from the adjacent region around a circular template, inevitably juxtaposes the 5' and 3' ends in DNA replication. Some prokaryotic viruses have linear DNA genomes with cohesive end regions or terminal redundancies, and consequently the termini of the DNA molecule are completely replicated through the temporary removal of the chromosome ends by circularization as in phage λ or concatemer formation as in phage T4 or T7 (114, 208). An animal virus, the herpesvirus pseudorabies, similarly replicates as circles or concatemers through the intramolecular ligation of complementary terminal sequences on the linear double-stranded DNA genome (15, 96). The novel mechanism of protein priming for initiation of DNA synthesis described for bacteriophage φ29 (158, 207) and adenovirus (44, 46, 81, 134, 161) obviates the requirement for a 5' terminal RNA primer. The 5' terminal proteins on the bacteriophage φ29 or adenovirus genomes bind the initiating nucleotides from which DNA polymerase can elongate in DNA replication. In an in vitro system for Adenovirus replication, the 80 kd precursor protein for the 5'-termini reacts with dCTP to give an 80 kd-dCTP protein-nucleotide primer. This interacts with the template DNA molecule at the origin of replication, located 9-18 bp from the terminus of the adenovirus genome, to form a 5' terminal complex on the daughter strand which is elongated by adenovirus DNA polymerase (45, 46, 81, 197).
Figure 1. The 5'-end Problem in DNA Replication and Some Possible Mechanisms Proposed as Solutions.

a. The 5' end problem in replication of a linear DNA molecule (208). The 5' ends of the daughter DNA strands remain unreplicated following removal of the terminal RNA primers.

b. Cavalier-Smith model(41) for the role of terminal palindromic sequences in completing the chromosome ends after the DNA replication. The 3' overhang on the parental strand folds back on itself due to intrastrand complementarity. Ligation of the parental strand with the daughter strand, a specific endonucleolytic cleavage, and extension of the parental strand in the 5'->3' direction completes replication.

c. Covalently closed loop model as proposed by Bateman (11). In this model, the termini are self-complementary and exist as covalently closed loops. DNA replication continues around the end and eliminates the requirement for a terminal primer.

d. Crossed strand exchange model proposed by Heumann (87). Completion of the 5' end of the daughter strands occurs by recombination between the the terminal repeat sequences and a similar internal repeat region on the chromosome.

e. Dancis and Holmquist fusion model (55, 91) for termini completion. Transient fusion and fission of chromosome ends are due to telomeric associations and direct repeats on all termini. Formation and resolution of a cruciform structure in the fission process results in the reproduction of hairpin loop ends.

f. Completion of chromosome ends by simple terminal repeat addition reviewed by Blackburn and Szostak (23, 25). Tandem, simple repeats containing specific single-strand gaps are present at chromosome ends. Further repeat unit addition during replication by recombination or a terminal transferase-like enzyme ensures complete replication of the ends.

Replication is equivalent for both daughter DNA molecules, hence only the completion of a single DNA molecule is presented for models b, d, e, f. The parental DNA strands are represented by solid lines (—), newly replicated daughter strands are broken lines (----), the RNA primers are vertical lines (»•»•), and the 5' -> 3' polarity of a parental strand is represented by an arrowhead (•*•). Repeat sequences are represented by the boxes and complementary nucleotides are represented by open (•) and closed (ESI) boxes. Endonucleolytic cleavages in the replication completion mechanisms are indicated by triangles (△).
Processing of the 80 kd protein to a 55 kd molecule results in the mature 5’ terminal protein covalently linked to either 5’-end of the Adenovirus DNA molecule (43).

Viruses provide relatively simple systems which are thought to reflect the replicative mechanisms utilized for completion of the host cell chromosomes. Consequently, models described for the complete replication of the DNA termini on eukaryotic chromosomes are based on terminal structures that have been characterized for eukaryotic viruses. All proposed schemes for the DNA replication of chromosome ends involve DNA strand exchange or temporary elimination of the extreme ends of the linear DNA strands and invoke specialized telomeric sequences or structures (Fig 1). The existence of palindromic DNA sequences that form hairpin structures at the chromosome ends (Fig. 1b) to complete DNA synthesis through strand exchange was proposed by Cavalier-Smith (41). Following gap-filling and ligation, a site-specific endonuclease cleavage, and hairpin transfer results in a 3' recessed end. Subsequently, the 5' to 3' elongation activity of DNA polymerase from the 3' OH completes the terminus. The rolling hairpin model for the replication of the linear single-stranded DNA of parvovirus was based on hairpin transfer (198). In this model a 5' terminal primer is not required because the priming function is provided by the terminal hairpin structures (44, 134). Direct support for this model was obtained with the DNA sequence analyses of hairpin termini of both viral and replicative form DNA molecules (6, 8, 9, 119). Bateman simplified the Cavalier-Smith model by proposing covalently closed or cross-linked termini on chromosomes (Fig. 1c) such that the chromosomal DNA molecule is regarded as circular, self-complementary, and single-stranded. DNA replication then continues around the ends and hence eliminates the terminal gap problem (11). Daughter strands are resolved by an endonucleolytic cut which is specific for the telomeric region, resulting in unfolding of the ends, followed by refolding of the covalently closed ends on the progeny DNA molecules. The terminal structures determined for some linear DNA genomes support the Bateman model. One terminus of the linear mitochondrial DNA molecule of Paramecium contains a cross-link, at least transiently during replication (76). Terminal loops or crosslinks were observed near the ends of the vaccinia virus genome, since denatured viral DNA strands do not separate at the ends (73). DNA sequence analysis of the termini (10) indicated that tandem repeats exist near the
ends of the vaccinia virus genome and the terminal 104 nt region exists as a single-stranded loop which is not completely base paired. Alternately, the termini of linear DNA molecules may be completed in DNA replication by a crossed-strand exchange mechanism between an internal repeat sequence and a terminal repeat sequence (Fig. 1d, (87) or a modified loopback mechanism described for Tetrahymena mitochondrial DNA (77). In this model, the internal repeat region is transferred to the incomplete terminus through recombination, and the resultant gap is completed by the 5' to 3' elongation activity of DNA Polymerase from the internal 3' OH. Cytological evidence of telomeric associations led Dancis and Holmquist (55, 91) to propose the completion of linear chromosome ends through the temporary removal of chromosome ends by a transient fusion followed by a fission process (Fig. 1e). This model predicts similar DNA sequences, in the same orientation on all telomeres, since this would facilitate the recognition of fused ends during the fission process. Telomeric associations and DNA sequence analyses of telomeric regions in eukaryotic chromosomes or extrachromosomal fragments (described below) provides support for their model. Recently proposed models for completion of eukaryotic chromosome ends are based on the structures characterized for cloned chromosome ends (Fig. 1f). The addition of terminal repeat sequences to chromosome ends, and single-stranded gaps within the terminal repeats are explained by either recombinational mechanisms (40, 203, 205) or by invoking a novel terminal transferase-like activity for the completion of chromosome ends in DNA replication (175).

C. Cytology of Telomeres

Transient telomeric associations with one another and with the nuclear membrane are observed cytologically. During mitosis, meiosis, or interphase of the cell cycle, telomeric associations (Reviews 25, 55, 91) may be essential for proper chromosome pairing and segregation as well as the non-random distribution of genetic material in interphase nuclei as shown in plants and insects (5, 74, 168, 214). Results are consistent with telomeres of both homologous and nonhomologous chromosomes becoming attached during interphase by chromatin connections. Possibly the termini of the DNA molecules associate by intermolecular ligation during DNA replication (55, 91).
Repetitive DNA sequences at telomeric regions in a given genomic complement, possibly resulting in heterochromatic structures at chromosome ends, may account for the transient telomeric associations. The heterochromatic structure of telomeres in the maize genome was observed as a knob at the chromosome end which lengthens the chromosome end but does not add necessary genetic material (120). Heterochromatin has been observed in other species but it is generally considered as a nonessential characteristic of telomeric structure (25). The primary structure of the repetitive DNA at the chromosome ends may be the necessary factor for telomeric functions and associations.

Telomeres in *Secale* species contain repeated sequences along with large blocks of heterochromatin, which display both intraspecies and interspecies heterozygosity (12, 14, 99). *In situ* hybridization of *Drosophila* polytene chromosomes with a telomeric probe, has revealed repeated sequences at chromosome ends and homology of telomeres with heterochromatin in the centromeric region (168, 214). Similarly, repetitive DNA was localized to the heterochromatin of the centromeric regions and some telomeric regions in *Rhynchosciara* polytene chromosomes through *in situ* hybridization with satellite RNA probes (66). Telomeric regions of *Xenopus* also contain repetitive DNA (151). During replication, amplification of telomeric repeat blocks occurs at the ends of the germ line chromosomes in *Ascaris* which may explain the heterochromatic region observed at the termini (167). Conclusive evidence of repetitive sequences at the telomeric regions of the chromosomes in a species was obtained using cloned telomeres from yeast (49, 175, 193), trypanosomes (24, 203), and the linear DNA fragments in the somatic macronuclei in ciliates (20, 22, 23, 113, 147, 212).

Telomere specific binding proteins or alternate DNA secondary structures may also be responsible for the associative behavior of telomeres. The chromatin structures of the telomeric regions on macronuclear fragments in the single-celled eukaryotes *Tetrahymena*, *Physarum*, and *Oxytricha* are non-nucleosomal (21, 50, 79). A deoxynucleoprotein complex exists at the termini of *Oxytricha* linear fragments (79) and the interaction between telomeric complexes is proposed. Z-DNA or poly-dG regions may explain the aggregation of telomeric regions since these structures will self-associate (4, 64, 162). Z-DNA may exist at telomeric regions, as demonstrated by the binding
of anti-Z DNA antibodies (4) and poly-dG regions have been sequenced at the extreme ends of telomeric regions (Reviews 23, 25).

D. Molecular Characterization of Telomeres

1. Extrachromosomal Linear DNA

In the ciliated protozoans, the germinal micronuclear DNA molecules are fragmented, the rDNA genes are amplified, and these linear rDNA gene fragments exist as short chromosome-like molecules in the somatic macronucleus. These naturally occurring amplified DNAs facilitated sequence and structural analyses of the termini on the linear DNA molecules (22). The ends of the amplified 21 kb rDNA palindrome in *Tetrahymena thermophila* (10^4 copies per macronucleus), were the first to be extensively studied (101), and these contain twenty to seventy tandem repeats of the sequence 5'-CCCCAA-3' (20), also referred to as 5'-C_4A_2-3' repeats, with specific single-stranded gaps in the terminal 100 bp of the repeat units (22). The extreme ends of the linear rDNA molecules may be blocked by hairpin loops, since they are not accessible to end-labelling techniques, and do not appear to be covalently bound with a terminal protein. The 5'-C_4A_2-3' repeat region varies in chromatin structure from bulk macronuclear DNA which is arranged into typical nucleosomes, as determined by nuclease protection experiments (21). The terminal repeat sequence is presumably required for telomeric function since other holotrichous ciliates such as *Glaucoma* (102) and *Paramecium* (212) have conserved the 5'-C_4A_2-3' repeat region which is added to the linear fragments during macronuclear development. The related hypotrichous ciliates, *Stylonychia* (147), *Oxytricha* (113), and *Euplotes* (113) have a similar terminal sequence on their macronuclear DNA fragments, which is poly-5'-CCCCAAAA-3', or more simply referred to as 5'-C_4A_4-3' repeat units. Conservation of telomeric repeat sequences in macronuclear and micronuclear genomes was demonstrated for *Oxytricha* (59) since the micronuclear chromosomal DNA molecules also have variable lengths of terminal 5'-C_4A_4-3' repeat sequences.

Amplified, palindromic linear rDNA molecules in the slime molds *Physarum* (98) and *Dictyostelium* (67) contain the related terminal repeat sequences 5'-C_3TA-3' and 5'-C_18T-3', respectively. In *Physarum*, the terminal restriction fragments display length and sequence
heterogeneity whereas the rDNA sequences are conserved (17). Single-stranded gaps are specifically located adjacent to the inverted repeat sequences, 5’-CCCTA-3′ or 5′-TAGGG-3′ within the one kilobase terminal region in Physarum (17, 98). This region also contains more complex terminal repeat units that may form hairpin ends (17, 98). The extrachromosomal linear fragments in both Oxytricha (79) and Physarum (50) have non-nucleosomal termini and terminal protein complexes.

2. Yeast Chromosomes

The molecular cloning of a yeast Saccharomyces cerevisiae telomere (193) provided sufficient DNA for molecular characterization of telomeres on chromosomes that participate in mitotic and meiotic events during the cell cycle. Yeast telomeres were cloned using a linear plasmid that had two Tetrahymena termini and which replicates in yeast (193). Yeast fragments that replaced one of the Tetrahymena ends were selected in yeast by assaying for the ability of the fragments to replicate the plasmid as a linear molecule (193). A yeast telomeric fragment derived from such a linear plasmid was extensively studied through Southern hybridization and DNA sequence analyses. The structure of yeast telomeres as they are presently understood is illustrated in Figure 2. Like the ends of extrachromosomal DNA molecules in simple eukaryotes, yeast termini may be covalently closed by a hairpin-like structure (71, 72, 193). The termini on yeast chromosomes contain simple repeat sequences that are tandemly arranged (T region), adjacent to a more complex repeat region (Y’ region and X region) which is referred to as the telomere associated region (49, 175, 193). Variable amounts of T region sequence (about 100 bp) exist between the Y’ and X regions (205). The 0.3 kb to 0.6 kb terminal T repeat sequence region, as well as the internal T region consists of 5’-C2-3A(CA)1-4-3’ units (175, 205, L.L.Button and C.R.Astell, in press), which are more simply described as 5’-C1-3A-3′ repeats. The T region at the terminus of the chromosome contains specific single-stranded gaps within the repeat units (49, 175). The telomere associated Y’ and X regions represent a family of ARS regions (47, 48, 49), which are presumptive origins of DNA replication in yeast (Review 210). The Y’ regions are conserved 6.7 kb regions that consist of 131 and Y regions, and are present in a tandem array of 1-4 copies on more than 50% of the chromosome ends in the yeast genome (49, 205, 206). Alternatively, X regions are heterogeneous in restriction map and
Yeast ARS elements of the Y' and X classes have been mapped to the telomere associated regions on yeast chromosomes. The arrangement of the repeat regions at yeast telomeres was described initially by Chan and Tye (49) and was modified by Walmsley et al. (205). The T region (WM) represents 5'-C-A-3' simple tandem repeat sequences located at the terminus of the chromosome as well as the Y'-X junction region. The first class of yeast telomeres described (a) has between 1 and 4 conserved 6.7 kb Y' elements, consisting of Y (■) and 131 (■) regions, adjacent to a single, heterogeneous X (■■■■) element. A variable length of T region separates the Y' and X elements on these telomeres. The second class of telomeres (b) contains a single heterogeneous X element adjacent to the T region at the chromosome end. Unique DNA sequences on the chromosome (---) map centromere proximal to the X element in both the T-Y'-X and the T-X types of telomeres.
length (0.3 - 3.75 kb), are present in single copy on all chromosome ends in the yeast genome, and have no homology with the Y' region (49). Consequently, there are at least two classes for yeast chromosome ends: (1) T-Y'-X telomeres, (Fig. 2a) and (2) T-X telomeres, (Fig. 2b). The telomere isolated on the linear yeast plasmid by Szostak and Blackburn (193) belongs to the T-Y'-X class and the T-X yeast telomere has been observed in Southern hybridization analyses with yeast telomeric probes (93, 205, 206).

3. Trypanosome Chromosomes

Telomeres of the hemoflagellate Trypanosoma brucei have been isolated by exploiting the known properties of chromosome ends: simple terminal repeats, single-stranded gaps near the ends, and Bal31 nuclease sensitivity of the termini (24, 203). The termini of T. brucei chromosomes consist of multiple tandem repeats of the sequence 5'-CCCTAA-3', also referred to as 5'-C3TA2-3' repeats, adjacent to a region of more complex repeats (24, 203). Other flagellate species apparently have retained the terminal simple repeat region (24). Progressive growth of telomeric restriction fragments (7-10 bp per generation) has been observed in T. brucei (18) through Southern hybridization analyses with probes from the 3' ends of telomERICally located variant surface-antigen genes (YSG). Proposals for the terminal addition of 5'-C3TA2-3' repeats during replication explain telomeric growth properties (24, 203).

E. Artificial Chromosomes in Yeast

1. Heterologous Telomeres in Yeast

The cloning of yeast telomeres was preceded by the demonstration that termini from Tetrahymena rDNA molecules provided replicative stability to a linear plasmid in yeast (193). Following the propagation of the linear plasmids in yeast, the Tetrahymena rDNA ends had been transformed into yeast-like telomeres in that yeast terminal repeat 5'-C1-3A-3' regions (100-300 bp), with yeast specific terminal gaps, were added to the ends of the Tetrahymena 5'-C4A2-3' repeats (175, 193, 204). Similarly, linear plasmids were stabilized in yeast with Oxytricha fallax termini.
(5'-C4A4-3' repeats), and these were capped by yeast terminal repeat addition (300-1000 bp of 5'-C1-3A-3' repeats), (156). The ability of termini from ciliate macronuclear DNA to function in the phylogenetically distinct yeast S. cerevisiae may reflect the conservation of a mechanism for DNA replication of chromosomal termini (56, 65, 140, 193, 195). Presumably terminal repeat structure, rather than recognition of a specific sequence, is essential for telomere replication and stability. Consistent with this idea, the Physarum polycephalum rDNA molecule is maintained in yeast in a linear form, in the absence of selection (117).

2. Artificial Chromosome Construction and Stability

The construction of artificial chromosomes in yeast was facilitated by the following observation. The terminus of the Tetrahymena rDNA molecule is not essential for a functional telomere in yeast since a circular clone containing an inverted repeat of Tetrahymena rDNA ends (ie. lacking the terminus region but containing a portion of the 5'-C4A2-3' repeat region) is resolved into a linear molecule with two functional telomeres in yeast (195). Cloned Tetrahymena rDNA ends were readily available and could be inserted as an inverted repeat in a circular plasmid construct, then resolved in yeast to render linear minichromosomes (65, 140, 194, 195).

The four known elements required for chromosome stability and replication are genes, ARS elements, centromeric region, and telomeres, and these were recombined in vitro to render artificial yeast chromosomes (56, 140, Reviews 26, 141). Natural yeast chromosomes are maintained in single copy in haploid cells and the mitotic stability (chromosome loss per generation) ranges from 10^{-4} to 10^{-5} (139). Centromeric minichromosomes, containing the LEU2 gene, CEN3 and ARS1 regions, with Tetrahymena rDNA termini are maintained in single copy in yeast cells if the length approaches the range of chromosomal DNAs (10^2 to 10^3 kb), (140) although the mitotic stabilities of long artificial chromosomes remain at least two orders of magnitude less than natural chromosomes. Possibly an unidentified element or specific spacing of elements may be required for stability. Artificial yeast chromosomes have not been constructed with natural yeast telomeres but these may be required for the proper mitotic segregation of artificial chromosomes due to requisite telomeric associations in the cell (56, 140, Review 25).
F. Stabilization of Linear Plasmids in Yeast

Yeast telomere associated Y' regions enhance chromosome end stability and provide healing functions to broken chromosome ends (65). A linear plasmid with a partial Y' region (i.e. broken artificial chromosome end) acquires additional Y' regions through a RAD52 dependent recombination mechanism and the mitotic stability of the linear plasmid increases with further Y' region addition (65). The yeast RAD52 gene product is required in recombinational events that involve double-strand break initiation (Review 196). Linear plasmids with *Tetrahymena* termini are stabilized or "healed" in yeast with the addition of T region repeats (5'-C1-3A-3') by a RAD52 independent mechanism (65, 215), followed by RAD52 dependent Y' region addition as observed with broken yeast chromosome ends (65). Addition of Y' ARS regions to *Tetrahymena* termini reflects the requirement for strong ARS activity at yeast chromosome ends (65). Increased ARS activity refers to a more efficient and regulated replicator which is defined by increased transformation frequency and mitotic stability on plasmids in yeast (33, 42, 104). Internal to the 5'-C4A2-3' repeat region on the *Tetrahymena* rDNA end there is a region which contains the ARS consensus sequence (33), and which functions as a yeast origin of replication (1, 105, 139, 140). However, the yeast Y' and X telomeric ARS regions have higher ARS activity than the region on the *Tetrahymena* rDNA end (65). The role of the telomere associated X region in telomere healing or chromosome stability has not yet been defined.

Y' region recombination occurs among chromosome ends within a given yeast strain. Although the telomere associated Y' region is highly conserved, there are restriction enzyme site polymorphisms among different yeast strains, and among meiotic segregants within a given strain (93). The extensive homology of the yeast chromosome ends (49) and the DNA repeat sequence units within the Y region (92) may result in the rearrangement of Y regions, along with the stabilization of broken chromosome ends, through gene conversion or other recombinational events.

6. Genetic Control of Telomere Length in Yeast

Different yeast strains display variation in the terminal restriction fragment lengths due to strain specific lengths of the T region (5'-C1-3A-3' repeat units), (40, 93, 206). All chromosome ends within a given yeast strain have similar amounts of telomeric T region repeats and apparently the
length is genetically controlled by a set of genes that are co-dominant in a diploid yeast strain (206). The cell division cycle CDC17 gene is at least partially responsible for the control of T region length at the chromosome end (40). Strains with temperature sensitive mutations in CDC17 demonstrate progressive growth in telomere length in a RAD52 independent manner, due to deficiency in functional CDC17 gene product (40).

H. Isolation and Characterization of the Telomeric Region from a Specific Yeast Chromosome

1. Chromosome Walking

Limited information was known about the structure of eukaryotic chromosome ends when this research project, involving the isolation a specific yeast telomere, was proposed. Tetrahymena rDNA macronuclear fragments were shown to terminate in variable lengths of 5'-C_{4}A_{2}-3' repeats and possibly with hairpin protected ends (20). Yeast S. cerevisiae chromosome ends were thought to exist as covalently closed loops (71, 72), but other terminal structures such as protein primers, palindromes, and repeat sequences (discussed earlier) were also plausible. Consequently, we decided to isolate a S. cerevisiae telomere by chromosome walking from a distal locus that was characterized on the chromosome end, since this cloning method does not select for a specific terminal structure or function. The technique of chromosome walking (13, 51) involves the use of a cloned chromosomal fragment as a hybridization probe to screen collections of genomic DNA clones for the isolation of novel fragments that overlap the original sequence. Hence through progressive steps, contiguous stretches of a particular chromosomal region can be analyzed. Chromosome walking is facilitated in yeast compared to higher eukarotes due to the relatively small amount of repetitive DNA, (about 5%), in the yeast genome (32, 69). Chinault and Carbon (51) were able to clone the centromere from yeast chromosome III (CEN3 region) by chromosome walking from LEU2 on the left end (III L) to CDC10 on the right end of chromosome III (III R). Identification of CEN3 in the isolated region was confirmed by assaying for the centromeric stabilization of autonomous plasmids in yeast (53).
A similar approach was adopted in this study to isolate the telomeric region on chromosome IIIL by chromosome walking from the most distal known gene on IIIL toward the telomere. The average length of the terminal regions on yeast chromosomes was estimated, given several assumptions. Firstly, the yeast genetic linkage map, which consists of seventeen linkage groups (136), is complete between the distal markers on any given yeast chromosome. If so, then any DNA that is not accounted for in the linkage map must exist at the terminus of the chromosomes. Secondly, the physical size and genetic linkage map proportionality of 2.7 kb/cM, determined for chromosome III by Strathern et al. (186), represents an average value for other regions of the yeast genome. Thirdly, equivalent lengths of terminal regions exist on all chromosome ends. The average terminal length was calculated as follows. The haploid yeast genome contains $1.4 \times 10^4$ kb of DNA which is equivalent to 5185 cM. The genetic linkage map encompasses about 3500 cM (136), hence 1685 cM is unaccounted for and may be distributed equally among the thirty-four chromosome ends. If these assumptions are valid, about 50 cM or 135 kb separates the distal genetic marker from the terminus on each yeast chromosome. Based on this estimated length, chromosome walking would appear to be a feasible approach to telomere isolation since at least 15-20 kb could be covered per step with clones prepared with lambda vectors (125). Since telomeres provide stability and complete replication to chromosomes, telomeric DNA must have distinctive characteristics recognizable through DNA sequence analyses, Southern hybridization and by assays for a functional telomere on linear plasmids in yeast. In this manner a yeast telomeric region could be isolated without prior knowledge of its structure, and the entire region between the telomere and the unique DNA on the chromosome end could be characterized.

2. Yeast Chromosome III

Yeast chromosome III was selected for the isolation of a telomere by chromosome walking for several reasons. The most distal known genetic marker on IIIIL is HMLa, one of the mating type genes. Mating type genes HMLa, MAT, and HMRa have been cloned (142), sequenced (7), and their orientation on chromosome III is known (187). Yeast strains containing circular derivatives of chromosome III were available (109) and were invaluable controls for progression towards the
telomere during chromosome walking experiments. Such circular III strains resulted from recombination between the mating type loci, as occurs during yeast mating type interconversion or switching events (143, 109). Information at the MAT locus situated on the right arm of III determines mating type. HML and HMR are unexpressed storage cassettes for α and α information respectively, and are repressed by the concerted action of unlinked loci known as SIR, MAR, or CMT genes. Mating type interconversion is a gene conversion event, and is initiated in homothallic (HO) yeast strains by the HO endonuclease double-stranded break (115, 116) within the recipient MAT locus (111, 188). In standard yeast strains (MAR+), only MAT switches mating types while the HML and HMR loci remain unchanged (106). However, mar1 or mar2 strains switch efficiently at HML loci as well as MAT (108). Circular chromosome III strains were derived from a mar1 parental strain by recombination events between HML and HMR (109). Presumably the telomeric regions of chromosome III are deleted in the circular III strains. Haploid circular III yeast strains are viable (109), indicating there are no essential genes distal to HML and that the distance between the III terminal and HML is relatively short. Electrophoretic karyotype analyses (36, 37, 173) indicate that chromosome III is 370 – 390 kb in length. Since 350 kb separate the chromosome III distal markers HML and MAL2 (107), the total amount of DNA distal to HML and MAL2 is probably 20 – 40 kb.

3. Characterization of the Telomeric Region on III

In this study it was found that the III terminus mapped 12 kb distal to HML as determined by Bal 31 nuclease sensitivity and the presence of a T region consisting of 5′-[C2-3A(CA)1-4]-3′ repeat units. Characterization of the cloned telomeric region from III by Southern hybridization and DNA sequencing analyses revealed that the III end is a T-X class telomere which extends distal to the III unique region with an average length of 1.8 kb and a size distribution of ± 0.2 kb for the terminal length. Telomeric properties described using probes from T-Y’-X telomeres (49, 65, 92, 93, 175, 193, 205, 206) were assayed with the novel T-X telomeric probe which hybridized exclusively to chromosome ends. The ability to transform yeast with cloned DNA (89) facilitated the
studies for telomeric function that were conducted by cloning the III L end on linear plasmids in yeast (193, 194, 195). The required information for a functional III L telomere was 50 bp (or possibly less) of the T region and the adjacent X ARS region. Linear plasmids containing deletions in the telomeric fragments from III L had T region addition but were never healed with Y' regions. Hence the T-X class of the III L telomere was maintained on linear plasmids in yeast and a rationale for the existence of two classes of yeast telomeres, T-X and T-Y'-X, is discussed. Hybridization analyses of linear III and ring III yeast strains with HMLqi distal probes indicated that the 9 kb terminal region on III L is retained in most circular III yeast strains through replacement recombination with an alternate homologous region in the yeast genome. HMLqi distal probes hybridized with an undefined yeast RNA species which led to the novel suggestion that there may be a unit of transcription in the HMLqi distal region which is functionally duplicated at the alternate region.
MATERIALS AND METHODS

A. Materials

All chemicals were analytical or reagent grade. Acrylamide, bisacrylamide and TEMED were purchased from Bio-Rad Laboratories; agarose (Type I) was from Sigma Chemical Company, and Low Melting Point (LMP) Agarose was from Bethesda Research Laboratories. Nitrocellulose filters (BA85) were from Schleicher and Schuell; Gene Screen or Gene Screen Plus was purchased from New England Nuclear. [α-32P]dNTPs (2000-3000 Ci/mmol) were either from New England Nuclear or Amersham Corp., deoxy NTPs and dideoxy NTPs were obtained from P.I. Biochemicals Inc. M13 oligonucleotide primers were synthesized on an Applied Biosystems oligonucleotide synthesizer. Restriction enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, or Boehringer Mannheim and used as specified by the supplier. E. coli DNA Polymerase I (Klenow fragment) was supplied by Bethesda Research Laboratories, Boehringer Mannheim, or Promega Biotec; E. coli DNA Polymerase I (Kornberg enzyme) was supplied by Boehringer Mannheim or Bethesda Research Laboratories. Nuclease Bal31 and Exonuclease III were supplied by New England Biolabs; S1 nuclease and T4 DNA Ligase were from Bethesda Research Laboratories. DNase I and Bacterial Alkaline Phosphatase (BAP) were purchased from Worthington, Calf Intestinal Alkaline Phosphatase (CIP) was from Boehringer Mannheim. Zymolase was from Kirin Breweries Co. Ltd. and Glusulase was from DuPont Pharmaceuticals. Bovine Serum Albumin (BSA) and Lysozyme were purchased from Sigma Chemical Co. BSA (DNase free) was supplied by Bethesda Research Laboratories.

B. Strains and Media

1. Yeast

Yeast strain AB20α XP8-10B (MATa hohis6leu1met1trp5-1gal2can7), described by Nasmyth and Tatchell (142), was used for cloning the IIIIL telomere. Strain K45 (HMLα MATα HMRα mar1hotrpl thr1 arg4 lys1 ade8 his2 ura1 met1), and the HML-HMR fusion derivatives, K191 and K192, as well as other circular III strains K193 (MATα HML-HMR fusion mar1 ade8-10lys1 met1), K195 (MATα HML-HMR fusion mar1 ura1 lys1), and K196 (MATα HML-HMR fusion mar1 ade8-10lys1 met1) were used.
were gifts from A. Klar, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (109). AB972 (MATa trp1-901 lys2-801 his3-112 leu2-3, 112 ura3-52, 112 can1) were provided by M. Olson, Washington University, St. Louis, Mo. (37, 169). Strains SR25-1A (MATa his4-912 ura3-52 and SR26-12C (MATa his4-912 ura3-52 leu2-3, 112) were gifts from S. Roeder, Yale University, New Haven, Conn. Strains T388 [(MATa leu2-3, 112 his3-11, 112 ura3-1) + pSZ216 (LEU2 his3)], (193), and A281 (formerly LL20-11-2), (63), a cir° derivative of A2 (MATa leu3, 112 his3-11, 15 can1) were gifts from J. Szostak, Harvard Medical School, Boston, Mass. (195). Yeast strains were grown at 30 °C with aeration in complete medium, YEPD (1% yeast extract, 2% peptone, 2% glucose), or in synthetic complete medium (0.67% yeast nitrogen base without amino acids, 2% glucose with all amino acids added except the selected marker amino acid), (176). Amino acids were prepared as a 100 x mixture containing 2 mg/ml of each of L-trp, L-ala, L-cys, L-met, L-pro, 3 mg/ml for each of L-ile, L-lys, L-tyr, L-leu, 5 mg/ml L-asp, L-glu, L-phe, and 10 mg/ml for L-ser, L-thr, L-val. The 100 x stock also contained 1 mg/ml uracil, and 2 mg/ml adenine sulfate, (166). Yeast Regeneration Agar was equivalent to selection medium, with the addition of 1M Sorbitol, and 2% Agar (177).

2. Bacteria

Escherichia coli strains DP50 [F−, tonA53, dawD8, lacY1, glnV44, (supE44), Δ(gal-uvrB 47, λ−, tyrR58, supF58 gyrA29, Δ(thyA57, hsdS3) or LE392 [F, hsdR51·rK− mR−], supE44·supF58·lacY1 or Δ(lacI2Y6, galK2, galT22, metB1, trpR55, λ−]] (126) were hosts for LCharon4A clones. Growth was in LBMgT medium which consists of LB medium [10 g/1 bactotryptone, 5 g/1 yeast extract, 5 g/l NaCl, (pH 7.2)], (57), supplemented with 10 mM MgCl2 and 50 mg/ml thymidine. LBMgT plates contained 1.5% agar, while top agar contained 0.7% agar or 0.7% agarose. E. coli RR1 [F−, hsdR52·rK−·mR−], ara-14, proA2, lacY1, galK2, rpsL20, (57), xyl1-5ml1-1, supE44], (126), DH1 [F−, recA1, endA1, gyrA96, thi, hsdR17·rK−·mR−], supE44, relA1?, λ−] or MM294 [F−, endA1, hsdR17·rK−·mR−], supE44, thi−, λ−], (130) were host strains for plasmid clones. Growth of RR1, DH1, or MM294 was in LB medium for minipreparations (5-10 ml), and in M9-minimal salts medium (6 g/l Na2HPO4, 3 g/l KH2PO4, 1g/l NH4Cl, 0.5 g/l NaCl, 1 mM
MgSO₄, 0.1 mM CaCl₂, 1 mM thiamine HCl, and 0.2% glucose), (57), for 0.5 - 1 liter cultures. For selective growth, ampicillin (AMP) was added to 50 mg/l, tetracycline (TET) was added to 12.5 mg/l, or chloramphenicol (CAM) was added to 20 mg/l. Amplification of plasmid DNA in cultures was with 170 mg/l chloramphenicol, or 10 mg/l for amplification on agar plates. E. coli JM101 (supE, thi-, lacproF, traD36proA8lacI92-M15), (132) was propagated in M9-minimal salts or YT media, [8 g/l Tryptone, 5 g/l yeast extract, and 5 g/l NaCl, (pH 7.2-7.4 )], (132). All strains were incubated at 37 °C, with aeration.

3. Phage and Plasmids

Lambda Charon4A vector DNA and phage packaging extracts were gifts from T. Snutch and I. Kovesdi, respectively, Simon Fraser University, Burnaby, B.C. The recombination screening system, IIAN7 vector and host strain MC1061 (p3), (H. Huang, Washington University, St. Louis, Mo.), was a gift from R. MacGillivray, University of British Columbia, Vancouver, B.C. The AMG14-Yeast (AB972) genomic library was kindly supplied by M. Olson, Washington University, St. Louis, Mo. The yeast genomic library in plasmid vector YEpl3, prepared by K. Nasmyth (142), was a gift from M. Zoller, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. Plasmids pSZ220 (193), pSZ93, pSZ221, pSZ222, (195, 150), and pSZ218 (194) were kindly provided by J.W. Szostak, Harvard Medical School, Boston, Mass. Plasmids YRp131A and YRp131B (48, 49) were gifts from C.Chen and B.-K. Tye, Cornell University, Ithaca, N.Y.

C. Basic Molecular Cloning Techniques

Most basic molecular cloning techniques were conducted as described by Maniatis et al. (126). Those routine methods include agarose and acrylamide gel preparation, restriction enzyme site mapping, isolation of DNA fragments from low melting point (LMP) agarose, phenol/chloroform (CHCl₃) extractions, ethanol precipitations, and quantitation of DNA using EtBr/agarose plates.
D. Yeast DNA Isolation

1. Large Scale DNA Preparation

High molecular weight yeast DNA for genomic library construction was isolated from *S. cerevisiae* AB20a XP8-10B cells by the spheroplast lysis - sucrose density gradient and EtBr cesium chloride equilibrium gradient purification method of Olson et al. (148). Cells from a 500 ml culture grown in YPD medium, at 30 °C, to late log or stationary phase (1 x 10^8 cells/ml), were pelleted at 4000 g, 10 min, 4 °C. After washing the cells with 30 ml distilled H2O, resuspension was with 2 ml spheroplasting mix per gram wet weight of cells, [1 M Sorbitol, 0.1 M NaCitrate, 0.06 M EDTA (pH 7.0), 1% 2-mercaptoethanol]. Zymolase 5000 (5 mg per gram of cells) was added and the suspension was incubated at 37 °C for 2 hr. Cells were lysed by slowly adding 3.5 ml lysis buffer per gram of cells, [3% sarkosyl, 0.5 M Tris, 0.2 M EDTA (pH 9.0)] and incubating at 65°C, 15 min. After quick chilling the lysed cell mixture on ice until the temperature was reduced to about 23°C, the mixture was loaded on sucrose step gradients (3 ml 50% sucrose cushion, 26 ml 10% - 30% w/v sucrose) prepared in Beckman polyallomer SW27 tubes. Gradients were centrifuged at 11000 rpm, 16 hr, 9 °C in a SW27 rotor. After removing the top layer, the viscous fraction at the 15 - 20% sucrose interface was collected with a wide bore pipet. The DNA solution was dialyzed against TE [10 mM Tris, 1 mM EDTA, (pH 7.5)] for about 12 hr at 4 °C, with at least one buffer change. Since there are no DNA pelleting and resuspension steps in this protocol, the probability of shearing DNA during the preparation is minimal. However, the preparation of sucrose gradients, subsequent fractionation, and dialysis of DNA fractions makes this method more tedious than later more efficient methods.

The preferred method for isolation of yeast high molecular weight DNA from 50 ml to 1 liter cultures was a modification of the Stiles protocol (183). For each 50 ml stationary phase culture (2x10^8 cells/ml), the cells were pelleted at 4000 g for 5 min, and washed with 3.5 ml TE [50 mM Tris, 50 mM EDTA, (pH7.5)]. Resuspension was in 3.5 ml spheroplast solution [1 M Sorbitol, 0.1 M EDTA (pH8.0), 14 mM 2-Mercaptoethanol], and Zymolase 60,000 (2.5 mg) was added. After incubation at 37 °C for 1 hr, spheroplasts were pelleted at 4000 g, 5 min, 4 °C. The spheroplasts
were resuspended in 3.2 ml TE [10 mM Tris, 1 mM EDTA, (pH 7.5)] followed by the addition of 0.32 ml 0.5 M EDTA (pH 8.0), 0.16 ml 2 M Tris base, 0.16 ml 10 % SDS. Cell lysis was completed by incubation at 65 °C for 30 min. Cell debris was removed by precipitation with 0.8 ml 5 M KAc (pH 4.8), incubation on ice for 1 hr, and centrifugation at 20,000 g for 15 min at 0°C. The supernatant was transferred to a fresh tube and DNA was precipitated with 2.5 volumes of EtOH (95%) at 23 °C. DNA was pelleted by 12,000 g centrifugation at 23 °C for 15 min, and resuspended in 5 ml TE [10 mM Tris, 1 mM EDTA, (pH 7.5)]. DNA was purified by centrifugation in EtBr-cesium chloride equilibrium density gradients.

Alternatively, yeast DNA was isolated by a similar spheroplast lysis protocol (177), however it was more time consuming and produced DNA that frequently gave partial digestion with restriction enzymes.

2. Small Scale DNA Preparation

Minipreparation yeast DNA (58) was used for the preliminary analysis of yeast transformants. Each transformant was grown in a 5 ml culture of selection medium if the selected amino acid marker was on an autonomously replicating (ARS) plasmid. If the selected marker was integrated into the genomic DNA of the yeast transformant, growth was in complete medium (YPD). Cells were pelleted for 5 min in a table top centrifuge (2500 g), then washed with 1 ml 1 M Sorbitol and transferred to eppendorf tubes. The cell pellet was resuspended in 0.5 ml of spheroplast solution (1 M Sorbitol, 50 mM KH₂PO₄, 14 mM 2-mercaptoethanol) and 0.2 mg Zymolase 5000 was added. Following incubation at 30 °C for 30 min, spheroplasts were pelleted, resuspended in 0.5 ml lysis solution [50 mM EDTA (pH8), 0.2% SDS], and incubated at 70 °C for 15 min. To remove cell debris and SDS, 50 µl 5 M KAc (pH 4.8) was added, tubes were left on ice 30 min, then centrifuged at 12,000 g at 4 °C for 15 min. Supernatants were added to 2 volumes EtOH (95%) at 23 °C, then centrifuged at 12,000 g at 23 °C for 15 sec. DNA pellets were dried in air, and resuspended in 50 µl TE [10 mM Tris, 1 mM EDTA, (pH7.5)] with 10 µg/ml RNaseA. If DNA was to be used for restriction enzyme digestion, it was first purified by Phenol/CHCl₃ (1:1) extraction and EtOH precipitation.
3. Yeast DNA Purification

Yeast DNA was purified by EtBr-cesium chloride equilibrium density gradient centrifugation. DNA isolated from a 500 ml culture was made to 9.4 ml with TE [10 mM Tris, 1 mM EDTA, (pH 7.5)], then 0.5 ml 100x TE was added, along with 10.02 g CsCl. After transfer to a Beckman polyallomer 16 x 76 mm quick seal tube, 0.15 ml ethidium bromide (10 mg/ml) was added. Centrifugation was at 60,000 rpm in a Ti70.1 rotor for 16 - 20 hr at 15 °C. The DNA band was visualized with long wave (366 nm) UV light, recovered with a syringe and 20G needle. EtBr was removed with n-butanol extractions, and the DNA solution was dialyzed against TE for 12 hr at 4 °C, with 1 or 2 buffer changes. DNA concentration was determined by A_{260} measurement, and by comparison with DNA standards using agarose/EtBr gel electrophoresis and UV fluorescence (126).

E. Lambda Phage DNA Isolation

1. Large Scale DNA Preparation

DNA from phage lambda clones was isolated using a modification of the reported procedures (126). Host LE392 bacteria (1.25 ml fresh stationary phase culture) were diluted with 1.25 ml of 10 mM CaCl₂, 10 mM MgCl₂, then infected with about 1.2 x 10⁸ pfu of a given phage clone. Following adsorption at 37 °C for 10 min, cells and phage were used to inoculate 250 ml LB/MgT medium that was preincubated at 37 °C. The culture was shaken vigorously (250 rpm) in a New Brunswick Scientific incubator until cells were lysed (6 - 8 hours). Complete lysis was ensured by adding 3 ml CHCl₃ and shaking (100 rpm) for an additional 3 min. Cells were pelleted at 8000 g at 4 °C for 15 min. The supernatant was added to 0.15 x volume 5 M NaCl, and 0.3 x volume 50 % PEG, mixed by inversion, and incubated overnight (16hr) at 4 °C. The precipitated phage were collected by centrifugation at 8000 g, at 4 °C for 15 min. The phage pellet was resuspended in 5 ml DNase I buffer [50 mM Tris (pH 7.5), 5 mM MgCl₂, 0.5 mM CaCl₂], and 50 μl DNase I (1 mg/ml) plus 100 μl RNase A (5 mg/ml) were added. After incubation at 37 °C for 30 min, the phage mixture was centrifuged at 14,000 g for 5 min. Phage supernatant was treated with 0.5 ml 10% SDS, 50 μl 0.5 M EDTA (pH 7.5), and 150 μl proteinase K (5 mg/ml), and incubated at 68 °C for 1 hr. DNA was purified by
phenol / CHCl₃ extractions and EtOH (95%) precipitation. DNA (about 0.5 mg) that was isolated from a 250 ml culture, was resuspended with 0.5 ml TE [10 mM Tris, 1 mM EDTA (pH 7.5)].

2. Small Scale DNA Preparation

The procedure described above was also used to isolate phage DNA from 10 ml cultures. A single plaque was used to infect 50 µl of host LE392 cells. Prior to removal of the phage coat with SDS, EDTA, and proteinase K, an aliquot (10%) was reserved at 4 °C for inoculation of large scale preparation. Phage DNA prepared in this manner was restriction enzyme digested in the presence of Bovine Serum Albumin, or BSA (50 µg/ml) and RNase A (50 µg/ml).

3. Phage DNA Purification

On occasion, phage DNA preparations were not readily digested with restriction enzymes. Such DNAs were purified by one of the following methods. (a) DNA was ethanol precipitated in the presence of ammonium acetate, with 0.5 volume 7.5 M NH₄Ac and 2 volumes ethanol (95%). For DNA concentrations greater than 50 µg/ml, incubation at -70 °C for 30 min was sufficient to precipitate DNA. DNA concentrations less than 50 µg/ml required -20 °C incubation for 12 hr. (b) Impurities were efficiently removed by CsCl density gradient centrifugation. Phage DNA (100 µg) was brought to 3.8 ml volume in TE [50 mM Tris, 10 mM EDTA, (pH 8.0)], then CsCl (3.9 g) and EtBr (0.3 ml of 10 mg/ml solution) were added. Centrifugation was in Beckman 13 x 51 mm polyallomer tubes in a VT165 rotor at 50,000 rpm at 20 °C for 16 hr. The phage DNA band was recovered, n-butanol extracted to remove EtBr, adjusted to 3 ml with TE [10 mM Tris, 1 mM EDTA, (pH 7.5)], and DNA was precipitated with 3 volumes ethanol (95%) at -20 °C for 12 hr.

F. Plasmid DNA Isolation

All solutions used for large or small scale plasmid DNA preparations were chilled on ice prior to use.

1. Large Scale DNA Preparation

Preparative scale plasmid DNA was isolated by the detergent lysis protocol (57) from 500 ml cultures of M9-minimal medium that were amplified with CAM. Cells were pelleted at 4000 g at 4 °C for 10 min, and washed with 25 ml TE [35 mM Tris, 100 mM EDTA, (pH 8.0)]. Cells were
resuspended in 10 ml STE [18% sucrose, 35 mM Tris, 100 mM EDTA, (pH 8.0)], and 2 ml fresh lysozyme solution (10 mg/ml in STE) was added. Following incubation on ice for 20 min, 2 ml 0.5 M EDTA (pH 8.0) and 0.5 ml RNaseA (2 mg/ml) were added. Following RT incubation for 5 min, cells were treated with 20 ml of lysis solution [1% Triton X, 15 mM EDTA, 50 mM Tris, (pH 8.5)] at 0 °C for 10 min. Cell debris was pelleted by centrifugation in a Ti45 rotor at 30,000 rpm at 4 °C for 1 hr. The supernatant was phenol extracted, then 2 or 3 volumes of ethanol (95%) were added, and left at -20 °C, overnight. Precipitated nucleic acid was pelleted at 2500 g at 4 °C for 10 min, and resuspended in 1 ml TE [10 mM Tris, 0.1 mM EDTA (pH 8.0)].

2. Small Scale DNA Preparation

Analytical preparations of plasmid DNA were isolated by the alkaline lysis procedure (19) from 5 ml stationary phase cultures. Cells from 1.5 ml aliquots were pelleted by centrifugation in an Eppendorf microcentrifuge, at 12,000 g at RT for 1 min. Cells were resuspended in 0.1 ml fresh lysozyme buffer [50 mM glucose, 25 mM Tris (pH 8.0), 4 mg/ml lysozyme]. After 5 min at RT, cells were lysed by adding 0.2 ml fresh lysis solution (0.2 N NaOH, 1% SDS). The tubes were inverted several times, then left 5 min at 0 °C. Cell debris and SDS were precipitated with 0.15 ml ice cold KAc (pH 4.8). Tubes were vortexed for 10 sec, left 5 min at 0 °C, then centrifuged at 12,000 g at 4 °C for 5 min. The supernatant was extracted with phenol/CHCl₃. Nucleic acid was precipitated by addition of 2 volumes RT ethanol (95%) with incubation at RT for 2 min and was pelleted with centrifugation at RT for 5 min. The precipitate was washed with 1 ml ethanol (70%), dried under vacuum, and resuspended in 50 μl TE [10 mM Tris, 1 mM EDTA (pH 7.5)]. RNaseA was added to 20 μg/ml and plasmid DNA was stored at -20 °C.

3. Plasmid DNA Purification

Preparative scale plasmid DNA was purified on EtBr cesium chloride equilibrium density gradients (1 or 2 gradients per DNA preparation from a 500 ml cell culture). For each gradient, the DNA solution was adjusted to 9.2 ml with TE [10 mM Tris, 1 mM EDTA (pH 7.5)], and 0.5 ml 100 x TE and 9.7 g CsCl were added. After transfer to a polyallomer 16 x 76 mm quick seal tube, about 0.8 ml EtBr solution (10 mg/ml) was added in the neck of the tube using a syringe and needle. The
tube was heat sealed, then shaken to mix the EtBr and CsCl solutions. Centrifugation was at 60,000 rpm in a T170.1 rotor for 16 hr at 15 °C. The closed circular band of plasmid DNA was recovered from the gradients as described for yeast DNA purification on CsCl gradients.

6. Yeast RNA Isolation

Total yeast RNA was isolated according A. Spence (manuscript in preparation), and RNA from strain GM3C2 or BM-CYC+ was provided by A. Spence and B. McNeil, U.B.C., Vancouver, B.C. Yeast cells from a 50 ml log phase culture (2 x 10^7 cells/ml) were treated with 0.25 ml cycloheximide solution (20 mg/ml in 95% ethanol), and cells were pelleted at 3000 g at 4 °C for 5 min. Cells were washed in 10 ml ice cold extraction buffer [0.1 M Tris (pH 7.5), 0.15 M NaCl, 0.1% DEP], pelleted, and resuspended in 0.25 - 0.5 ml extraction buffer at 0 °C. Cells were broken after adding 0.05 volume VRC (0.2 M) and acid treated glass beads (0.45 - 0.50 mm diameter) to below the surface level of the liquid in the tube. Vortexing at maximum speed for 5 times in 15 sec pulses, with 45 sec intervals on ice between each pulse, served to break the cells open as determined by phase contrast microscope analysis of an aliquot taken from the supernatant. The supernatant was transferred to a sterile Eppendorf tube along with 3 x 100 μl rinse solution from beads. The combined supernatant was centrifuged at 12,000 g in an Eppendorf microcentrifuge for 5 min at 4 °C. Following transfer of the supernatant to a fresh tube, SDS was added to make the solution 0.5% SDS. VRC was added to 0.05 x volume and proteinase K was added to 0.6 mg/ml. After incubation at 37 °C for 60 min, the mixture was extracted with phenol/CHCl₃ (1:1 mix), and ethanol (95%) precipitated. The pellet was rinsed with ethanol (70%), and resuspended with 0.5 ml 10 mM EDTA (pH 8.0), then 0.5 ml 4 M LiCl was added. RNA was precipitated at 0°C for 16 hr, and pelleted with centrifugation at 12,000 g at 4 °C for 15 min. Following resuspension of RNA in 10 mM EDTA (pH 8.0), undissolved material was removed with a second centrifugation at 12,000 g. The RNA in the supernatant was ethanol precipitated and resuspended in 0.15 - 0.25 ml H₂O. The RNA yield was estimated by A₂₆₀ measurement (126).
**H. Yeast Genomic DNA Library Preparation**

Lambda Charon4A DNA was isolated by the formamide extraction method from phage prepared by the PDS procedure and purified on CsCl gradients (28, 126). Following EcoRI digestion of λCh4A phage DNA, the cohesive ends of the 20 kb and 10 kb EcoRI vector arms were annealed by incubation at 42°C for 2 hr. The resultant 30 kb vector DNA fragment was separated from the 6.6 kb and 7.8 kb phage DNA stuffer fragments by electrophoresis in a 0.5% Low Melting Point (LMP) agarose gel at 1.5 V/cm for 40 hr. Isolation of the 30 kb band was by electrophoresis onto a Whatman 3MM filter paper strip, backed by a piece of dialysis membrane, (75) placed in a slit in the gel that was cut directly below the 30 kb band. DNA was recovered from the filter paper and membrane with several washes of elution buffer [10 mM Tris (pH 7.6), 10 mM NaCl, 1 mM EDTA, 0.2% SDS]. The isolated vector DNA was ethanol precipitated and quantitated by agarose gel electrophoresis. A second preparative 0.5% LMP agarose gel purification of the 30 kb fragment which encompasses the 20 kb and 10 kb vector arms was performed to ensure the complete removal of stuffer fragments and a 1:1 ratio of vector 20 kb (left) and 10 kb (right) arms.

Yeast DNA 16-20 kb partial EcoRI digest fragments were prepared by digesting high molecular weight yeast DNA with a range of EcoRI concentrations (0.25 - 1.0 u/μg), with aliquots taken at 15 min, 30 min, and 60 min intervals from each reaction. The aliquots were combined, and were fractionated alongside lambda DNA size markers on a 0.5% LMP agarose gel at 2 V/cm for 16 hr. Fragments in the 17 kb range were isolated by the filter paper strip method (75). Fragments larger than 21 kb were prevented from contaminating the 16-20 kb fraction by inserting an additional filter paper-dialysis membrane strip at the 21 kb region to collect higher molecular weight fragments.

Concentrations of λCh4A vector and yeast DNA fragments were determined by the EtBr agarose plate method (126). Ligation mixes required a 2:1 molar ratio of λCh 4A vector arms to yeast EcoRI fragments, and a minimum DNA concentration of 0.25 μg/μl in the ligation mixture (125). The 10 μl ligation mix contained 2 μg of the vector arm fragment (30 kb) and 0.5 μg yeast EcoRI fragments (15 kb). The vector arm fragment was incubated at 42°C to ensure that all 20 kb and 10 kb arm fragments were joined at the cohesive ends. The yeast DNA fragments and ligation buffer were added
along with 2 u T4 DNA Ligase. Incubation was at 4 °C for 20 hr. The extent of ligation was assayed by analysis of 5% of the ligation mix on a 0.3% agarose minigel alongside undigested λCh 4A vector (0.2 µg) and EcoRI digested vector (0.2 µg). In vitro packaging was carried out with ligated vector plus yeast insert (68, 90). One packaging extract (50 µl, stored at −70 °C) was thawed on ice for 3 min and the ligation mix (9.5 µl) was added along with 1.5 µl ATP (0.1 M), and 20 µl CH buffer [40 mM Tris (pH 8.0), 10 mM spermidine, 0.1% 2-mercaptoethanol, 7% DMSO, 1.5 mM ATP]. The suspension was mixed with a glass rod at 15 min intervals during incubation in a 37 °C H2O bath for 1 hr. A second packaging extract aliquot was thawed 3 min on ice, and 10 µl DNasel (1 mg/ml) plus 2.5 µl MgCl₂ (1 M) were added. Half of the extract (25 µl) was added to the λCh4A/yeast DNA packaging reaction, and 37 °C incubation was continued for 30 min, with stirring at 10 min intervals. Finally, 0.9 ml SM [0.1 M NaCl, 0.01 M Tris (pH 7.5), 0.01 M MgCl₂, 0.02% gelatin] was added along with a few drops CHCl₃. The genomic library was stored at 4 °C.

I. Bacteriophage Lambda Techniques

Host bacteria cells (DP50 SupF or LE392) were grown to stationary phase (10⁹ cells/ml) in a 10 ml culture and pelleted in a table top centrifuge at 2000 rpm for 10 min. Cells were resuspended in 0.5 x volume of λ dll [10 mM Tris (pH 7.5), 10 mM MgSO₄]. Plating bacteria (0.15 ml) were mixed with the appropriate number of phage and incubated for 15 min at 37°C to allow phage adsorption. Cells and phage were added to 3 ml of 0.7% top agar or agarose at 42 °C, and the mixture was poured onto a 37 °C agar plate. The top agar layer was hardened at RT for 10 min, then plates were inverted and incubated at 37 °C for 10-14 hr.

Phage stocks were prepared by plating about 10⁵ phage per 85 mm plate. After 37 °C incubation to obtain confluent lysis, 5 ml of λ dll were added to each plate, and plates were incubated (right side up) for 12 hr at 4 °C. The overlay solution was removed with a pasteur pipet and 0.1 ml CHCl₃ was added. Bacterial and agar debris were removed with a brief spin in the table top centrifuge. The supernatant was made to 0.3% CHCl₃, and stored at 4 °C.
J. Plasmid Dephosphorylation and Ligation

Reaction conditions with bacterial alkaline phosphatase (BAP), (30), for plasmid DNA dephosphorylation consisted of restriction enzyme digested vector (0.01 μg/μl), and BAP (0.05 u/μg) in 50 mM Tris (pH 8.0). After 30 min at 37 °C, the reaction was stopped with the addition of 1 μl 0.25 M EDTA (pH 8.0), followed by phenol extraction and ethanol precipitation. Conditions used for calf intestinal alkaline phosphatase (CIP) were restriction enzyme digested DNA (0.05 μg/μl) and CIP (0.05 u/μg) in 1 x CIP buffer [50 mM Tris (pH 9.0), 1 mM MgCl₂, 0.1 mM ZnSO₄, 1 mM spermidine]. Incubation was at 60 °C for 30 min and at 68 °C for 1 hr. Dephosphorylated DNA was ethanol precipitated before the subsequent ligation reactions.

Intermolecular ligations with plasmid DNA typically contained 0.1 - 0.2 μg of linearized plasmid DNA and 2-5 x molar excess of insert fragment in 20-25 μl reaction volumes with 1-2 u T4 DNA Ligase, and incubation at 16°C for a minimum of 12 hr. Intramolecular ligations were carried out in large volumes (100-1000 μl) under otherwise similar conditions. Ligation buffer used for initial cloning reactions contained 66 mM Tris (pH 7.6), 6.6 mM MgCl₂, 10 mM DTT, 1 mM ATP. Later work indicated better ligation efficiencies were obtained with T4 DNA Ligase using ligation buffer containing 50 mM Tris (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 1 mM spermidine, 0.1 mg/ml BSA, and 1 mM ATP.

K. M13 Cloning

1. Preparation of M13 Replicative Form DNA

M13 Replicative Form DNA (RF) was prepared by either the in vivo or the in vitro methods (132), however in vitro preparations were preferred due to the simplicity and rapidity of the method. In vitro M13 RF DNA was prepared by annealing 2-4 μg M13 template clone DNA with 4 pmole M13 universal primer in a 20 μl reaction mixture containing 10 mM Tris (pH 8.0) and 5 mM MgCl₂ at 55 °C for 10 min. The primer was elongated with DNA Polymerase I, Klenow fragment (2u), and 0.25 mM dNTPs for 20 min at RT. The reaction was stopped by heat inactivation of the DNA Polymerase I, Klenow fragment at 70 °C for 10 min. In vivo M13 RF DNA (132, 213) was prepared from a 500 ml culture of JM101 cells infected with the M13 clone of interest. Stationary phase
JM101 cells grown in M9 medium (about 10^8 cells) and a M13 clone plaque picked from a fresh plate were used to inoculate 5 ml of 2 x YT medium. Growth at 37°C for 4 hr with vigorous shaking produced sufficient phage for infection of a 500 ml culture. The 5 ml phage supernatant and 5 ml of stationary phase JM101 cells were added to 500 ml of 2x YT medium and incubated at 37 °C for 4-5 hr with high aeration. Cells were harvested by centrifugation at 4000 g at 4 °C for 10 min. M13 RF DNA was isolated by the alkaline lysis procedure described for plasmid DNA isolation (126), and purified by EtBr-cesium chloride equilibrium density gradient centrifugation.

2. Subcloning Fragments in M13 Vectors

Fragments to be subcloned in the Smal site of the M13RF vector were made blunt-ended using DNA Polymerase I, Klenow Fragment (126). After digesting the DNA with restriction enzymes, it was treated with Klenow fragment (2u), in a 50 µl reaction mixture [50 mM Tris (pH 7.4), 10 mM MgSO4, 0.01 mM DTT, 50 µg/ml BSA], containing 8 µM dNTPs. After incubation at RT for 20 min, the DNA Polymerase I Klenow fragment was heat inactivated at 70 °C for 10 min. The resultant blunt ended fragments were purified following electrophoresis in 0.7% LMP agarose by extraction with phenol/CHCl3 (126). M13 cloning ligation mixes usually had 20-30 µl volumes and routinely contained 0.8 pmoles/ml M13 vector DNA, 5x molar excess of insert fragment, ligation buffer and 1-2 u T4 DNA ligase. Reactions were incubated at 16 °C for 16-20 hr (132).

3. M13 Template DNA Preparation

The single-stranded M13 template DNA was isolated after infecting 1.2 ml JM101 cells (early log phase) with a selected M13 plaque, and growing for 4 hr at 37 °C, with vigorous shaking. Cells were pelleted in an Eppendorf microcentrifuge for 3 min. The phage supernatant (800 µl) was added to 200 µl PEG solution (15% PEG 4000, 2.5 M NaCl) and incubated for 15 min at 23 °C. Precipitated phage were pelleted by centrifugation at 12,000 g at RT for 10 min in an Eppendorf microcentrifuge and the supernatant was removed by aspiration. After resuspension of phage with 100 µl TE [10 mM Tris, 1 mM EDTA (pH 7.5)], phage DNA was isolated with phenol/CHCl3 extractions and EtOH precipitation. DNA was resuspended in 40 µl TE and stored at -20 °C.
4. M13 Clone Complementation Test

To establish the orientation of the insert in a given M13 clone, hybridization screening (132) using a clone of defined insert orientation as reference was performed on selected M13 clone templates. Hybridization buffer consisted of 10 mM Tris (pH 7.5), 0.1 M NaCl, 0.1% SDS and 1 mM MgCl₂. The number of clones tested for insert orientation (x) determined the volumes of premix (+) and premix (-) reagents. The volume of the premix (+) or (-) reagent was 8(x+2) μl, and each contained (x+2) μl of 10x hybridization buffer. The premix (+) reagent also had 2(x+2) μl of template DNA from a homologous M13 clone of known insert orientation. For each clone to be tested, 2 μl of template DNA was added to 8 μl of each premix reagent. Hybridization was for 1 hr at 65 °C. The reactions were terminated with the addition of 5 μl of 60% sucrose, 0.02% Bromophenol Blue, 0.02% Xylene Cyanol, and 0.025 M EDTA (pH 8.0). For each tested clone, premix (+) and (-) reaction mixes were run in adjacent lanes on a 0.7% agarose gel at 150 V for 3 hr. Tested M13 clones having the opposite insert orientation to the standard M13 clone migrate as a single band in the premix (+) lane, with slower mobility than the premix (-) sample due to the double stranded region of the hybridized insert region. M13 clones having the same insert orientation as the standard clone have equivalent mobilities in premix (+) and (-) lanes.

L. Plasmid and M13 Transformations

Plasmid or M13 DNA was introduced into bacteria cells that were made competent by incubation in CaCl₂ (54, 57, 126, 132). An overnight culture of bacteria was diluted 100-fold in culture medium (0.5 ml in 50 ml) and incubated at 37 °C with shaking, until mid-log phase or about 2 hr. Cells were pelleted in sterile 30 ml tubes at 4000 g at 4 °C for 5 min. Cells were resuspended in 0.5 x volume of cold CaCl₂ (50 or 100 mM) and kept on ice 15 min. After pelleting the cells by centrifugation as done previously , the cells were resuspended in either 0.5 ml 100 mM CaCl₂ or 4 ml 50 mM CaCl₂ . For RR1, DH1, or MM294 cells, plasmid DNA transformation efficiencies were improved by incubating the resuspended cells in the CaCl₂ solution at 4 °C for 12-16 hr prior to use. Freshly prepared JM101 competent cells were used for M13 transformations.
For plasmid DNA transformation, 0.1 ml of 100 mM CaCl₂ cells or 0.2 ml of 50 mM CaCl₂ cells were used per reaction. After adding DNA in TE [10 mM Tris, 1 mM EDTA, (pH 7.5)] or ligation buffer, cells were incubated on ice for 30 min. Cells were heat shocked at 37 °C for 5 min, and 1 ml of LB or YT medium was added per tube. Cell wall recovery was at 37°C for 1 hr without shaking. Aliquots of the transformation mix were plated on selection plates and colonies were visible after 12-16 hr at 37 °C.

For transformations with M13 DNA (ss template of RF DNA), 0.3 ml 50 mM CaCl₂ treated JM101 cells and M13 DNA (2 ng), or ligation mix aliquots, were incubated on ice for 40 min. Following heat shocking at 45 °C for 2 min, cells were added to 3 ml YT top agar that contained 50 μl Xgal (2%), 10 μl IPTG (100 mM), and 0.2 ml exponential JM101 cells. Cells and top agar mixtures were plated on YT agar plates and plaques were visible after incubation at 37 °C for 10 - 14 hr.

M. DNA Probe Preparation

1. Nick Translation

Nick translated probes (126, 163) were used predominantly for isolating clones by plaque hybridization or colony hybridization. A typical nick translation reaction contained 0.5 μg DNA, 5 μl 10 x nick translation buffer [0.5 M Tris (pH 7.4), 0.1 M MgCl₂, 1 mM DTT, 0.5 mg/ml BSA], 5 μl 2 mM CaCl₂, 5 μl each of 0.1 mM dGTP and 0.1 mM dCTP, 0.2 μl each of 0.1 mM dATP and 0.1 mM dTTP, 2.5 μl each of [α 32 P] dATP and [α 32 P] dCTP, 0.5 μl DNase I (25 pg/μl, freshly diluted from 1 μg/μl stock), 0.5 μl DNA polymerase I, Kornberg enzyme (2.5u) and H₂O to 50 μl total volume. Incubation was at 16 °C for 2.5 hr. The reaction was terminated with the addition of 2 μl 0.5 M EDTA (pH 8.0) and incubation at 68 °C for 10 min. After chilling the reaction mix on ice, the unincorporated radioactivity was removed by gel filtration chromatography using AcA 54 (5000 - 7000) matrix, and a BioRad dispocolumn (17 x 1 cm). The column buffer was 0.2 M NaCl, 10 mM Tris, 0.25 mM EDTA (pH 7.8). Fractions (500 - 700 μl) were collected in Eppendorf tubes. The probe eluted in the void volume (fractions 3-4) and had an average specific activity of 10⁷ cpm/μg, (Cerenkov counts). The probe was denatured by adding 0.04 volume of 5 M NaOH and heating at 90-
100 °C in a H₂O bath for 2 min. and quick chilling at 0 °C. The probe solution was neutralized with 0.04 volume of 5 M HCl and 0.1 volume of 1 M Tris (pH 7.4).

2. M13 Primer Extension

Most Southern hybridization probes were prepared from fragments cloned in M13 vectors using a modification of the M13 sequencing protocol (132). The M13 universal sequencing primer was annealed to the template clone DNA in a 10 μl reaction mixture containing 0.5 – 1 μg single-stranded template DNA and 2 pmole primer in reaction buffer [10 mM Tris (pH 8.0), 5 mM MgCl₂]. The annealing reaction was incubated at 55 °C for 15 min and cooled at room temperature for 10 min. After adding 5 μl dNTP mixture (100 μM dGTP, 100 μM dCTP, 15 μM dATP, 15 μM dTTP), 20 μCi each of [α³²P] dATP and [α³²P] dTTP, plus 2 μg DNA polymerase I Klenow fragment, incubation was at 23 °C for 20 min. The primer extension reaction was stopped with incubation at 68 °C for 10 min. The unincorporated radioactivity was removed from M13 probe preparations by gel filtration chromatography on AcA54 columns then probes were denatured with alkali and heat, as described for nick translated probes. Specific activities of 10⁷–10⁸ cpm/μg (Cerenkov counts) were routinely obtained for M13 primer extended probes.

N. Gel Hybridization Analyses

1. Agarose Gel Electrophoresis and Southern Hybridization

Agarose gel electrophoresis of DNA samples (126) was conducted using between 0.5% to 0.75% agarose gels, containing 1 μg/ml EtBr, and Tris borate buffer [TBE: 50 mM Tris, 50 mM borate acid, 1 mM EDTA, (pH 8.3). Sucrose-dye stop solution [50% sucrose, 25 mM EDTA (pH 7.4), 0.02% Bromophenol Blue and 0.02% Xylene Cyanol] was added to samples prior to gel loading such that the final sucrose concentration was between 5% and 10% (w/v). Yeast genomic DNA or high molecular weight DNA fragments were subjected to electrophoresis at low voltage gradients (1-2.5 V/cm) for 13 – 17 hr. Visualization of DNA samples by UV fluorescence was conducted with either short wave UV (254 nm) or long wave UV (300 –360 nm).
Southern hybridizations were conducted on DNA samples in situ on dried agarose gels (201) or DNA was transferred, either bidirectionally (179) or unidirectionally (129), to nitrocellulose, NEN Gene Screen, or NEN Gene Screen Plus filters. NEN Gene Screen Plus was the preferred membrane for Southern analyses, due to its ease of handling and its efficient binding capacity. Prehybridization and hybridization conditions were routinely 6 x SSC [1 x SSC: 0.15 M NaCl, 0.015 M NaCitrate (pH 8.3)]; 10 x Denhardt's mix (0.2% Ficoll, 0.2% polyvinyl pyrrolidone, 0.2% BSA); 0.5% SDS, and 0.1 mg/ml carrier salmon sperm DNA (sheared and denatured). Filters were incubated in sealed bags at 68 °C, in a H2O bath for 12 - 24 hr. Following hybridization, the initial washes were in 2 x SSC, 0.5% SDS at RT for 2 x 15 min. Stringent washes were conducted in 1 x SSC, 0.5% SDS at 68 °C, with slow shaking in a H2O bath for 2 x 1 hr. Filters were exposed to Kodak XAR-5 film at -20 °C or -70 °C, with intensifying screens. For secondary hybridization, initial probes were removed from the transfer filters as follows: (1) Nitrocellulose filters were washed in 0.01 x SSC, 0.1% SDS, with 5 - 10 min incubation at 100 °C; (2) Gene Screen was washed for 1 to 3 hr with constant agitation at 68 °C, in 250 - 300 ml of manufacturer's suggested buffer [5 mM Tris, 0.2 mM EDTA (pH 8.0), 0.05% sodium pyrophosphate, 0.1 x Denhardt's mix]; (3) Gene Screen Plus membrane was not completely dried following hybridization, if subsequent probe removal was required. The probe was efficiently removed with incubation in a 68°C shaking H2O bath, for 1 hr using 100-200 ml of 0.4 N NaOH followed by 100-200 ml of 0.1 x SSC, 0.1% SDS, 0.2 M Tris, (pH 7.5). Filters were exposed to X-ray film to assay for the removal of the initial probe prior to hybridization with a second probe.

2. RNA Dot Blot and Northern Hybridization

Yeast RNA was subjected to dot blot analysis (199, 200) using a BRL Hybri-Dot Manifold to spot RNA (1-20 μg) on Gene Screen Plus filter that was wetted with H2O and 20 x SSC. Prehybridization and hybridization conditions were those used for Southern analyses. Northern analysis was performed on RNA samples (199, 200) fractionated in 1% agarose - formaldehyde gels with 1 x MOPS buffer [5 x MOPS: 0.2 M morpholinopropanesulfonic acid (MOPS, pH 7.0), 50 mM sodium acetate, 1 mM EDTA (pH 8.0)], (126). Following electrophoresis at 30 V for 20 hr, a portion
of the gel was stained with 33 μg/ml acridine orange in 10 mM Na₂HP0₄ (pH 6.5) for 10 min, then
destained with 3 x 20 min washes in 10 mM Na₂HP0₄ (pH 6.5) (129). RNA was visible in the
stained gel by UV fluorescence at 300 nm. RNA was transferred to NEN gene screen plus (126) and
baked in vacuo at 80 °C for 2 hr. Filters were prehybridized at 60 °C for a minimum of 6 hr in
prehybridization mix containing 5 x Denhardt’s mix, 1 M NaCl, 0.5% SDS, 10% dextran sulfate.
Denatured, sheared salmon sperm DNA (0.1 mg/ml) and denatured, nick-translated probe were added.
Hybridization was at 60 °C, in a H₂O bath for 15 - 20 hr, with constant agitation. Washes were
conducted with continuous agitation of filters for 2 x 5 min at RT, in 300 ml 2 x SSC, followed by 2 x
45 min washes at 60 °C in 300 ml 2 x SSC, 1% SDS. Finally, filters were washed in 300 ml of 0.2 x
SSC at RT. Membranes were dried at RT and exposed to CIUREX film, with an intensifying screen, for 1
week.

0. Plaque and Colony Hybridization

1. Plaque Hybridization

Bacteriophage lambda recombinant libraries were screened by plaque hybridization (16,
126). A maximum 10⁴ plaques per plate, in LB MgT top agarose, were transferred to nitrocellulose
filter discs, by laying a sterile filter on a plate for 5 min. India ink spots, positioned asymmetrically
on the periphery of the agarose surface, served to orient the filter on the plate. Phage were denatured
and fixed to filters by submerging the filters in petri plates filled with 1.5 M NaCl and 0.5 M NaOH,
for 5 min. Filters were transferred to plates containing 3 M NaCl, 1 M Tris (pH 7.0) for 5 min and
this wash was repeated. After filters were rinsed in 2 x SSC for 5 min, they were blotted on Whatman
3MM paper and dried at 68 °C for 1 hr.

Bacteriophage M13 were similarly transferred to nitrocellulose filters for screening. Denaturation and neutralization steps were carried out in duplicate. Rather than submerging filters in
petri plates, cells and phage were denatured on Whatman 3MM paper soaked with 0.5 M NaOH, 1.5 M
NaCl and neutralized on filters soaked with 1 M Tris (pH 8.0), 1.5 M NaCl. Nitrocellulose filters
were washed in 6 x SSC for 2 min, and filters were dried on Whatman 3MM paper and baked in a
68 °C oven for 1 hr.
2. Colony Hybridization

Bacterial colonies which contained recombinant plasmid DNA were screened by the high colony density method (84, 126). Colonies were plated on sterile nitrocellulose filters (maximum 10^4 per filter) on LB-AMP plates. Following growth at 37 °C for 12 hr, colonies were transferred to replica filters and grown on LB-AMP at 37 °C for 8 hr. Colonies were amplified on LB-AMP/CAM plates at 37 °C for 12 hr. Alternatively, colonies were transferred to LB-AMP grid pattern plates, grown at 37 °C until colonies were 0.2 - 0.5 cm diameter, and transferred to sterile nitrocellulose filters as for plaque hybridization. Colonies were lysed by laying filters on buffer saturated Whatman 3MM paper as described for M13 plaque hybridization. Filters were transferred to Whatman 3MM wetted with 2 x SSPE [0.36 M NaCl, 20 mM NaH_2PO_4 (pH 7.4), 2 mM EDTA] for 5 min, then filters were dried briefly on Whatman 3MM paper at RT and baked for 2 hr at 68 °C. Bacterial debris was removed from the filters by rinsing with 100 ml of prewash solution [50 mM Tris (pH 8.0), 1 M NaCl, 1 mM EDTA, 0.1% SDS], at 42 °C, for 1 to 2 hr followed by a brief rinse in 6 x SSC prior to prehybridization. Filter hybridization and wash conditions were equivalent to those described for Southern hybridization analyses, using 2 to 3 ml of hybridization mix per filter. Filters were dried at RT on Whatman 3MM and exposed to XAR-5 film with intensifying screen at -70 °C.

P. Recombination Screening

In vivo recombination selection (126, 174) was used to screen the λCh4A/yeast genomic library. In this screening method the probe fragment is cloned in a microplasmid vector (PAN7), which contains an amber suppressor tRNA gene. The genomic library, which has amber mutations in the phage vector, is grown in a recombination proficient bacterial strain that carries the probe clone. Genomic clones which have insert fragments homologous to the probe may acquire the amber supressor tRNA gene by reciprocal recombination with the microplasmid clone. The subsequent growth of the genomic library in a suppressor deficient bacterial strain selects for genomic clones which have an amber suppressor tRNA gene integrated in the probe fragment region.

The PAN7 (885 bp microplasmid which contains the ColE1 replicon, supF gene, and polylinker region) was isolated from E. coli MC1061 (p3) (PAN7) by alkaline lysis (126). The
microplasmid was separated from *E. coli* genomic DNA by electrophoresis in 0.7% LMP agarose at 30 V for 16 hr and was purified by phenol/CHCl₃ extraction of the isolated gel band (126). The probe fragment was cloned in the polylinker region of IIAN7, using 0.3 µg microplasmid vector and 1 or 3 x molar excess of insert fragment. Aliquots from the ligation mixture were assayed via miniagarose gel electrophoresis before and after the addition of 2 u T4 DNA Ligase and 16 hr incubation at 15°C. Ligation mixes were transformed in MC1061(p3) cells made competent with 100 mM CaCl₂ (54) and transformants were plated on LB-AMP (12.5 µg/ml) - TET (7.5 µg/ml) plates. DNA was isolated from selected transformants by the small scale alkaline lysis preparation (19) and analyzed for IIAN7 microplasmid containing the insert probe fragment by agarose gel electrophoresis. Bacteria containing the desired recombinant microplasmid were infected with either the amplified or nonamplified ACh4A-yeast library using 10⁵-10⁶ phage per 2.5 ml overnight cell culture (2 x 10⁸ cells/ml). Phage were plated in LB-AMP (12.5 µg/ml) - TET (7.5 µg/ml) top agar and grown at 37°C for 12-16 hr. Phage stocks were prepared and plated on Su⁺ host LE392 (5 x 10⁴ dilution) or Su⁻ host MC1061 (nondilated) to determine the Su⁺ phage resulting from microplasmid and phage recombination. True Su⁺ recombinant phage were distinguished from amber revertants by plaque hybridization of phage grown on MC1061. The hybridization probe was either the fragment that was used for the recombination screening or a fragment that mapped centromere proximal to that region on the chromosome.

Q. S1 and Bal31 Nuclease Digestion of Yeast DNA

Yeast high molecular weight DNA (10 µg) was digested with S1 nuclease (1 u) in 100 µl reaction volume, containing 1 x S1 buffer [30 mM NaOAc (pH 4.6), 150 mM NaCl, 1 mM ZnSO₄, 5% glycerol]. Following incubation at RT for 30 min, reactions were stopped by phenol/CHCl₃ extraction.

Yeast high molecular weight DNA (10 µg) was treated with the ds exonuclease Bal31 (1 u) in 100 µl reaction volume containing Bal31 buffer [600 mM NaCl, 12 mM CaCl₂, 12 mM MgCl₂, 20 mM Tris (pH 8.0), 1 mM EDTA] with incubation was at 30°C. Aliquots were removed at periodic intervals between 0 and 30 min and added to 0.1 volume of 0.2 M EGTA (pH 8.0) to terminate the
reaction. Prior to restriction enzyme digestion, aliquots were phenol/CHCl₃ extracted and ethanol precipitated.

R. DNA Sequence Determination

1. Preparation of Deletions with Exonuclease III and S1 Nuclease

The terminal IIII EcoRI fragment was isolated from MTLB6411 RF, made blunt ended with DNA Polymerase I Klenow fragment, then ligated to Smal digested M13mp18 RF (213). Sets of deletion clones were prepared with Exonuclease III (ExoIII) and S1 nuclease for both orientations of the insert present in the subclones MTLB6S12 and MTLB6S21, as described by Henikoff (86). For each clone, 10 µg DNA was treated with 800 u ExoIII in 100 µl buffer [66 mM Tris (pH 8.0), 0.66 mM MgCl₂], at 37 °C. Aliquots (7.5 µl) were taken at 30 sec intervals for 5 min, added to 22.5 µl ExoIII stop mix [0.2 M NaCl, 5 mM EDTA (pH 8.0)] per time point, and incubated at 70°C for 10 min. DNA was precipitated with 3 volumes EtOH (95%) per aliquot, resuspended in 50 µl S1 nuclease buffer [0.25 M NaCl, 30 mM KAc (pH 4.8), 1 mM ZnSO₄, 5% glycerol] and treated with 1 u S1 nuclease per aliquot for 30 min at 23 °C. The reaction was terminated with 6 µl S1 stop solution [0.5 M Tris, 0.125 M EDTA, (pH 8.0)], followed by phenol/CHCl₃ extraction and EtOH precipitation. The termini were repaired by treatment with 0.1 u DNA Polymerase I Klenow fragment per aliquot in 10 µl Klenow repair buffer [20 mM Tris (pH 8.0), 7 mM MgCl₂], at 37 °C for 2 min, followed by the addition of 1 µl dNTP mix (0.125 mM) and incubation at 37 °C for an additional 2 min. The deletion clones were religated in 40 µl ligation buffer per time point aliquot containing spermidine, BSA, and 1 u T4 DNA Ligase and incubated at 23 °C for 4 hr. Ligation mix aliquots (10 µl) were transformed in CaCl₂ treated JM101 cells. The extent of deletion was determined by agarose gel electrophoresis of a sample of template DNA clones for each ExoIII time point aliquot and comparison of the clone length with the parental clone and the M13mp18 vector.

2. DNA Sequence Determination

Deletion clone sets were sequenced by the dideoxy chain terminator method with single-stranded DNA templates (132, 170, 171). Template DNA (5 µl or 1-2 µg) was annealed with 2 pmoles M13 universal sequencing primer (or insert sequence specific primer) in 10 µl annealing
buffer [10 mM Tris (pH 8.0), 5 mM MgCl$_2$] at 55 °C for 5-10 min. After 10 min at 23 °C, 1 μl [α$^{32}$P] dATP (10 μCi) and 1 μl 12.5 μM dATP were added to the annealed mixture. In sequencing poly dC-dA repeat region, i.e. poly dG-dT template strand, the concentration of dATP was doubled by adding 25 μM dATP rather than 12.5 μM dATP. Four tubes were labelled C, T, A, or G, and 2 μl of DNA / primer mix plus 2 μl of each respective dNTP/ ddNTP mix were added. The following nucleotide mixes were used:

- dC/ddCTP: 0.014 mM dCTP, 0.25 mM ddCTP, 0.11 mM dGTP, 0.11 mM dTTP.
- dT/ddTTP: 0.0055 mM dTTP, 0.50 mM ddTTP, 0.11 mM dCTP, 0.11 mM dGTP.
- dA/ddATP: 0.05 mM ddATP, 0.075 mM dCTP, 0.075 mM dTTP, 0.075 mM dGTP.
- dG/ddGTP: 0.055 mM dGTP, 0.30 mM ddGTP, 0.11 mM dCTP, 0.11 mM dTTP.

Tubes were preincubated in a 30 °C H$_2$O bath for 5 min. DNA Polymerase I Klenow fragment was diluted to 0.25 μl/μl in 1 x Kpn buffer [6 mM NaCl, 6 mM MgCl$_2$, 6 mM Tris (pH 7.5), 6 mM 2-mercaptoethanol]. At time 0 min, 2 μl of the diluted Klenow fragment (0.5 μl) was added to each CTA6 tube. Following incubation at 30 °C for 15 min, 2 μl of the diluted Klenow fragment (0.5 μl) plus 2 μl dNTP (0.5 mM) chase mix were added per tube. Following a second 15 min, 30 °C incubation, 5 μl formamide dye stop mix (95% deionized formamide, 10 mM EDTA, 0.1% Xylene Cyanol, 0.1% Bromophenol Blue) was added. Samples were heated to 90-100°C for 2-3 min in a H$_2$O bath to denature DNA and were immediately quick chilled on ice. Electrophoresis conditions were 6% acrylamide, 7 M urea gels, 30 W (constant power), 30 mA maximum current. Samples (2 μl) were loaded at times 0, 1.5, and 3.5 hr, and electrophoresis was stopped at 4.5 hr. Gels were dried on Whatman 3MM paper using a vacuum gel dryer. Exposure of XRP-1 film was at RT for 12-16 hr.

S. Construction of Plasmids to Assay for Telomere Function

1. Clones with Requisite Restriction Site Arrangements

DNA sequence and Southern hybridization results indicated clone MTLB6S12 (Fig. 16) contained the IIIIL telomere, however it was necessary to determine whether it functioned as a yeast telomere. Plasmids replicate as linear molecules in yeast if the ends are stabilized by telomeric fragments (193) and circular plasmids which contain inverted telomeric repeats are resolved into
linear plasmids in yeast (194, 195). The IIIIL telomere in MTLB was tested for telomere function by subcloning it as an inverted repeat in the yeast plasmid pSZ218 (194), introducing the plasmid into yeast by transformation, and assaying for presence of linear plasmids. In the same manner, deletion derivatives of the IIIIL telomeric fragment were assayed for the stabilization of linear plasmids in yeast.

Initially, the telomeric fragments in MTLB6S12 and the derivative ExoIII/S1 nuclease deletion clones (MTLB6SD12 clones) were subcloned into the Smal site of M13mp19 to provide the requisite flanking restriction sites. For example, the pSZ218 vector has a single BglII site for cloning BamHI fragments as inverted repeats (194), hence it was necessary that the telomeric fragments have a BamHI site on the centromere proximal end and an EcoRI site on the distal end (Fig. 25). Telomeric fragments in MTLB6S12 and MTLB6SD12 clones were excised from the M13mp18 vector by digestion of the M13RF clone DNAs with EcoRI/BamHI for MTLB6S12 or EcoRI/HindIII for the ExoIII/S1 deletion MTLB6SD12 clones. Following treatment with DNA PolI Klenow fragment, blunt ended fragments were isolated from 0.7% LMP agarose gels. These fragments were ligated in the Smal site of M13mp19RF (213) and clones were selected by M13 clone complementation tests with the MTLB6S12 clone as the reference orientation clone. The selected clones had the telomeric fragments oriented with the BamHI/Sall sites from the M13 vector on the proximal end, adjacent to the X region and the HindIII/EcoRI sites from the M13 vector on the distal end, adjacent to the T region. All subclones were sequenced to ensure that the correct orientations and deletions of the IIIIL telomeric region had been obtained. The M13mp19 subclone which contained the full length telomeric fragment from MTLB6S12 was named TF1. The subclones TF2 to TF7 contained the set of MTLB6SD12 fragments with progressive deletions of the telomere in M13mp19 (Fig. 25). A deletion clone constructed for DNA sequence analysis of the opposite orientation clone (MTLB6S21) was used to construct a plasmid to assay for the requirement of an ARS region on the telomere. Clone MTLB6SD21-66 lacks the ARS region of the IIIIL telomere, but retains the 5'-C1-3A-3' repeat region. It did not require subcloning into M13mp19 because the telomeric fragment has the necessary
flanking RE sites in the M13mp18 vector but MTLB6SD21-66 was referred to as TF8 for the linear plasmid constructions (Fig. 25).

2. Design of Linear Plasmids

For each telomeric subclone (TF1 - TF8), double-stranded M13 clone DNA was prepared by the *in vitro* RF method. For cloning in pSZ218, BamHI/EcoRI telomeric fragments were isolated from telomeric clones TF1 - TF7. The *LEU2* gene was isolated from pSZ218 as a Sall/Xho1 fragment (2.2 kb) or a Sall/HindIII fragment (2.5 kb) for preparing a set of linear yeast plasmids which lacked the pBR322 region of pSZ218 (65). Telomeric fragments were isolated from clones TF1 - TF8 as Sall/EcoRI or HindIII/EcoRI fragments for the *LEU2* plasmid constructions. As an experimental control for testing telomeric function, the telomere of the *Tetrahymena* rDNA linear molecule was cloned as an inverted repeat in the yeast plasmid pSZ93, (195) since the *Tetrahymena* rDNA end is known to function in yeast (56, 65, 139, 140, 193, 194, 195, 215). The *Tetrahymena* rDNA end fragment (0.7 kb) which contains 0.33 kb of 5'-CCCCAA-3' terminal repeat units and 0.36 kb of rDNA unique DNA, was isolated from pSZ222 (195) by Xhol/HhaI digestion (Fig. 24). All fragments were isolated by 0.7% LMP agarose gel electrophoresis and purified by phenol/CHCl₃ extraction.

Ligation mixes were similar to those described for previous linear plasmid constructions in yeast (194, 195). Yeast telomeric fragments were ligated in 10x molar excess to yeast plasmid vector pSZ218/BgIII (0.2 µg) or to the *LEU2* fragment (0.1 µg) in 30 µl reaction volumes. The *Tetrahymena* rDNA end fragment was ligated in 10x molar excess to yeast plasmid vector pSZ93 (0.2 µg) in a 30 µl reaction volume. Ligation buffer contained 50 mM NaCl, 25 mM Tris (pH 7.4), 10 mM MgCl₂, 10 mM DTT, 1 mM spermidine, 0.1 mg/ml BSA, and 1 mM ATP. When ligation involved joining fragments with isochizomer restriction site ends, the corresponding restriction enzymes (RE) were included in the ligation mixture. This reduced the percentage of unwanted ligation events in the reaction since the joining of ends produced by the same enzyme is followed by RE cleavage, while joints from isochizomer ends are not recognized by either enzyme. For example, in ligations of pSZ218/BgIII and BamHI/EcoRI telomeric fragments, BamHI (2 u) and BgIII (2 u) were included in ligation mixes to enhance the proportion of BamHI/BgIII ligations. Ligations were
incubated for 20 hr, at 16 °C after the addition of 1.5 u T4 DNA Ligase, and 2 u of each required restriction enzyme. Enzymes were heat inactivated by incubation at 70 °C for 10 min. For ligation mixes which contained restriction enzymes, further RE aliquots (2 u) were added and reactions were incububated for an additional 2 hr at 37 °C to ensure the complete digestion of undesirable ligation products, and reduce the frequency of background transformants. Ligation mixes were stored at -70°C and used in yeast transformations.

T. Yeast Transformation

Yeast spheroplasts were transformed with plasmids as described by Sherman et al. (177) and modified by Orr-Weaver et al. (150). Cells were pelleted at mid log phase (10^7 cells/ml) in a table top centrifuge at 2000 g. For each 50 ml culture, cells were washed twice with 10 ml 1 M Sorbitol, then resuspended in 5 ml 1 M Sorbitol. Spheroplasts were generated with 150 u1 glusulase and 5 u1 2-Mercaptoethanol, at 30 °C for 1 hr. Gentle washing was critical since spheroplasts were easily disrupted. Spheroplasts were washed twice with 10 ml 1 M Sorbitol, then once in Sorbitol buffer [0.9 M Sorbitol, 10 mM Tris (pH 7.4), 10 mM CaCl2]. The final resuspension was in 1.5 ml STC [1 M Sorbitol, 10 mM Tris (pH 7.4), 10 mM CaCl2] and 0.3 ml were used per transformation. For each transformation, the 25 u1 ligation mix and 5 u1 sheared E. coli carrier DNA (8 ug) were added to the spheroplast aliquots and incubation was at 23 °C for 15 min. A 3 ml solution of 45% PEG 4000, 10 mM Tris (pH 7.4), 10 mM CaCl2 was added to each transformation mix, followed by incubation at 23 °C for 15 min. The PEG was removed after pelleting the cells in a table top centrifuge. Resuspension was in 0.5 ml STC. Cells (0.3 ml) were plated in 30 ml regeneration agar with selection for LEU^+ prototrophs and incubation at 30 °C for about 4 days. Transformants were purified by streaking individual colonies onto selective medium plates. After removal of the PEG solution from the pelleted spheroplasts, resuspension of the spheroplasts was difficult, and this may reduce the yeast transformation frequency. To test this, an alternate set of transformations was performed in which the final spheroplast pelleting step was omitted and the PEG/spheroplast mixture was plated directly in regeneration agar. The volumes of the transformation mixtures were reduced such that the PEG concentration in the spheroplast / regeneration agar mixture was low. In this alternate protocol,
spheroplasts were resuspended in 0.5 ml buffer per 50 ml cell culture and 60 μl were used per transformation with the addition of 10 μl of DNA. Following incubation at 23 °C for 15 min, 0.6 ml PEG solution was added, and incubation was continued at 23 °C for 15 min. Cells (0.3 ml) were plated directly in 30 ml regeneration agar. Fewer spheroplast washing steps made this modified protocol more efficient, however the transformation frequencies obtained were equivalent using either version of the procedure (average 25 transformants per ligation mixture).

I. Mitotic Stability Determination

Mitotic stabilities were reported as the fraction of cells containing a plasmid after growth on nonselective medium (65, 140, 195). Following growth of transformants to log phase in selection medium (SC-leu), aliquots were diluted and plated on complete medium (YEPD) to render about 100 colonies per plate. Plates were incubated for 2 days at 30 °C, then colonies were replica plated to selective medium plates using a transfer block and sterile Whatman No. 1 filter paper. The percentage of colonies growing on selection versus complete medium was recorded as the mitotic stability for a given transformant.
RESULTS

A. Chromosome Walking from HMLΔ

1. Hybridization of Probe 1* with Yeast Genomic DNA

The strains of yeast S. cerevisiae that contain a linear chromosome III (Fig. 3a) or a circular chromosome III, also referred to as ring III, (Fig. 3b) differ in the HMLΔ and the HMRΔ distal regions as these regions are deleted with the HMR-HMLΔ fusion event in ring III strains (109). The initial probes used for chromosome walking were HindIII/Xhol and HindIII/XbaI fragments (probe 1*) isolated from the cloned region (142), and were distal to HMLΔ on chromosome IIII (Fig. 3c). Southern hybridization characteristics of probe 1* with restriction enzyme digested and electrophoretically fractionated DNA from linear chromosome III and circular III yeast strains are shown in Figure 4. Two EcoRI fragments hybridized with equal intensity to the HMLΔ distal probe (1*) in linear III strains AB20α, XP8-10B (Fig. 4a, lane 1), AB972 (Fig. 4a, lane 2), and K45 (Fig. 4a, lane 3 and Fig. 4b, lane 1). The lack of restriction enzyme site polymorphisms in these two regions is evidenced by the conserved 8.5 kb and 4.9 kb EcoRI fragments homologous to probe 1* in the three linear III yeast strain genomic DNAs. Similarly, there were two HindIII and two Sall fragments that were homologous with probe 1* in the linear III strain K45 (Fig. 4b, lane 1). The hybridization of a HMLΔ distal probe with two regions in the haploid yeast genome has been reported (109), but the map position of the alternate region is unknown. For each RE digest, one fragment in the linear III strain K45 that hybridized with probe 1* was missing in the circular III strains, i.e. the 4.9 kb EcoRI, 6.5 kb HindIII and >15 kb Sall fragment (Fig. 4b). Presumably each fragment that was missing in the ring III strains maps distal to HMLΔ and consequently these fragments were referred to as "IIIIL distal fragments". Alternatively, the fragments that were homologous to probe 1* and that were retained in the circular III strains, i.e. the 8.5 EcoRI, 5 kb HindIII, and 0.4 kb Sall fragments (Fig. 4b) must map to the alternate region in the haploid yeast genome and will be referred to as "IIIIL alternate fragments". One IIIIL alternate fragment, the 0.4 kb Sall fragment, had a slower mobility in the ring III strains K191 and K196, and is missing in K193, a ring III strain. The explanation for this heterogeneous hybridization pattern with different circular III strains was not obvious since
Figure 3. Organization of Chromosome III in the Yeast S. cerevisiae.

a. The relative map positions for the distinguishing markers on chromosome III are indicated. The most distal known genetic markers on the left and right ends of chromosome III, HMLα and MAL2 respectively, are ca. 350 kb apart, if physical and genetic map distances are equated at 2.7 kb/cM (107, 186). About 12 kb separate HMLα and the left telomere (underlined), but the MAL2 to right end distance remains to be determined. Electrophoretic karyotype analysis of yeast chromosomes (37) indicates that chromosome III is approximately 370 kb in length. Since the region between the IIIIL terminus and MAL2 region accounts for about 360 kb, the distance between MAL2 and the IIIIR telomere must be in the range of 10–20 kb. It is unknown if the IIIIL and IIIIR telomeres are identical, so they are distinguished by different symbols. The solid dot on the chromosome represents the centromere.

b. Yeast strains which contain this circular version of chromosome III result from recombination between the homologous regions in the mating type cassettes, HMLα and HMRα (109). The fusion cassette HML–HMRα is the product of this recombination event, and the regions which are distal to HMLα and HMRα, i.e. the IIIIL and IIIIR telomeres, are apparently deleted during the fusion event.

c. Initial probes used for chromosome walking. Probe 1* is the 2.2 kb HindIII/Xbal fragment or the 1.4 kb HindIII/Xhol fragment which maps distal to HMLα. Probe 1* was isolated from the 6.47 kb HindIII fragment in the pHMLα clone (7, 142). The 1.4 kb Xbal and 2.3 kb HindIII/Xbal fragments map within the HMLα cassette region and were used as probes in restriction mapping experiments. The W, X, Ya, Z1, and Z2 regions of the HMLα cassette, and the orientation of HMLα on IIIIL are indicated. Restriction sites: H, HindIII; X, Xbal; Xh, Xhol.
Figure 4. Southern Hybridization of Linear III and Ring III Yeast Strains with the HMLα Distal Probe 1*.

a. Yeast genomic DNA from the linear chromosome III strains AB20α XP8-10B, AB972, K45, and the circular III strain K191, was digested with EcoRI, fractionated on a 0.7% agarose gel, transferred to nitrocellulose, and probed with a nick-translated 2.2 kb HindIII/XbaI pHMLα fragment (probe 1*). The 4.9 kb EcoRI fragment that was absent in ring III strain K191, but present in the linear III strains, must map distal to HMLα on III. The 8.5 kb EcoRI fragment that hybridized with probe 1* maps to the III alternate region.

b. Identification of fragments that map distal to HMLα on III by using various circular III yeast strains. DNA from linear III strain K45, (lane 1) and circular III strains K191, (lane 2), K192, (lane 3), K193, (lane 4), K195, (lane 5), and K196, (lane 6) were digested with EcoRI, HindIII, or Sall as indicated. After fractionation on a 0.65% agarose gel, the DNA was transferred bidirectionally to gene screen plus filters. One filter was hybridized with an M13 probe that contained the 2.6 kb EcoRI/Sall fragment which maps to the probe 1* region, (MTeLB2-2.6, or probe E in Fig. 8). The HMLα distal fragments were those absent in circular III strains but present in linear III strain K45. Size markers are λ/HindIII/EcoRI fragment positions.
restriction fragment length polymorphisms were not detected with other restriction enzymes. Later
hybridization analyses suggested that a recombination event occurred in the HMLɛ distal region during
formation of the ring III strains, likely in the vicinity of the 0.4 kb Sall fragment (see discussion).

2. Hybridization Screening of the Lambda Charon4A-Yeast DNA Library

Genomic DNA libraries are constructed with lambda or cosmid vectors for chromosome
walking studies, since the average length of the insert fragments is greater than that in libraries made
with plasmid vectors. A lambda Charon4A (hereafter referred to as λCh4A) rather than a cosmid
vector–yeast genomic DNA library was prepared for isolating the IIII telomere by chromosome
walking for two reasons: (1) The probability of cloning noncontiguous chromosomal DNA in a given
clone is higher for cosmid vectors since the average length of the insert fragments (30-45 kb) is
double that for lambda vectors (15-20 kb). Consequently, the possibility of "chromosome hopping"
rather than "chromosome walking" is enhanced with a cosmid vector library. (2) Restriction
mapping the yeast DNA inserts would be simpler for the lambda vector versus the cosmid vector clones
due to the shorter insert lengths.

The genomic DNA library of yeast strain AB20α XP8-10B was constructed by ligating 16 -
20 kb EcoRI partial digest fragments with the EcoRI arm fragments of the λCh4A vector (28) and
packaging the resulting DNA concatamers with lambda phage extracts in vitro (68, 90). A titer of
10^7 pfu/ml was obtained when the yeast library was plated on LE392 cells. An aliquot from the
library was grown in culture and the phage DNA was digested with EcoRI and fractionated by agarose
gel electrophoresis. Prominent λCh4A stuffer fragments, (6.6 kb and 7.8 kb EcoRI fragments),
indicated that nonrecombinant λCh4A phage DNA was present in the yeast genomic DNA library. The
proportion of λCh4A nonrecombinant phage to λCh4A phage with yeast inserts could not be estimated
since the nonrecombinant λCh4A phage may have a growth advantage over recombinant phage in liquid
culture. The number of recombinant phage having an average insert length of 17 kb that should be
screened for a 99% probability of recovering a unique yeast genomic sequence was calculated as 4 x
10^3 phage (52). Plaque hybridization of 5 x 10^3 phage using the initial nick-translated probe (1*)
identified three recombinant phage, named \( \lambda 4\text{ALB}1-4, \lambda 4\text{ALB}2-4, \) and \( \lambda 4\text{ALB}3-4 \) (Fig. 5a). The recombinant phage named \( \lambda 4\text{ALB}4-4 \) was isolated from the yeast genomic library in a subsequent hybridization screening experiment using probe 1*. These phage clones were purified through a series of four plaque hybridizations since the background \( \lambda \text{Ch}4\text{A} \) nonrecombinant phage present in the library outgrew the selected recombinant phage in liquid culture. The DNA isolated from the phage clones was analyzed by restriction enzyme digestion and agarose gel electrophoresis. The insert fragments in the phage clones differed only in the presence or absence of the 0.5 kb EcoRI fragments that flank the 16.1 kb region containing the 4.6 kb, 4.9 kb, 5.7 kb, and 0.9 kb EcoRI fragments (Fig. 5a). Phage clones \( \lambda 4\text{ALB}1-4 \) and \( \lambda 4\text{ALB}3-4 \) contained the 16.1 kb region while \( \lambda 4\text{ALB}2-4 \) and \( \lambda 4\text{ALB}4-4 \) contained the 16.1 kb region flanked by the two 0.5 kb EcoRI fragments.

The restriction map of the HML\( \text{ex} \) distal region in the phage clones (Fig. 5b) was derived by restriction enzyme analysis using single and double enzyme digestions and Southern hybridization using the probe 1* or the adjacent 2.3 kb XbaI/HindIII fragment from the pHML clone (Fig. 3c). The fragments that were mapped distal to the HML\( \text{ex} \) region in the Southern hybridization experiment with yeast genomic DNA and probe 1* (Fig. 4b) were present in the phage clones, i.e. the 4.9 kb EcoRI, 6.5 kb HindIII, and >15 kb SauI fragment. None of the clones isolated from the \( \lambda \text{Ch}4\text{A} \)/yeast library had insert fragments that mapped to the IIII alternate region. The lack or absence of the IIII alternate region in the \( \lambda \text{Ch}4\text{A} \)/yeast library may be explained by an unfavourable arrangement of EcoRI sites in that region for cloning 16–20 kb partial digest fragments (125). The EcoRI fragments in \( \lambda 4\text{ALB}4-4 \) were shotgun cloned into pBR325 which had been EcoRI digested and dephosphorylated with Bacterial Alkaline Phosphatase (BAP). (29, 30). The subclones with the yeast EcoRI fragments that mapped to the region distal to HML\( \text{ex} \), were referred to as pTel-1 (4.6 kb insert) and pTel-2 (4.9 kb insert). The subclones pTel-3 (5.7 kb insert), pTel-4 (0.9 kb insert), and pTel-5 (0.5 kb insert) mapped to the centromere proximal side of HML\( \text{ex} \) (Fig. 5c). The 0.5 kb EcoRI fragment that mapped distal to pTel-1 in the phage clone \( \lambda 4\text{ALB}4-4 \) was not cloned, due to the problem of distinguishing it from the upstream 0.5 kb fragment. The restriction map established for the region distal to HML\( \text{ex} \) in the phage
**Figure 5. Chromosome Walking Steps From HMLα on III.I.**

a. Genomic clones isolated from the λCh4A/ yeast library with a nick-translated pHMLα probe 1* (2.2 kb HindIII/XbaI). The extent of the yeast DNA inserts in the phage are indicated, and the region homologous with probe 1* is depicted by the bar above the insert DNA. Phage λ4ALB1-4 and λ4ALB3-4 were identical (16.1 kb partial EcoRI digest fragment), while phage λ4ALB2-4 and λ4ALB4-4 had identical 17.1 kb inserts of EcoRI fragments.

b. Restriction map of the region isolated on chromosome III.I. Restriction sites were mapped using multiple restriction enzyme digestions and Southern hybridization analysis. Restriction sites: R, EcoRI; H, HindIII; B, BamHI; S, Sall; Xh, Xhol; and X, XbaI. The orientation of the map, with respect to the centromere on chromosome III, is indicated as distal at the telomeric end or proximal at the centromeric end.

c. EcoRI fragments from phage λ4ALB2-4 were subcloned into the EcoRI site of pBR325. The position of the insert fragments for the subclones pTel-1, pTel-2, pTel-3, and pTel-4 on the restriction map of the III.I region is indicated. Further restriction sites were mapped in the pTel-1 and pTel-2 subclones that were distal to HMLα: T, BstEII; P2, Pvull; N, Ncol; K, KpnI; and P, PstI. The distal 1 kb EcoRI/Sall fragment or the 0.5 kb and 1.2 kb EcoRI/Pvull fragments in pTel-1 were referred to as probe 2* and used for further chromosome walking experiments.

d. Genomic phage clones λ4ALB12-5 and λ4ALB102-3 were isolated from the λCh4A/yeast library using probe 2*. The insert fragments in both recombinant phage extended no further toward the telomere on III.I than probe 2*. Phage λ4ALB102-3 contained an additional centromere proximal 3.8 kb EcoRI fragment.

e. The phage clone, λ4ARLB301, was isolated by in vivo recombination selection from the λCh4A/yeast genomic library, using the recombinant microplasmid ΠpT-T. Other phage clones isolated by this screening method, λ4ARLB302-λ4ARLB312 were identical to λ4ARLB301. The 1.9 kb EcoRI fragment that mapped distal to probe 2* was present in all recombination selection clones, and it resulted from the integration of the ΠpT-T microplasmid into the phage and the duplication of the target 1 kb EcoRI/Sall fragment (probe 2*) upon integration.
clones was confirmed through Southern hybridization of restriction enzyme digested DNA from the \( \lambda \)4ALB phage clones using the pTel subclones as probes. The most distal probe, pTel-1, extended 9.6 kb distal to HML\( ^e \) on III\( ^L \). The second set of chromosome walking probes, referred to as probe 2*, were isolated from the distal end of pTel-1. The probe 2* fragments extended 7.3 kb from probe 1* toward the left end of chromosome III and included the 1 kb EcoRI/SalI, 3.8 kb EcoRI/BamHI, and 0.5 kb EcoRI/PvuII fragments (Fig. 5c). The \( \lambda \)Ch4A–yeast DNA library was screened by plaque hybridization with probe 2* using at least ten times the required number of recombinant phage to encompass the yeast genome (5 x 10\(^4\) pfu). The phage clones \( \lambda \)4ALB12-5 and \( \lambda \)4A102-3 were isolated using probe 2* (Fig. 5d), however probe 2* was the most distal yeast fragment in both of these clones.

3. Recombination Screening of the Lambda Charon4A–Yeast DNA Library

To determine whether phage clones that contained inserts from the III\( ^L \) region that was distal to probe 2* were present but at very low frequency in the yeast library, the \( \lambda \)Ch4A–yeast DNA library was screened with probe 2* using In vivo recombination selection (126, 174). The sensitivity of this screening method allows the selection of a single recombinant phage from a population of 10\(^6\) pfu. A probe 2* fragment (1 kb EcoRI/SalI) was cloned into the EcoRI/SalI sites in the polylinker region of the \( \Pi \)AN7 microplasmid which contains the amber suppressor gene supF, to produce the recombinant \( \Pi \)pT-T. The \( \lambda \)Ch4A–yeast DNA library was amplified and both the unamplified (10\(^5\) pfu) and amplified (10\(^6\) pfu) phage populations were passed through the \( E. \) coli strain MC1061(p3) (Su- cells) that had been transformed with \( \Pi \)pT-T. The resultant phage titers were determined on the \( E. \) coli strain LE392 (Su+ cells), then 10\(^6\) - 10\(^7\) phage were plated on MC1061(p3) to select for phage containing the supF gene by a prior recombination event with \( \Pi \)pT-T. The expected recombination frequency for the probe 2* and the phage library is 10\(^{-6}\) (126, 174) but the recombination frequency of the unamplified library was 4 x 10\(^{-5}\) and the amplified library was 4 x 10\(^{-3}\). The elevated recombination frequencies obtained for the \( \lambda \)Ch4A library presumably reflect the reversion of the amber mutations in the \( \lambda \)Ch4A phage since amber
revertants can also grow on MC1061(p3). The proportion of amber revertants in the λCh4A-yeast DNA library was determined by plating the amplified and the unamplified λCh4A/yeast library (10^4 pfu) on the MC1061(p3) Su− strain without prior passage through the microplasmid bearing strains. There was no phage growth for the unamplified phage library, but a 10−2 reversion frequency was obtained for the amplified library. Apparently the amber revertants had a growth advantage during the amplification of the λCh4A-yeast DNA library. To distinguish between revertant Su+ phage and true recombinant Su+ phage, probes from the region that was distal to HIMG on IIII were used for secondary screening of the Su+ phage by plaque hybridization. The SupF recombinant phage isolated from the unamplified λCh4A-yeast DNA library were selected by growth on the Su− host MC1061(p3), and screened by plaque hybridization with either probe 2* or the 1.2 kb EcoRI/SalI fragment that mapped upstream from probe 2* in pTel-1 (Fig. 5c). About 75% of the Su+ phage hybridized with both probes. A set of the positive phage clones named λ4ARLB and numbered 301 to 312 were purified and characterized by restriction enzyme digestion of the isolated DNA. All of the recombinant Su+ clones had identical restriction maps to the phage clone λ4ALB1-4 except for an additional 1.9 kb EcoRI fragment that mapped adjacent to pTEL-1, (Fig. 5e). However, this extra fragment resulted from the recombination event between the IIII distal 1 kb EcoRI/SalI fragment and the homologous probe 2* fragment in IIpT-T which results in the duplication of the target site upon integration of the IIpT-T microplasmid.

4. Hybridization Screening with other Yeast Genomic DNA Libraries

The probe 2* distal region was apparently not represented in the yeast genomic DNA library that was constructed with yeast EcoRI fragments and the EcoRI arm fragments of the phage λCh4A. Presumably a genomic DNA library prepared using a restriction enzyme that has a four base pair recognition site would have a more random distribution of insert fragments and may contain clones with further distal fragments from the IIII telomeric region than the λCh4A library that was constructed using EcoRI which has a six base pair recognition site. A yeast genomic DNA library had been prepared by partially digesting the DNA of the strain AB972 (169) with Sau3A to obtain
fragments that were 15 kb average length. The phage vector that was used for cloning the Sau3A fragments was λMG14 (M. Olson, personal communication) which consisted of the left arm of λ1059 (100) and the right arm of λCh30 (164). Southern hybridization of restriction enzyme digested DNA from yeast strain AB972 with a HMLα distal fragment (probe 1*) did not reveal any restriction fragment length polymorphisms between AB20α XP8-10B (λCh4A-yeast DNA library) and AB972 (λMG14-yeast DNA library), (Fig. 4a). The λMG14-yeast DNA library initially had 100% recombinants but had been amplified prior to plaque hybridization with the HILL distal fragments. Hybridization screening of about 1-2 x 10^4 pfu in total, (10^3 pfu/85 mm plate), resulted in the identification of multiple recombinant phage (designated λMGBO1 to λMGBO14) that were homologous with probe 1*. Mapping studies indicated that the inserts in the λMGBO phage clones had different restriction fragments than those contained in the recombinant phage isolated from the λCh4A-yeast DNA library with probe 1*. The restriction maps that were derived for the λMGBO1 to λMGBO14 clones (Fig. 6) show that all phage have insert regions about 17 kb long, which differ only in the presence or absence of the distal HindIII or EcoRI sites. The 2.2 kb HindIII/XbaI/pTel-2 fragment, (probe 1*), hybridized with the 5 kb HindIII fragment and the 1.0 kb EcoRI/SalI pTel-1 fragment (probe 2*) did not hybridize with any of the λMGBO clones. Southern hybridization analysis of yeast DNA from linear and circular III strains (Fig. 4b) revealed that a 5 kb HindIII fragment was homologous with probe 1* but it mapped to the IIII alternate region. Presumably the λMGBO clones that were isolated contain fragments from the IIII alternate region, not the IIII distal region. Plaque hybridization of the λMG14-yeast DNA library with probe 2* did not detect homologous recombinant phage, even when 50 genomic equivalents of phage (5 x 10^4 pfu) were screened. Possibly the IIII distal region was poorly represented in the λMG14-yeast library and consequently it was eliminated during the amplification of the library.
Figure 6. Restriction Endonuclease Maps of Recombinant Phage λMGB01-14.

Restriction maps the yeast DNA inserts in the recombinant phage isolated from the λMG14/yeast library, using nick-translated probe 1* fragments (pHMLα, 2.2 kb HindIII/XbaI or 1.4 kb HindIII/XhoI illustrated in Fig. 3c). All clones contained similar partial Sau3A fragment inserts (about 17 kb), with HindIII, (H) and EcoRI, (R) sites indicated. The four identified clone sets differed only in the presence or absence of flanking HindIII and EcoRI sites. Probe 1* hybridized to a 5 kb HindIII fragment, and a >5 kb EcoRI fragment in all clones.
As an alternate approach, a yeast genomic library that was prepared with 15 kb Sau3A fragments of yeast DNA and the yeast plasmid vector Yep13 (142), was screened with pTel-1 fragments (probe 2*) by high-density screening of at least 2 x 10^4 clones (84, 85) and by grid plate screening using sufficient clones to include the entire yeast genome (5000 colonies). As a positive control for the hybridization, the HMLα 1.4 kb XbaI/HindIII fragment (Fig. 3c) hybridized with clones on duplicate filters, with the expected frequency. Probe 2* did not hybridize to any colonies, indicating the IIII distal region was not represented in the Yep13-yeast DNA library which has been used successfully to isolate many yeast genomic segments including the yeast mating type genes (142).

5. Southern Hybridization of Yeast Genomic DNA with Probe 2*

The absence of probe 2* distal fragments in yeast genomic DNA libraries suggested that probe 2* was in the proximity of the IIII telomere and the structure of the telomere may have precluded the cloning of the probe 2* distal region in the libraries. To establish whether probe 2* which mapped 8.6 kb distal to HMLα was near the chromosome IIII end, its hybridization characteristics with yeast genomic DNA were studied. Probe 2* hybridized exclusively to the IIII distal region, as evidenced by the single fragment homologous with probe 2* for most restriction enzyme digestions (Fig. 7a). Two fragments (3.0 kb and 1.2 kb) hybridized with probe 2* in Pvull digestion (Fig. 7a, lane 4), due to the Pvull restriction site within the probe 2* (1 kb EcoRI/Sall) fragment (Fig. 7b). The restriction fragments that were homologous with probe 2* in the yeast genomic DNA digestions (Fig. 7a) agreed with the fragment lengths that were mapped on the IIII clones isolated from the λCh4A-yeast genomic DNA library (Fig. 5b). It was possible to predict the distance between probe 2* and the distal restriction enzyme sites on IIII since the sites on the proximal side of probe 2* had been mapped (Fig. 5b). For example, the 10 - 12 kb PstI fragment (Fig. 7a, lane 3) must extend 1.7 to 3.7 kb on the telomeric side of probe 2*, since the upstream PstI site mapped 8.3 kb centromere proximal from the EcoRI site on the distal end of probe 2* (Fig. 7b). In the same manner, the distal Pvull site was estimated to be 2.5 kb from probe 2* (Fig. 7b), since the 3.0 kb Pvull fragment (Fig. 7a, lane 4) encompasses the distal 0.5 kb of probe 2*. None of the distal restriction enzyme sites that were estimated in this manner extended further than 3.5 kb from probe 2* toward the IIII telomere.
Figure 7. Southern Hybridization with Probe 2* and Yeast Genomic DNA.

a. Aliquots of DNA from yeast AB20α XP8-10B (3.3 μg) were digested with restriction enzymes, fractionated on a 0.65% agarose gel, and transferred to gene screen plus filter. Probe 2* was a 1 kb EcoRI/SalI fragment isolated from pTel-1 and cloned in M13mp8. This probe 2* clone was called MTeLB1-1, and it mapped 8.6 kb distal to HMLα. Restriction enzyme digestions: lane 1, EcoRI; 2, HindIII; 3, PstI; 4, PvuII; 5, BstEII; 6, KpnI; 7, BamHI; 8, XbaI; 9, SalI; and 10, XhoI. Size markers are λ/HindIII/EcoRI fragment positions.

b. Estimated positions for the restriction enzyme sites in the region that is distal to probe 2*. The RE map in the region between HMLα and probe 2* was determined from the genomic phage clones and the plasmid subclones (Fig. 5b,c). The length of the fragments homologous with probe 2* were determined from Southern hybridization (Fig. 7a). For each restriction enzyme, this fragment length was the distance between the centromere proximal site and the centromere distal site on either side of probe 2*. Fragments 1–10 in the restriction maps for the probe 2* region correspond to lanes 1–10 in the Southern blot.
In the Southern hybridization with probe 2*, heterodisperse bands were observed in the PvuII (Fig. 7a, lane 4) and Sall (Fig. 7a, lane 9) digestions. Studies have shown that telomeric fragments in yeast are heterogeneous in length due to variable amounts of simple 5'-C1-3A-3' repeat units at chromosome ends (49, 175, 206). This suggested that the Sall and PvuII fragments homologous with probe 2* contained the variable repeat lengths of the IIIIL telomeric region and hence probe 2* was proximal to telomeric DNA. However, probe 2* hybridized to a unique region of the yeast genome and therefore it was apparently not included in the telomeric repeat region that contains the complex X or Y' ARS regions and the simple T region repeats (48, 49).

B. The IIIIL Distal Region is Retained in Some Circular III Strains

The region between probes 1* and 2* that was isolated from the yeast genomic library was hybridized to the genomic DNA from yeast strains that were similar except for the presence of a linear chromosome III or circular chromosome III in the haploid genome. The rationale was that the differences in the Southern hybridization patterns for the circular III and linear III strains would show that the IIIIL distal region rather than the IIIIL alternate region had been isolated in the phage clones since it was expected that the entire HMLε distal region was deleted in ring III strains (109). However, results from the Southern hybridization analysis indicated that the region distal to probe 1* is retained in most of the circular III strains examined. A single fragment was homologous to the distal 1 kb EcoRI/Sall fragment (probe A) in the linear III strain K45 and all circular III strains except K192 (Fig. 8A, lane 3). The lengths of the EcoRI, HindIII, and Sall fragments were consistent for K45, a linear III strain, (Fig. 8A, lane 1) and for those fragments retained in the K191, K193, K195, and K196 ring III strains (Fig. 8A, lanes 2, 4, 5, 6). The 2.4 kb Sall fragment (probe B) hybridized with two regions in the linear III strain K45, and to a single fragment in all of the circular III strains (Fig. 8B). For most circular III strains, the hybridization pattern for probe B was contrary to the expected result. The IIIIL distal fragments that were homologous with probe B were retained in the circular III strains K191, K193, K195, and K196, while the IIIIL alternate fragments that were homologous with probe B were absent (Fig. 8B, lanes 2, 4, 5, 6). Only circular III strain K192 had the expected hybridization pattern since the IIIIL distal fragments were absent and the IIIIL alternate...
Figure 8. Southern hybridization of Linear III and Ring III Yeast Strains with Probes from the IIIL Distal Region.

Fragments that mapped to the region distal to HMLα, between probe 1* and probe 2*, were cloned as Sall or Sall/EcoRI inserts in M13mp8. Probes A to E refer to the following M13 clones: A, MTeLB1-1.0; B, MTeLB1-2.4; C, MTeLB1-1.2; D, MTeLB2-2.3; and E, MTeLB2-2.6; and the number following the hyphen in each name indicates the length of the insert fragment. Genomic DNA from the linear III yeast strain K45 and the circular III yeast strains K191, K192, K193, K195, and K196 was digested with EcoRI, HindIII, or Sall, (3.3 μg DNA/digest), fractionated on a 0.65% agarose gel, and transferred bidirectionally to gene screen plus filters. Southern hybridization with probes A, B, C, and D resulted in the blots labelled A to D respectively. The Southern blot with probe E was included with the probe 1* hybridization results (Fig. 4b). Lanes: 1, K45; 2, K191; 3, K192; 4, K193; 5, K195; and 6, K196; and EcoRI, HindIII, or Sall enzyme digestions are indicated. The restriction fragments which map to the IIIL distal region are indicated by the symbols on the right side of the K45 lane (lane 1) for EcoRI (■), HindIII (○), and Sall (▲) digestions. Size standards were λ/HindIII/EcoRI fragment positions which are indicated by the horizontal bars at the left, and the positions of λ/HindIII fragments at the right side of the southern blots for A-D.
A

B

C

D

EcoRI  HindIII  Sall

 kb

 237  50  67  43  20
region fragments were retained and hybridized with probe B for each of the EcoRI, HindIII, and Sall digestions (Fig. 8B, lane 3). These hybridization characteristics for the various ring III strains were also observed with the adjacent 1.2 kb Sall/EcoRI (probe C) fragment. The IIIIL distal region was deleted in circular III strain K192, but retained in all of the other circular III strains, K191, K193, K195, and K196 (Fig. 8C). The IIIIL alternate region was retained in K192, and was deleted in K191, K193, K195, and K196 (Fig. 8C). Since both the IIIIL distal and the IIIIL alternate region had a 3.6 kb Sall fragment homologous with probe C, no difference in hybridization was detected between K192 and the other ring III strains for Sall digested DNA (Fig. 8C-Sall, lanes 2-6). The pTel-2, 2.3 kb EcoRI/Sall fragment (probe D) had a mixed hybridization pattern for the IIIIL distal and the IIIIL alternate region fragments that were retained in the circular III strains (Fig. 8D). With EcoRI digestion, the IIIIL alternate region 8.5 kb fragment was retained, and the IIIIL distal 4.9 kb fragment was deleted in all circular III strains (Fig. 8D-EcoRI, lanes 2-6). With HindIII digestion, K192 was the only circular III strain that had deleted the IIIIL distal 7 kb HindIII fragment and retained the IIIIL alternate 6 kb HindIII fragment (Fig. 8D-HindIII, lane 3). The other ring III strains had the reverse HindIII fragment pattern which was similar to that observed with probes A, B, and C. The IIIIL distal fragment (7 kb HindIII) was retained in the circular III strains K191, K193, K195, and K196, while the IIIIL alternate fragment (6 kb HindIII) was deleted (Fig. 8D-HindIII, lanes 2, 4, 5, 6). The pTel-2, 2.6 kb Sall/EcoRI (probe E) hybridized exclusively to IIIIL alternate region fragments in all circular III strains examined (Fig. 4b). Possibly, that portion of the IIIIL distal region was deleted during the HMLe - HMRe fusion event (109) that produced the circular III strains. These Southern hybridization results are summarized in Table I. The presence of the IIIIL distal fragments in a given yeast strain is indicated by closed symbols and the IIIIL alternate region fragments are indicated by open symbols. Since K45 has a linear chromosome III, both the IIIIL distal and alternate region fragments were present for probes B to E. Probe A was unique in the yeast genome and hybridized to only the IIIIL distal region. Strains with a circular chromosome III (K191, K192, K193, K195, K196) have deleted either the IIIIL distal or IIIIL alternate fragments that hybridized with probes B to E. Circular III strain K192 was unique in having the entire IIIIL distal region deleted while the
Table I. The IIII Distal Region is Retained in most Circular III Strains.

The Southern hybridization data from Figures 4 and 8 are presented in this table to demonstrate the pattern of hybridization for the probes from the IIII distal region. Fragments homologous with probes A to E are represented by the symbols, where the closed symbols are fragments which mapped to the IIII distal region, and the open symbols are fragments that mapped to the IIII alternate region. Both IIII distal and IIII alternate regions are present in the linear III strain K4S for all the probes except for A which maps exclusively to the IIII distal region. The presence of the fragments from the IIII distal or IIII alternate regions in the circular III strains (K191, K192, K193, K195, and K196) is indicated by closed or open symbols. A hybrid pattern of open and closed symbols is evident in most circular III strains and this may reflect the position of a recombination or conversion event. The fragments from the IIII distal region that were retained in the genomes of the ring III strains K191, K193, K195, and K196 and the complete deletion of the IIII distal region in the strain K192, is sketched on the map below the table.
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**Diagram:**

- **III End**
  - **K191**
  - **K193**
  - **K195**
  - **K196**

- **HML**
  - **CEN3**

- **K192**
- **DELETE**
- **RETAIN**
- **DELETE**
IIIL alternate region was conserved. Circular III strains K191, K193, K195, and K196, had a break point in the hybridization pattern for the IIIL distal versus IIIL alternate fragments in the probe D region. These circular III strains retained IIIL distal fragments in the probe A to D region, presumably at the IIIL alternate region since the IIIL alternate fragments were absent for the probe A to D region. Possibly gene conversion events occurred concomitantly with the HMLα - HMRα fusion events, replacing a portion of the IIIL alternate region with the IIIL distal region (Table I).

C. Transcription in the IIIL Distal Region

To investigate the reason for the strong homology between the IIIL distal and IIIL alternate regions and for the retention of the IIIL distal region in most circular III strains, I asked whether there is a transcription unit in the IIIL distal region that is repeated at the IIIL alternate region in the genome of haploid yeast strains. An essential, but functionally duplicated gene in the HMLα distal region could answer these problems and also explain why circular III strains that contain only one of these loci are viable as haploids (109). Preliminary dot blot analysis of total yeast RNA isolated from linear III or circular chromosome III strains hybridized with the HMLα distal probe pTel-1, which includes the probes A, B, and C used in the previous Southern analysis (Fig. 8), indicated that there are RNA transcripts homologous with this region (Fig. 9a). Northern analysis of the total RNA from linear III strain K45 and circular III strain K191 (Fig. 9b) indicated that the pTel-1 probe hybridized to a single transcript sized at approximately 1.4 kb. Since pTel-1 maps 5 kb distal to HMLα and it is homologous to a 1.4 kb transcript, a transcription unit in the IIIL distal region must map at least 4-5 kb distal to HMLα and this corresponds to the IIIL distal region that is retained at the IIIL alternate region in four of the five circular III strains studied. Considering that such a transcript is separated from HMLα by more than 4 kb, it cannot be a run-off transcript from HMLα. In the mar1 strains K45 and K191 and it maps to the middle of the fragment that was retained in the circular III strains. Since a RNA transcript is homologous with the pTel-1 probe in the circular III strain K192 which lacks the IIIL distal region (Fig. 8, Table I), there must also be a transcription unit at the IIIL
Figure 9. Hybridization Analysis of Yeast RNA with a IIII Distal Probe.

a. RNA Dot Blot Analysis. Total yeast RNA was spotted on a Gene screen plus filter using a BRL Hybridot Manifold. Yeast strains AB20α, XP80-10B, K45, K191, K192, and GM3C2 (ΔCYC) had 1, 3, 5, 10, and 20 µg RNA as indicated, but strain BM-CYC+ had only the 1, 3, 5, and 10 µg RNA samples. Probe pTel-1, the distal 4.6 kb EcoRI fragment on IIII which includes probe 2* in the vector pBR325, was nick-translated to a specific activity of $3.5 \times 10^7$ cpm/µg (Cerenkov counts). A pBR322 probe that was used as a hybridization control did not show homology with any of the RNA samples except for strain BM-CYC+.

b. RNA Northern Analysis. Total yeast RNA from the yeast strains K45 and K191 was fractionated on a 1% agarose – formaldehyde gel, transferred to a gene screen plus filter, and was hybridized with pTel-1, that was nick-translated to a specific activity of $4 \times 10^7$ cpm/µg (Cerenkov counts).

Lanes: 1, K45, (7.5 µg); 2, K45, (15 µg); 3, K191, (7.5 µg); 4, K191, (15 µg). Size standards were the positions for denatured λ/HindIII/EcoRI fragments.
alternate region. If the IIII distal region and the IIII alternate region have equivalent transcription units, then the IIII distal region was retained in some of the circular Illl strains as a consequence of the recombination event with a broken chromosome end rather than as a functional selection.

D. Probe 2* is Adjacent to the IIII Telomere

1. Bal31 Nuclease Sensitivity of the Probe 2* Region

Chromosome ends in yeast (175, 206) and trypanosomes (18, 24, 60, 203, 209) are sensitive to digestion with the double-stranded exonuclease Bal31 (80). The rationale behind the following set of experiments was that if probe 2* from the IIII distal region mapped to within a few kilobase pairs of the IIII terminus, some restriction fragments would contain both the IIII end and probe 2*. Such fragments would be sensitive to Bal31 nuclease digestion and would hybridize with probe 2* in Southern hybridization analysis. Determination of the Bal31 nuclease sensitivity of yeast genomic DNA and the length of time required to shorten the restriction fragments that are homologous with probe 2* would reflect the distance between the IIII terminus and probe 2*.

Yeast DNA was digested with Bal31 nuclease for increasing periods of time (Fig. 10). Aliquots of DNA digested with Bal31 nuclease were subsequently digested with the restriction enzymes Sail or PvuII, and analyzed by Southern hybridization with probe 2*. Since variable lengths of chromosome ends had been demonstrated in yeast genomic DNA (40, 49, 93, 175, 206) and since probe 2* hybridized to heterodisperse Sail and PvuII fragments (Fig. 7b), I asked whether the IIII terminus was included in these fragments. The 3.3 kb Sail and the 3.0 kb PvuII fragments were readily digested with Bal31 nuclease and the region homologous with probe 2* was completely deleted after 15 min of Bal31 nuclease treatment. With the digestion conditions used for Bal31 nuclease, about 150 bp/min were deleted from the IIII end, hence probe 2* was a minimum of 2.3 kb from the IIII end. The sensitivity of the 3.0 kb PvuII fragment to Bal31 nuclease digestion and the resistance of the upstream 1.2 kb PvuII fragment that was homologous with probe 2*, provided an internal control for the specificity of Bal31 nuclease digestion at the termini of chromosomes. The length heterogeneity and the Bal31 sensitivity of the 3.3 kb Sail and 3.0 kb PvuII fragments suggested that these fragments contain the IIII chromosome end, and that the terminus maps 2.3 kb distal to probe 2*. 
Figure 10. Length Heterogeneity and Bal31 Nuclease Sensitivity of the IIII Distal Region.

Yeast genomic DNA (AB20α XP8-108) was treated with exonuclease Bal31 (0.1 u/μg) for up to 30 min. DNA was digested with Sall or PvuII for each time point aliquot, fractionated on a 0.65% agarose gel (3.3 μg/lane), and transferred to gene screen plus filters. Hybridization was with probe 2* (MTeLB1-1.0). Size markers are λ/HindIII/EcoRI fragment positions.
2. The IIIIL Alternate Region Is Telomere Proximal

Since the IIIIL distal region consisted of 12 kb between HMLΔE and the IIIIL telomere, I asked whether the homologous IIIIL alternate region was also near a chromosome end. Bal31 nuclease digestion with yeast genomic DNA from the linear III strain K45 and the circular III strains K191 or K192 provided an answer to this question. The 1.2 kb SalI/EcoRI fragment from the pTel-1 clone (Fig. 5) was used as probe for Southern hybridization analysis since it hybridized to both the IIIIL distal and the IIIIL alternate region (Fig. 8C). The 7 kb HindIII fragment from the IIIIL distal region and the 6 kb HindIII fragment from the IIIIL alternate region were sensitive to Bal31 nuclease digestion (Fig. 11), suggesting that both IIIIL distal and IIIIL alternate regions are telomeric. The hybridization signal was lost after 10 min of Bal31 nuclease digestion for the IIIIL alternate region, while the IIIIL distal region remained for at least 20 min of digestion with Bal31 nuclease. Assuming the probe mapped 5.7 kb from the end of the IIIIL distal region and the rate of Bal31 digestion was equivalent for all chromosome ends in the genome of K45, the IIIIL alternate region had the homologous region only 2.8 kb from a chromosome end. For circular III strain K191 which had retained a large portion of the IIIIL distal region, the kinetics of digestion of the 7 kb HindIII fragment with Bal31 nuclease was equivalent for K45 and K191 indicating the distance from the probe to the chromosome end was equivalent for each (5.7 kb). If the IIIIL distal region replaced the IIIIL alternate region in strain K191 by a recombination or conversion event, then either the IIIIL distal telomere replaced the IIIIL alternate telomere or the distance between the probe and the telomere at the IIIIL alternate region was increased. Alternatively, strain K192 which did not retain the fragments from the IIIIL distal region and which contained the 6 kb HindIII fragment from the IIIIL alternate region, had the same kinetics of Bal31 nuclease digestion as the IIIIL alternate region in K45.

3. The IIIIL Telomere Associated Region Maps Distal to Probe 2*

The length of telomeres on yeast chromosomes is genetically controlled (40), hence the length of the terminal DNA restriction fragment on a chromosome is strain specific (93, 206). A 3.3 kb (+/- 0.2 kb) SalI fragment hybridized with probe 2* in the yeast strain AB200X XP8-10B (Fig. 12c, lane 2), but a 3.0 kb (+/- 0.2 kb) SalI fragment hybridized with probe 2* in strain K45.
Figure 11. The IIL Alternate Region Is Bal31 Nuclease Sensitive.

DNA from the linear III yeast strain K45, and the circular III strains K191 or K192 was digested with Bal31 nuclease (0.32 u/μg) for up to 20 min. Following HindIII digestion, fractionation on a 0.6% agarose gel, and transfer to gene screen filters, hybridization was with MTeLB1-1.2 (probe C, Fig. 8C) since it shares homology with both the IIL distal and IIL alternate regions in the yeast genome. For each yeast strain the DNA samples were U, Bal31 nuclease untreated, followed by 0 min, 5 min, 10 min, 15 min, and 20 min Bal31 nuclease digestions as indicated. Lane M contains the λ/HindIII fragments that were used as size markers.
Figure 12. Probe 2* is not Homologous with Telomere Associated Regions in Yeast.

a. Restriction maps of the yeast telomere associated region in clones: pSZ220, (193); YRp131A or YRp131B, (48,49). The extent of the X region, Y region, and 131 region in each clone is indicated. Restriction sites: N, Ncol; P1, Pvul; S, Sall; Sc, Sacl.

b. DNA from yeast, phage λ clones, and plasmids was digested with restriction enzymes and fractionated on a 0.65% agarose gel which was stained with EtBr. The banding pattern for the DNAs was observed by UV fluorescence. Lanes 1-12: 1, λ/HindIII/EcoRI; 2, A820α XP8-10B/Sall, (4 μg); 3, K45/Sall, (4 μg); 4, K191/Sall, (4 μg); 5, K192/Sall, (4 μg); 6, pSZ220/Pvul/Sacl, (0.5 μg); 7, pTel-1/EcoR1/Sall, (0.25 μg); 8, λ4ALB4-3/ EcoRI, (0.25 μg); 9, λ4ALB102-3/EcoRI, (0.25 μg); 10, YRp131A/Sall/Ncol, (0.25 μg); 11, YRp131B/ Sall/Ncol, (0.25 μg); 12, λ/HindIII, (0.5 μg).

c. DNA was transferred to gene screen plus filter, and hybridized with the III+ distal probe 2* (MTelB1-1.0).
a.

pSZ220

Yrp131A

Yrp131B

b.

c.

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65a
(Fig. 12c lane 3). The length heterogeneity observed for different yeast strains supports the idea that the Sall fragment homologous with probe 2* includes the IIIL terminus. Probe 2* was unique to the IIIL distal region and did not hybridize with the ring III strain K192 (Fig. 12c, lane 5) which has deleted the entire IIIL distal region. However, the ring III strain K191 has a Sall fragment that is homologous with probe 2* and it has an equivalent length to that in the parental linear III strain K45 (Fig. 12c, lanes 3&4). If this 3.0 kb (+/- 0.2 kb) Sall fragment does contain the IIIL telomere in K191, then the conversion event that replaced the IIIL alternate region with the IIIL distal region (Table I) also converted the telomere at the IIIL alternate region to the telomere from the IIIL distal region.

Since probe 2* hybridized to yeast fragments which included the IIIL terminus, I asked whether probe 2* also contained sequences that are homologous to the telomere associated region which consists of X and Y' repeats (49). The clones pSZ220 (193), YRp131A, and YRp131B, (47, 48, 49), (Fig. 12a) isolated from the telomere associated regions in yeast were restriction enzyme digested, fractionated by agarose gel electrophoresis, and analyzed by Southern hybridization with probe 2* (Fig. 12b, 12c, lanes 6,10,11) alongside control clones that are homologous with probe 2* (Fig. 12b, 12c, lanes 7,8,9). However, probe 2* was not homologous with the type X ARS clone, YRp131A (Fig. 12c, lane 10) or the type Y ARS clones, YRp131B and pSZ220, (Fig. 12c, lanes 6&11). Furthermore, Southern hybridization analyses using type X or Y probes to screen the cloned IIIL distal region did not reveal any telomere associated sequences in the 9.6 kb HMLe distal region (data not shown). The telomere associated region on IIIL must be distal to probe 2* and must be within the terminal 2.3 kb on IIIL.

**E. Attempts to Clone the IIIL Telomere by Marker Rescue**

One strategy that I used in attempting to isolate the IIIL end involved the introduction of a yeast selectable marker in the proximity of the IIIL telomere. Cloning of the closely linked IIIL telomere would conceivably be possible on a linear or circular plasmid by recovery of the plasmid through selection for the marker on the vector, as outlined schematically (Fig. 13). This strategy was analogous to the marker insertion and eviction method that was developed to clone several yeast genes.
Figure 13. Strategy to Clone the IIIIL Telomere by Marker Insertion and Excision.

a. Integrative plasmid pLB21-17 contained the 3.8 kb EcoRI/BamHI fragment from pTel-1 in the Ylp5 vector (189). pLB21-17 was digested with Xhol, which cuts 0.75 kb from the BamHI end of the insert fragment, to target pLB21-17 for insertion in the IIIIL distal region.

b. Transformation of yeast strain SR25-1A (uracil auxotroph) with pLB21-17/Xhol using the lithium acetate, alkali cation method (94) produced mitotically stable transformant YeLB21-17-26. The expected map for integration at the IIIIL distal region is indicated.

c. Excision of \textit{URA3} from the IIIIL distal region and co-isolation of the IIIIL telomere. Transformant DNA is partially digested with HindIII or Xhol under conditions such that the IIIIL end, probe 2* region, and the AMP to \textit{URA3} region on the vector are contained on the same restriction fragment.

d. Recovery of the fragment as a linear plasmid could be accomplished by adding \textit{Tetrahymena} rDNA termini to the HindIII or Xhol end, as 0.7 kb HindIII/HhaI or Xhol/HhaI fragments from pSZ221 and pSZ222 respectively (195). The linear plasmids would then be introduced into yeast strain SR25-1A and uracil prototrophic transformants would be characterized for linear plasmids containing the IIIIL end.

e. Recovery of the AMP marker along with part of the IIIIL end is possible on a circular plasmid. YeLB21-17-26 DNA is partially digested with HindIII or Xhol, and treated with S1 nuclease to render blunt ends on the fragment, followed by intramolecular ligation and selection for AMP in \textit{E. coli}. Colony hybridization with a IIIIL distal probe would identify clones with a fragment from the IIIIL telomeric region. Since the yeast telomeric single-stranded breaks are sensitive to S1 nuclease digestion (175), some of the terminal region will be deleted in cloning the IIIIL telomere in a circular vector.

Restriction sites: B, BamHI; H, HindIII; P, PstI; R, EcoRI; S, Sall; X, Xhol.
Plasmid pLB21-17 (Fig. 13a) was constructed by cloning the 3.8 kb EcoRI/BamHI fragment from pTel-1 (includes probe 2* region) in Ylp5, which is a yeast vector that contains the URA3 marker and lacks a yeast origin of replication (189). Insertion of the URA3 marker in the IIIL distal region was achieved through the genetic transformation of yeast strain SR25-1A (which contains the non-reverting ura3-52 mutation) with pLB21-17 that was digested with Xhol to target the insertion (149, 150) and selection for uracil prototrophic transformants. Since Ylp5 cannot replicate autonomously in yeast, URA3 transformants presumably had pLB21-17 integrated by site directed, homologous recombination into yeast genomic DNA (149, 150, 196), (Fig. 13b). Transformants were obtained at a low frequency (5 transformants per 2.5 µg DNA), and these transformants had a mitotically stable URA3 marker which is indicative of vector integration. Transformant YeLB21-17-26 appeared to have the plasmid pLB21-17 integrated in the IIIL distal region, as determined by restriction enzyme mapping and Southern hybridization analysis (Fig. 14). Sizes of the fragments that were homologous with probe 2* in transformant YeLB21-17-26, (Fig. 16c) differed by the predicted lengths from fragments on the IIIL distal end in yeast strain AB20α XP8-10B (used for cloning and mapping the IIIL distal region) or host strain SR25-1A (Fig. 14a, 14b). An exception to the predicted fragment pattern was the extra 7 kb HindIII fragment in YeLB21-17-26 where only the >15 kb fragment was expected to hybridize with probe 2* (Fig. 14b). Also the low molecular weight fragments that hybridized with reduced intensity to probe 2* in the PstI, HindIII, and EcoRI digests of AB20α XP8-10B and SR25-1A were not previously detected. Two additional transformants isolated in the same experiment had the same Southern hybridization pattern as YeLB21-17-26.

To establish whether the extra HindIII fragment in transformant YeLB21-17-26 was a result of vector integration at the IIIL alternate region in the yeast genome, the probe 2* was removed from the filter (Fig. 14c), and it was used for Southern hybridization with the 1.2 kb Sall/EcoRI fragment (probe C*) that mapped proximal to probe 2* on IIIL, since this fragment shares homology with both
Figure 14. Characterization of the URA3 Integration Site in IIII.

a. Restriction map of the integration site in the probe 2* region for the yeast strain AB20α XP8-10B, and the lengths of the fragments which are homologous with the probe 2* are indicated. Control probe, C*, is a 1.2 kb SalI/EcoRI (Probe C, Fig. 8C) fragment which hybridizes to both IIII distal and alternate regions in AB20α XP8-10B.

b. Expected restriction map following integration of pLB21-17, which results in the duplication of the target 3.8 kb EcoRI/BamHI fragment in the IIII distal region. Positions of homology for probe 2* and probe C* and the fragment lengths expected in the URA3 transformant YeLB21-17-26 are indicated.

Restriction fragment lengths are indicated in kb and restriction enzyme sites are B, BamHI; H, HindIII; P, PstI; R, EcoRI.

c. Southern hybridization of probe 2*, (MTeLB1-1.0), with DNA from AB20α XP8-10B, (lane 1), SR25-1A, (lane 2), and YeLB21-17-26 (lane 3) digested with PstI, BamHI, HindIII, and EcoRI as indicated. Following hybridization with probe 2*, and development of the probe 2* autoradiogram, probe 2* was removed, and secondary hybridization of probe C* was conducted. Size standards are λ/HindIII/EcoRI fragment positions.
the IIIIL distal and IIIIL alternate regions in the genome of other yeast strains (probe C\*, Fig. 8C). Results from this Southern blot were difficult to interpret since the IIIIL alternate region fragments that were observed in strain AB20α XP8-10B were absent in the host strain SR25-1A that was used in the transformation. The faint bands that hybridized with probe C\* in the genomic DNA digests of SR25-1A and AB20α XP8-10B were also present in the probe 2* Southern blot (Fig. 14c), and were not previously detected in Southern hybridization. The reduced or lack of homology between probe C\* and a IIIIL alternate region in the host strain SR25-1A suggested that integration did occur at the IIIIL distal region in the SR25-1A transformants, the region homologous to the target site in pLB21-17 (180). In addition, the fragments that were homologous with probe C\* in YeLB21-17-26 (Fig. 14c) agreed with those predicted for integration of pLB21-17 in the IIIIL distal region (Fig. 14b) except for HindIII digestion where both 15 kb and 7 kb fragments hybridized with probe C\*.

Genetic linkage analysis (176, 177) would conclusively show whether URA3 was linked with the IIIIL distal region in YeLB21-17-26, however I decided to first test the feasibility of recovering the marker along with the chromosome end in Ye21-17-26. Since both the IIIIL distal and IIIIL alternate regions are proximal to telomeres as shown in the previous section by their sensitivity to Bal31 nuclease digestion, the URA3 marker along with the flanking chromosome end should be recovered for insertions at either region. Genomic DNA from transformant YeLB21-17-26 was digested partially with HindIII or Xhol such that the yeast URA3 marker, the bacterial AMP marker, the probe 2* region, and the chromosome IIIIL terminus would be contained on the same restriction fragment (Fig. 13c). Tetrahymena rDNA termini were isolated as HindIII/Hhal or Xhol/Hhal fragments from pSZ221 or pSZ222 respectively (195). Ligation mixes containing the partially digested DNA fragments from YeLB21-17-26 and the Tetrahymena rDNA end fragments (Fig. 13d) were introduced by genetic transformation into yeast strain SR25-1A, but no URA3 prototrophic transformants were produced. The second approach to marker recovery involved the circularization of the HindIII or Xhol partial digest DNA fragments from YeLB21-17-26 and selection for the AMP marker in bacteria. Blunt chromosome ends were generated on the partial RE digest fragments using S1 nuclease (20, 24, 175). These were circularized by intramolecular ligation, introduced in E. coli.
DH1 cells by transformation, and AMP resistant colonies were selected (Fig. 13e). No transformants were obtained with either HindIII or XhoI digested and ligated DNA (5 μg) from YeLB21-17-26, however the control digestion and intramolecular ligation with DNA from the host strain SR25-1A produced 60 colonies. Southern hybridization analysis demonstrated that pBR322 homology exists in the genomic DNA isolated from strain SR25-1A and possibly that region was recovered in the selection for AMP resistant colonies. The question remains as to why no AMP resistant colonies were produced with YeLB21-17-26 DNA, since the genome of this URA3 yeast transformant contained the host pBR322 sequences of SR25-1A plus pLB21-17 vector sequences. Problems with identifying the marker insertion region in the transformants isolated from host strain SR25-1A along with difficulties encountered with marker recovery suggested that this strategy was not an efficient method to use for the isolation of the IIIIL telomere.

F. Attempt to Clone the IIIIL Telomere by Vector Addition at the Terminus

A variation of the marker insertion strategy for IIIIL telomere cloning was the addition of the vector fragment that contains a selectable marker at the chromosome ends, analogous to the method developed by VanderPloeg et al. (203) for cloning Trypanosome telomeric regions. This method involves the ligation of a plasmid vector to the ends of double-stranded DNA in high molecular weight genomic DNA which presumably are chromosome ends, followed by intramolecular ligation, and selection in E. coli for the antibiotic resistance marker on the plasmid vector (Fig. 15). AB20α XP8-10B DNA was treated with Bal31 nuclease for increasing periods of time (20 μg aliquots at 1, 3, 5, 7, and 9 min digestion) to ensure that chromosomal DNA was blunt ended. The pBR322 vector (3 μg) was digested with PvuII, dephosphorylated to prevent self-ligation of the plasmid, and ligated to Bal31 digested AB20α XP8-10B DNA (7 μg), and the ligation mixture was partially digested with EcoRI (1u/μg for 10, 20, and 30 min digestion). Intramolecular ligation of the products followed by transformation in DH1 cells resulted in about 1200 AMP resistant colonies of which 5 hybridized to probe 2*. The plasmids in these transformants consisted of a 2 kb and a 4 kb HindIII fragment that both hybridized with probe 2*. However a single HindIII fragment that is >5 kb is homologous with probe 2* on chromosome IIIIL indicating that deletion or rearrangement events occurred in the clones.
Figure 15. Strategy for Cloning the IIII Telomere by Vector Addition.

High molecular weight DNA from yeast strain AB20α XP8-10B was treated with Bal31 nuclease, to produce blunt ended chromosomes. pBR322 was Pvull digested, CIP treated, and ligated to Bal31 treated genomic DNA. Following partial digestion with EcoRI, fragments were intramolecularly ligated, and transformed in E. coli DH1 cells. Selection was initially for AMP resistance clones, followed by colony hybridization with the IIII distal probe 2*. Restriction sites: B, BamHI; H, HindIII; P2, Pvull; R, EcoRI; and S, Sall.
Chromosomal DNA

- Bal31 Nuclease Digestion
  - pBR322 Ligation

- EcoRI Partial Digestion

- Intramolecular Ligation
  - AMP Selection
  - Probe 2 Screen
6. Cloning the IIIL Terminal Fragment in a Circular Vector

Southern hybridization results indicated that probe 2* hybridized with a 3.3 kb (+/- 0.2 kb) SalI fragment that contained the IIIL terminus (Fig. 10). The method chosen to clone the IIIL terminus was to prepare blunt-ended chromosomes with Bal31 nuclease, such that the terminal restriction fragment on IIIL could be cloned in a circular vector (24, 175, 193, 203), (Fig. 16a). Some of the telomeric region is deleted in generating blunt-ended chromosomes with Bal31 nuclease, however reports indicate that the terminal 5'-C_3A-3' repeat sequence extends for 0.3 -0.5 kb on yeast chromosomal termini (49, 175, 204, 206).

High molecular weight DNA from yeast strain AB20α XP8-10B was treated briefly with Bal31 nuclease (2 and 4 min), and repaired with DNA Polymerase I, Klenow fragment such that the IIIL end could be ligated with a blunt-ended vector. DNA was digested to completion with SalI, and Bal31 nuclease/SalI digested fragments in the 3-4 kb range (including the 3.3 kb IIIL terminal SalI fragment) were isolated by preparative 0.7% LMP agarose gel electrophoresis. Fragments were directionally cloned into SalI/Smal digested M13mp9RF, hence telomeric fragments were selected since only they should contain the necessary Bal31 blunt-end for cloning in the Smal site. Following hybridization with probe 2*, clone MTLB6411 was purified (Fig. 16b). The 3 kb insert had not undergone any significant recombination events in cloning since its restriction map was identical to that established through mapping of the region distal to probe 2* on IIIL (Fig. 7b). Apparently 0.3 kb (+/- 0.2 kb) were removed in the Bal31 nuclease treatment during cloning of the 3.3 kb (+/- 0.2 kb) SalI fragment on the intact IIIL terminus. MTLB6411 contained the 0.5 kb EcoRI fragment distal to probe 2*, as well as a 1.5 kb EcoRI fragment, presumably the IIIL terminal fragment. Preliminary sequencing analysis confirmed that the 1.5 kb EcoRI fragment contained the 5'-C_3A-3' repeat sequence adjacent to the Smal site in MTLB6411, which is indicative of yeast telomeric structure.

The terminal 1.5 kb EcoRI/Bal31 fragment was subcloned to remove probe 2* sequences that might confuse the characterization of the IIIL terminus (probe 3*). Also M13mp9 was not an appropriate vector to use for hybridization probes, since it contains a short region that is homologous
Figure 16. Cloning the IIIL Terminal Fragment in a Circular Vector.

a. High molecular weight yeast AB20a XP8-10B DNA was treated with Bal31 nuclease (0.1 u/µg, 2 min and 4 min aliquots) and repaired with DNA Polymerase I, Klenow fragment to yield blunt chromosome ends which can be cloned. Following complete digestion with Sail, fragments which include the 3.3 kb Sail/chromosome IIIL end were isolated by preparative 0.7% LMP agarose gel electrophoresis and electroelution.

b. Purified Bal31/Sail fragments were cloned in the M13mp9RF vector which was digested with Sail and Smal. JM101 transformants were screened with a nick-translated probe 2* fragment and this resulted in the identification and purification of the IIIL telomeric clone MTLB6411. The 3 kb Sail/END fragment in MTLB6411 corresponds to the 3.3 kb (+/-0.2 kb) IIIL end with 0.3 kb (+/-0.2 kb) excised by the prior Bal31 nuclease treatment.

c. The IIIL terminal fragment, probe 3*, was subcloned for DNA sequence determination and analysis by Southern hybridization. The EcoRI ends on probe 3*, one derived from yeast DNA IIIL end and the other from the polylinker region of M13mp9, were repaired with DNA Polymerase I, Klenow fragment. Probe 3* was cloned into the Smal site of M13mp18RF to result in MTLB6512, which has the same orientation of the insert as MTLB6411. Symbols: telomeres (⧫, □); blunt-ended chromosome IIIL (■E); M13 universal sequencing primer (——); and the M13mp9 or M13mp18 vectors (——). Restriction sites: B, BamHI; H, HindIII; P, PstI; S, Sail; R, EcoRI; Z, Smal.
with the pBR322 plasmid (131) and hence hybridizes with the vector regions of clones which could lead to problems in interpreting Southern hybridization results. The IIIIL terminal fragment (probe 3*), (Fig. 16b), was released from MTLB6411 by EcoRI digestion which made use of both the M13mp9 poolinker site and the EcoRI site on the yeast IIIIL terminal fragment. Probe 3* was subcloned in the Smal site of M13mp18RF, after DNA Polymerase I, Klenow fragment repair of the EcoRI ends (Fig. 16c). Consequently, probe 3* was flanked by several restriction sites in the poolinker region of M13mp18, such that deletion clones could easily be generated with Exonuclease III (also referred to as ExolII) for DNA sequence determination (86). MTLB6S12 had the same insert orientation in the M13 vector as MTLB6411, which contained 3'-G_1-T_5'- repeat units adjacent to the binding site in M13 of the universal sequencing primer. The IIIIL terminal fragment was also cloned in the opposite orientation, as an EcoRI fragment in the EcoRI site of M13mp18, (referred to as MTLB6S21) such that deletion clones could be generated with ExolII and S1 nucleases from the opposite end of the IIIIL telomeric fragment and the sequence of both DNA strands could be established.

H. The IIIIL End Conforms to the T-X Class of Yeast Telomeres

The arrangement of yeast telomeric regions is conserved. Southern hybridization analysis indicates that yeast telomeres contain a telomere associated region that consists of complex repeat units referred to as type X and Y', and these repeat units contain ARS regions (49, 93, 193, 206) which are thought to be origins of replication in yeast (210). As expected for a telomeric fragment, the IIIIL terminal EcoRI fragment (probe 3*) hybridized with multiple fragments in the yeast genome, of either discreet or heterodisperse lengths (Fig. 17, lanes 1–3). Southern hybridization was used to determine whether the IIIIL telomeric region (probe 3*) was homologous with clones YRp131A, YRp131B, and pSZ220 from the yeast telomere associated region (Fig. 17a, 17b). The IIIIL telomeric probe was homologous with the type X ARS region of YRp131A (48, 49), hybridizing with the 0.9 and 3.3 kb Sall/Ncol fragments (lane 5) and the 3.8 kb Sall/HindIII fragment (lane 6). The IIIIL telomeric probe did not hybridize with the type Y' ARS region in either YRp131B (lane 4) or pSZ220 (lane 7), (48, 49, 193). Since probe 3*, but not the adjacent probe 2* (Fig. 12), was homologous with a telomere associated region clone, the telomeric region must be contained within the
a. Southern hybridization using MTLB6S12 as the probe for the III telomere (probe 3*) with the following DNA samples: 1, AB20α XP8-10B/BamHI, (4 μg); 2, AB20α XP8-10B/Pvull, (4 μg); 3, AB20α XP8-10B/HindIII, (4 μg); 4, YRp131B/Sall/Ncol, (0.12 μg); 5, YRp131A/Sall/Ncol, (0.12 μg); 6, YRp131A/Sall/HindIII, (0.12 μg); 7, pSZ220/Alul, (0.25 μg). Size markers on the right of the autoradiogram indicate positions of λ/HindIII/EcoRI fragments. Chromosome III terminal fragments are distinguished from the other bands by the open triangles (△).

b. Restriction maps of the yeast telomere associated regions in clones pSZ220 (92, 193), YRp131A, and YRp131B (48, 49). Restriction sites: A, Alul; H, HindIII; N, Ncol; P1, Pvul; S, Sall; Sc, ScaI.
a.

Yeast Y * V'

b.

pSZ220

0.8 kb

Yrp131A

10 kb

Yrp131B

6.7 kb
terminal 2.3 kb on IIIL and it is reasonable that the type Y′ ARS repeat region which is usually 6.7 kb in length, is not present within the short telomeric region on the IIIL end. The IIIL telomere contains only the simple repeat T region distal to the X ARS region and was therefore classified as a type T-X telomere, as opposed to the previously isolated and characterized type T-Y′-X telomere (48, 49, 175, 193).

Restriction enzyme digestions of yeast genomic DNA were probed with the IIIL telomeric fragment, probe 3*, (Fig. 18) to determine whether both class T-X and T-Y′-X telomeres are homologous with the IIIL telomeric fragment. The estimated lengths for the components of yeast telomeres (49, 205) are: (1) simple repeat T region, 0.3-0.5 kb, (2) conserved Y′ ARS region, 6.7 kb, (3) internal simple repeat region, 0.1-0.2 kb, and (4) the heterogeneous X ARS region, 0.3-3.75 kb, (Fig. 2). Hence yeast telomeres conforming to the T-Y′-X class are at least 7.5 kb in length and the distance between the X region and the terminus of the chromosome is about 7 kb on the T-Y′-X telomeres. Consequently, the heterodisperse, low molecular weight (<7 kb), and intensely hybridizing bands in the Southern hybridization analysis with the IIIL terminal probe (Fig. 18) must represent telomeres that lack a Y′ ARS region, and therefore can be categorized as type T-X telomeres (49, 93, 175, 205, 206). Such T-X telomeres represent about 30% of the yeast chromosome ends that hybridized with probe 3*. The heterodisperse, low molecular weight (<7 kb), and weakly hybridizing bands, evident in PstI digestion (1.0 kb), KpnI digestion (1.1 kb) and XhoI digestion (1.4 kb), (Fig. 18, lanes 3, 6, 10), probably shared homology with only the simple repeat region (T) of probe 3* and therefore represent type T-Y′-X telomeres. The sizes of these fragments correspond to the PstI, KpnI, and XhoI restriction sites within the conserved Y′ ARS region on most yeast telomeres (175, 193, 204). The low molecular weight fragments with defined fragment lengths must represent chromosome ends which contain the specific restriction enzyme site between the X region and the terminal heterodisperse (T) region (Fig. 18, lanes 1, 3, 4, 6, 7, 9). It was not possible to categorize those fragments into T-Y′-X or T-X class telomeres, since the proximities of the X regions to the respective chromosomal termini were unknown. The telomeres from the seventeen chromosomes in the haploid yeast genome were not distinguishable by different fragment lengths in
**Figure 18. The IIII Terminal Fragment is Homologous with Yeast Repetitive DNA.**

Southern hybridization of yeast AB20α XP8-10B DNA with MTLB6S12, the terminal probe from IIII. DNA (3.3 μg) was digested with each of the following restriction enzymes: Lane 1, EcoRI; 2, HindIII; 3, PstI; 4, PvuII; 5, BstEI; 6, KpnI; 7, BamHI; 8, XbaI; 9, SalI; and 10, XhoI. Open triangles on the autoradiogram distinguish the IIII end fragments from the other telomeric bands. Size markers are λ/HindIII/EcoRI fragments on the left and λ/HindIII fragment positions on the right edge of the autoradiogram, as indicated by the horizontal bars.
any of the restriction enzyme digestions (Fig. 18). The maximum number of fragments that hybridized with IIIIL T-X probe (12-16 fragments) was observed for Pvull (lane 4) or BstEII (lane 5) digestions. Similar restriction site arrangements in the type X ARS flanking regions must preclude the generation of different RE fragment lengths for each telomere.

1. The IIIIL Terminal Probe Is Homologous Exclusively with Telomeres

Bal31 nuclease digestion was used to estimate the distance from the X region (probe 3*) to the respective chromosome ends. AB20α XP8-10B genomic DNA was digested with Bal31 nuclease for up to 30 minutes, digested with Sail or Pvull, and analyzed by Southern hybridization with probe 3* (Fig. 19). A subset of Sail fragments that hybridized with the T-X probe was sensitive to digestion with Bal31 nuclease whereas all of the Pvull fragments that hybridized with the T-X probe were digested progressively with Bal31 nuclease. Assuming that Bal31 nuclease initiates digestion at chromosome ends in high molecular weight DNA (18, 24, 60, 175, 203, 206, 209), all Pvull fragments detected by the probe 3* must include the terminus of a yeast chromosome. The difference in sensitivity to deletion with Bal31 nuclease observed for Sail and Pvull fragments is a consequence of the restriction site differences in the type X and Y' ARS regions. The representative X and Y' clone maps (48, 49) have no Pvull sites distal to the ARS region in any of the heterogeneous type X clones isolated, and the conserved Y' region is void of Pvull sites. A Sail recognition site separates the type X and Y' regions in all of the clones, hence all type T-Y'-X telomeres are digested with Sail at the Y'-X junction. As a result, all Pvull fragments homologous with the X region probe contain the Bal31 nuclease sensitive chromosome end. Alternatively, Sail fragments that contain an X region and which map to T-X telomeres belong to the subset of Sail fragments that contains the Bal31 nuclease sensitive region. The other Sail fragments that hybridize to the X region probe are from T-Y'-X telomeres and these fragments display Bal31 nuclease sensitivity only after the T-Y' region (>7 kb) is completely deleted by Bal31 nuclease. These assumptions are valid only if all X regions are void of Sail and Pvull recognition sites in the region distal to ARS, which is the case for all X regions that have been isolated
Figure 19. The IIII Terminal Probe Hybridizes with other Chromosome Ends.

Yeast AB20α XP8-1OB DNA was treated with Bal31 nuclease (0.1 u/μg) for up to 30 min, (deletes 125-150 bp per minute), and was digested with Sall or PvuII as indicated. After fractionation on a 0.65% agarose gel, the DNA fragments were transferred to gene screen plus filters, and hybridized with MTLB6S12 (probe 3*). The Sall or PvuII fragments on the IIII end are indicated by open triangles. Size markers are λ/HindIII/EcoRI fragment positions on the left side and λ/HindIII fragments on the right edge of the autoradiogram.
If T-Y'X telomeres have a minimum length of 7.5 kb, then all PvulI fragments that are homologous with the T-X region from IIII and shorter than 7.5 kb (Fig. 19) must represent telomeres of the alternate T-X category. Based on this reasoning, together with the proportion of Sall fragments that display Bal31 nuclease sensitivity, I estimate that at least 30% of chromosome ends in the haploid yeast genome belong to the T-X class of telomeres.

J. Length Heterogeneity of the IIII Telomere Among Yeast Strains

Restriction endonuclease site polymorphisms and DNA rearrangements among telomeric regions in various yeast strains have been examined using a Y' probe, pSZ220, in Southern hybridization analysis (93, 193). Extensive differences in hybridization patterns, both in the size and relative abundance of restriction fragments have been observed and it was concluded that the number of Y' homologous regions varies considerably among different yeast strains (93). The T-X probe from the IIII telomere hybridized to both yeast telomere classes, and consequently was used to examine strain differences in the T-X region of yeast telomeres.

The unique fragment on the distal end of chromosome IIII (probe 2*) was homologous with fragments that had conserved lengths among different yeast strains, if the fragments did not include the IIII terminus (Fig. 20a, lanes 1-6, PvulI, EcoRI). The 1.2 kb PvulI fragment and the 4.6 kb EcoRI fragment extend in the centromere proximal direction from probe 2*, and both fragments were conserved in the various strains. The identity of the extra bands in SR25-1A (lane 5, PvulI, EcoRI, Sall) is unknown. DNA from this strain hybridized with pBR322 vector sequences, hence the extra bands may be due this foreign DNA of unknown origin that was introduced into this strain. Fragments homologous with probe 2* that contained the IIII terminus in strain AB20α XP8-10B varied in their average length with a distribution +/- 200 bp compared to other yeast strains (lanes 1-6, PvulI, Sall). The average length of the PvulI 3.0 kb (+/- 0.2 kb) fragment and Sall 3.3 kb (+/- 0.2 kb) fragment in AB20α XP8-10B (lane 1) was different in other strains, however these telomeric fragments remained equally heterodisperse (+/- 0.2 kb) in all strains. Since heterodisperse Sall and PvulI fragments hybridized with probe 2* in each of the various strains, the length heterogeneity in
Figure 20. Southern Hybridization Indicates Length Heterogeneity of the IIIIL Telomere Among Yeast Strains.

a. Yeast DNA (4 †g/lane) was digested with either PvuII, EcoRI, or Sall, and fractionated on a 0.65% agarose gel. Lanes: 1, AB20α XP8-10B; 2, K45; 3, T388; 4, AB972; 5, SR25-1A; 6, SR26-12C. Following unidirectional transfer of DNA to gene screen plus, the filter was hybridized to probe 2*, (MTeLB1-1.0).

b. Secondary hybridization of the filter was with probe 3*, (MTLB6S12), after removing probe 2*. Fragments that are expected to map to the IIIIL end are distinguished by open triangles, while open circles indicate restriction fragment length polymorphisms observed in the X or Y' regions among the various yeast strains.

Size markers are positions of λ/HindIII/EcoRI fragments and are indicated in the center section separating Southern blots a and b.
the IIIL terminal region among the strains is not due to differences in Sall or Pvull restriction sites in the T-X region of the IIIL telomere. Presumably the length differences are due to different average amounts of simple repeat T sequences (5'-C_1A_3-3' units) at the chromosome IIIL end in the various strains (40, 175, 206).

Southern hybridization of the various yeast DNAs that were restriction enzyme digested, and probed with the T-X region from IIIL (probe 3*) suggested that the variation in the length of the T repeat region was a characteristic of most T-X telomeres (Fig. 20b). For yeast DNA digested with Pvull, EcoRI, and Sall, the low molecular weight (<4 kb) heterodisperse bands varied in average length by about 0.2 kb among the different strains. Such relatively small length differences were not detectable for the large Pvull fragments (>7 kb), which presumably represent T-Y'-X telomeres, due to the limitations of the assay system. Variations in terminal repeat length at both T-X and T-Y'-X chromosome ends, either within a given yeast strain or among different strains, have been described recently (40, 93, 206).

There were some restriction enzyme site polymorphisms or DNA rearrangements in the telomeric regions of the various yeast strains that were detected with the T-X probe from IIIL (probe 3*). The Pvull fragments that are polymorphic among the various strains are larger than 7 kb, the EcoRI fragments are larger than 4.5 kb, and the Sall fragments are larger than 4 kb (Fig. 20b). As described in a previous section, Pvull fragments shorter than 7.5 kb were categorized as T-X telomeres, and those 7.5 kb or longer were classified as T-Y'-X telomeres. Apparently, the polymorphisms that were detected with the T-X probe, (probe 3*), map to T-Y'-X telomeres. It is also conceivable that the polymorphisms detected, exist in the Y' region of these telomeres, and the X region is void of strain specific variations. The hybridization pattern of the T-X region probe 3* with the genomic DNA from various yeast strains is similar, and the proportion of high and low molecular weight fragments that hybridized with the T-X probe is conserved among the yeast strains (Fig. 20b). This suggests conserved ratio of T-X class to T-Y'-X class telomeres in the genomes of the various haploid yeast strains.
K. The IIIIL Telomere is Retained in Some Ring III Yeast Strains

Southern hybridization of genomic DNA from linear and circular chromosome III strains with the IIIIL telomeric probe served to investigate the fate of the IIIIL end in the HMLα-HMRα fusion event that formed the circular chromosome III (Fig. 21). The average length of the IIIIL terminal fragment in yeast strain K45 was about 0.3 kb shorter than that in strain AB20α XP8-10B, as described in the previous section (Fig. 20a). In K45, the 1.5 kb (+/- 0.2 kb) EcoRI, 1.6 kb (+/- 0.2 kb) HindIII, and 3.0 kb (+/- 0.2 kb) Sall fragments (Fig. 21, lane 1) mapped to the IIIIL end and these fragments were absent in strain K192 in which the IIIIL distal region is deleted, (lane 3). The telomeric fragments from chromosome IIIIL were retained in the ring III strain K191 (lane 2), which supports the proposal that the IIIIL distal region was retained in K191 by replacing the IIIIL alternate region in a telomere conversion or recombination event. Both K191 and K192 were derived from K45, hence the telomeric fragments, other than those on chromosome III in K45, should be conserved in these ring III strains. Other than the differences in the telomeric fragments from IIIIL between the linear III and ring III strains, the fragments from the right end of chromosome III (IIIIR) should hybridize with the T-X probe in K45 and these should be absent in the ring III strains. For each restriction enzyme digestion of the genomic DNA from linear III and ring III strains, one fragment was identified that may map to the IIIIR end: a >10 kb EcoRI fragment, a 8 kb HindIII fragment, and a 4.5 kb Sall fragment (lane 1), but these have not been characterized. A 3.6 kb EcoRI fragment that was conserved in K45 and K192, but absent in K191 may correspond to the telomere of the IIIIL alternate region that was replaced by the IIIIL telomere in K191. Although strains K193, K195, K196 are circular III strains which were derived from an alternate host strain than K45, the majority of fragments that were homologous with the IIIIL telomeric probe in the K191 and K192 strains were maintained in the K193, K195, and K196 strains. Similar to ring III strain K191, the restriction fragments which contain the IIIIL telomere were retained in the ring III strains K193, K195, and K196 (lanes 4, 5, 6).
Figure 21. Southern hybridization of Linear III and Ring III Yeast Strains with the IIIL Terminal Probe.

Yeast DNA was digested with EcoRI, HindIII, or Sall as indicated, then fractionated on a 0.65% agarose gel and transferred to gene screen plus filters. Lanes: 1, K45; 2, K191; 3, K192; 4, K193; 5, K195; and 6, K196. This Southern hybridization filter was used for the IIIL distal probes in Figure 8. After removing the other IIIL distal probes, hybridization was with the IIIL terminal probe 3* (MTLB6S12). The length of the IIIL terminal fragment in linear III strain K45 is indicated for EcoRI (■), HindIII (●), and Sall (▲) digestions by closed symbols. The estimated positions for the IIIR terminal fragments in the EcoRI, HindIII, and Sall digestions are indicated by the equivalent open symbols (□, ○, ▲).

Size Marker positions are indicated by horizontal bars at the left for λ/HindIII/EcoRI fragments and positions at the right are for the λ/HindIII fragments (23.7 kb, 9.5 kb, 6.7 kb, 4.3 kb, 2.3 kb, 2.0 kb, and 0.6 kb).
L. DNA Sequence Analysis of the Chromosome IIIIL Telomere

To establish the extent of the terminal repeat region on IIIIL, to compare the DNA sequence with that reported for a Y' containing telomere, to locate the position of the ARS consensus sequence within the X region on IIIIL, and to search for tandem units of repeat sequences in the X region on IIIIL, the complete DNA sequence of the cloned fragment from the T-X telomere on IIIIL was determined. Sets of deletion clones were prepared with Exonuclease III (referred to ExoIII) and S1 nuclease according to Henikoff (86) from the subclones MTLB6S12 and MTLB6S21 (both insert orientations) with processive ExoIII digestion from the BamHI site in each clone. Dideoxynucleotide chain terminator sequence determination (170, 171) of both sets of deletion clones (Fig. 22a) gave the complete DNA sequence for the IIIIL telomeric fragment (Fig. 22b). The set of deletion clones for MTLB6S21 was incomplete in the T region, possibly due to problems with ExoIII digestion in the G-rich region of the template strand of MTLB6S21. To resolve this problem, a sequence specific primer was used to complete the sequence of the T simple repeat region of MTLB6S21. Repeat DNA sequences from yeast telomeres (175) had been previously determined using the Maxam and Gilbert chemical sequencing method (128), however simple terminal repeat sequence for the IIIIL end was completely determined with the enzymatic method for DNA sequence determination (170, 171), (Fig. 22c). The repeat sequence 3'-G13T-5' was obtained using a MTLB6S21 deletion clone and the synthetic primer that was specific for the insert in the clone, while the alternate strand 5'-C13A-3' repeat sequence was determined from a MTLB6S12 deletion clone using the M13 universal sequencing primer. The 356 nt sequence of the T region in MTLB6S12 was more accurately defined as 5'-[C23A(CA)1-4]-3' and the distal end of the cloned 1539 nt IIIIL telomeric fragment contained fifty-three repeat units. An 80 nt repeat region from a Y' containing telomere was sequenced by Shampay et al. (175) and it has the same simple repeat unit (5'-C13A-3') as the IIIIL telomere. However, the defined formula for this telomere is 5'-[C23A(CA)1-3]-3' since it has a maximum of three tandem CA dinucleotides whereas the repeat units at the IIIIL end are defined by 5'-[C23A(CA)1-4]-3' since a repeat unit in the fragment from the IIIIL telomere had four tandem CA repeats. The simple repeat region on a telomere is thought to extend to the terminus of that chromosome (22, 23, 24, 25, 175, 203, 206). This
Figure 22. DNA Sequence of the IIIL Terminal Fragment.

a. Extent and orientation of DNA sequence determination for the cloned 1539 nt EcoRI/Bal31 nuclease treated fragment from the IIIL terminus. Position 0 refers to the initial nucleotide in the sequence of the cloned telomere. The 0.3 kb (+/-0.2 kb) which extends distal to this 1539 bp fragment on the end of chromosome III were deleted during cloning of the IIIL telomere. Dotted lines represent the M13mp18 vector sequences which flank the insert fragment. Arrows below represent the start point and extent of sequence obtained from each ExonI/I/S1 nuclease deletion clone. The sequence of the MTLB6S12 clones extend toward the 5' end of the insert fragment, while the MTLB6S21 clones extend toward the 3' end, and hence the entire insert is covered in both directions. The arrow with an open circle at its base in the MTLB6S21 deletion set distinguishes the sequence obtained using a site-specific, synthetic oligonucleotide primer.

b. Nucleotide sequence of the IIIL telomeric region, that extends 1539 bp from the IIIL terminal EcoRI site, as determined by dideoxynucleotide chain terminator sequencing (170,171). The 5' to 3' DNA strand on the chromosome contains the 5'-C1-3A-3' repeat sequences. A perfect ARS consensus sequence (33, 42, 104) is underlined at positions 1036-1048 nt. The simple repeat region differs from the reported (175) formula 5'-C2-3A[CA]3-3' at nt 152-163 by having a [CA]4 block. The only 6 bp recognition restriction enzyme site in the terminal IIIL cloned fragment is BstEII at nt 1175-1181.

c. IIIL telomeric repeat sequence determined by dideoxynucleotide chain terminator method (170, 171). Repeat sequences 3'-G1-3T-5' and 5'-C1-3A-3' were determined from the reverse orientation clones, MTLB6S21 and MTLB6S12 respectively, from the IIIL telomere. The sequence was determined with the site-specific synthetic primer, and the sequence prior to the 3'-G1-3T-5' repeat region in MTLB6S21 stems from the IIIL X region which flanks the T region. The MTLB6S12 sequence was determined using the M13 universal forward primer, and the sequence prior to the 5'-C1-3A-3' repeat region in MTLB6S12 is the M13mp18 polycloning site. The only C3A(CA)4 repeat unit sequenced in the IIIL T region is indicated at the top of the MTLB6S12 sequence.
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proposal is supported by the finding that a section of the 5'-C1-3A-3' repeat region adjacent to a telomere associated ARS region is sufficient to provide telomere function in yeast (65, 175, 195, L.L. Button and C.R. Astell, manuscript in preparation). It is assumed that the 0.3 kb (+/-0.2 kb) of DNA removed from the IIIIL end with Bal31 nuclease in cloning the IIIIL telomere consisted of additional 5'-C1-3A-3' repeat sequences.

Adjacent to the T region on IIIIL was the type X ARS region (Fig. 22b). Distal portions of X regions that were derived from T-Y'-X telomeres were sequenced by Walmsley et al. (205) and the distal 260 bp of such X regions are 80-90% homologous with the X region which flanks the T region on IIIIL. The consensus sequence for yeast presumptive origins of replication, or ARS regions, is 5'-A/T-T-T-T-A/T-Pu-T-T-T-A/T-3' (34, 42, 104, 182). A perfect ARS consensus sequence, 5'-T-T-T-T-A/T-G-T-T-T-T-3', is located at nt 1036-1047 on the cloned IIIIL end or 1.2 kb (+/-0.2 kb) from the terminus on the intact chromosome IIIIL end. The ARS consensus in the Y' region of a T-Y'-X telomere that was partially sequenced (175) is 700 bp from the chromosome end, and in the opposite orientation with respect to the 5'-C1-3A-3' containing strand compared with the ARS region on the IIIIL end. The effect of the orientation of the ARS consensus sequence on ARS activity has not been reported. The flanking regions are known to participate in ARS function and the sequences adjacent to the consensus sequence vary to a large extent in the ARS regions that have been characterized (34, 42, 104, 182). Consequently, the strength of the ARS regions in X and Y' cannot be estimated simply by DNA sequence analyses. Instead, comparison of the X and Y' ARS regions must involve assays for ARS function.

Nucleotide sequence studies with the Y' containing clone pSZ220 (92, 193) revealed a region of tandem direct repeats which were proposed to have a role in telomeric replication, maintenance, and homogenization of telomeric sequences. In contrast to the Y' region, no direct repeat region was apparent in the X region on IIIIL and the lack of G residues in the 5'-C1-3A-3' containing strand was its only distinguishing feature (Fig. 22b).
**M. The IIII Telomere Includes 1.8 kb at the Terminus**

The extent of the X region on the end of IIII, and the effect of the T region on the hybridization pattern obtained with the T-X probe, were measured by Southern hybridization analysis with probes that contained progressive deletions in the IIII telomeric region (Fig. 23). The full length clone, MTLB6S12, (probe A, 1539 nt insert) was homologous with multiple fragments in restriction enzyme digestions of yeast genomic DNA and with the X region fragments in YRp131A (Fig. 23A). The removal of the entire 5'-C_1-A_3-3' repeat region in probe B did not significantly alter the hybridization pattern or the relative bend intensities (Fig. 23B) from those detected with probe A. The overall reduction in hybridization intensity with probe B (Fig. 23B) compared with probe A (Fig. 23A) was the result of the asymmetric transfer of DNA in bidirectional Southern analysis. In transferring DNA from an agarose gel to two filters, I have consistently found that the top filter (Fig. 23, A,C) contained a greater percentage of DNA than the bottom filter (Fig. 23, B,D). All of the T region and a portion of the X region was deleted in probe C, which had a total deletion of 1051 nt compared with probe A (MTLB6S12). This deletion completely eliminates the ARS consensus sequence in MTLB6S12 (Fig. 22b) and probe C did not hybridize with the 0.9 kb Sall/NcoI fragment in YRp131A (Fig. 23C, lane 5) which maps adjacent to the ARS region in YRp131A (Fig. 17b).

However, the 500 nt fragment from IIII in probe C contained further X region sequence since probe C hybridized with the 3.3 kb Sall/NcoI and 3.8 kb Sall/HindIII fragments (Fig. 23C, lanes 5&6) which include the region upstream of the ARS consensus sequence in YRp131A (Fig. 17b). A subset of genomic DNA fragments (high molecular weight) that were homologous with probes A and B, did not hybridize with the X region in probe C. Possibly these fragments represent yeast telomeres that are homologous with the region between the ARS consensus sequence and the T region on the IIII telomere, since they hybridize with probes A and B but not with probe C. The hybridization intensity of the fragments from the IIII telomere has increased relative to the other telomeric fragments that are homologous with probe C (Fig. 23C, lanes 1−4), due to greater heterogeneity on the centromeric side of the ARS consensus sequence than on the telomeric side for the X regions. In support of this idea, the restriction maps of the heterogenous X region clones (48, 49) display more conservation in
Bidirectional Southern hybridization analysis, with deletion fragments from the IIIL telomere as probes, was conducted on restriction enzyme digested yeast and plasmid DNA samples that were fractionated on duplicate 0.65% agarose gels. Lane: 1, AB20α• XP8-10B/Sall, (4 μg); 2, AB20α• XP8-10B/HindIII, (4 μg); 3, AB20α• XP8-10B/BamHI, (4 μg); 4, AB20α• XP8-10B/XhoI, (4 μg); 5, YRp131A/Sall/NcoI, (0.1 μg); 6, YRp131A/Sall/HindIII, (0.06 μg). Probes A, B, C, and D were M13 EcoII/S1 nuclease deletion clones that were prepared for DNA sequence analysis. For probes A, B, C, and D, approximately 300 bp, 650 bp, 1350 bp, and 1550 bp respectively were removed from the intact 1.8 kb (+/-0.2 kb) EcoRI fragment at the chromosome IIIL terminus. The map below the Southern blots indicates the positions of probes A, B, C, and D on the IIIL end. The heavy arrow on the IIIL end represents the 600 (+/-200)bp of 5'-C3A-3' simple repeat region. Open triangles on the autoradiogram indicate the fragments on the IIIL end and the closed triangles at the right indicate the positions λ/HindIII/EcoRI fragments.
restriction sites on the telomeric side of ARS compared to the centromeric side. Finally, probe D had a 1239 nt total deletion compared with probe A (MTLB6S12). The 300 nt insert in probe D had no significant homology with the X region in YRp131A (Fig. 23D, lanes 5&6). For each restriction enzyme digestion, there were only two or three genomic fragments that were homologous with probe D (Fig. 23D, lanes 1-4). Since X regions are homologous with one another by definition (48, 49), there must be minimal X region sequence remaining in probe D. The boundary between DNA that is unique to chromosome IIIIL and the DNA that is repeated on all yeast telomeres must be near the terminal EcoRI site on chromosome IIIIL, about 1.8 kb from the IIIIL terminus. The fragments that are homologous with probe D, which do not map to the IIIIL terminal region (Fig. 23D, lanes 1-4), presumably represent other chromosome ends that contain X regions which are highly homologous to that on IIIIL. It is unknown whether these chromosome ends are also T-X telomeres.

N. Construction of Clones to Study the Function of the IIIIL Telomere

I determined whether the cloned IIIIL telomere, which had the terminal 0.3 kb (+/-0.2 kb) DNA deleted, contained sufficient information to maintain a linear plasmid in yeast (193). The required amount of telomeric DNA for recognition as a chromosome end by the cell's replication machinery was also analyzed, through using progressive deletions of the IIIIL end fragment. The assay system was analogous to that developed by Szostak et al. (65, 140, 194, 195) to study replication and resolution of Tetrahymena thermophila macronuclear rDNA ends on linear plasmids in yeast S. cerevisiae.

Since the cloned Tetrahymena termini function as telomeres on linear plasmids in yeast, I prepared a yeast plasmid with Tetrahymena rDNA end fragments to use as a control in yeast for the function of clones constructed with IIIIL ends (Fig. 24). The Tetrahymena rDNA end in pSZ222 is a 0.7 kb Xhol / Hhal fragment, which has 330 bp of Tetrahymena 5'-C4A2-3' terminal repeat sequence adjacent to 360 bp of rDNA which contains a yeast ARS consensus sequence and displays low ARS activity in yeast (1, 65, 105, 139, 195). The vector used to prepare the linear plasmid was pSZ93 (Fig. 24a), (150). Its salient features include the LEU2 gene (160) for selection in yeast, and the
Figure 24. Construction of a Linear Plasmid with *Tetrahymena* rDNA Termini.

**a.** The 700 bp Xhol/Hhal fragment from the end of *Tetrahymena* rDNA was isolated from pSZ222, (195). The 330 bp *C4A2* region is oriented toward the Hhal end of the fragment as indicated by the arrow, and the adjacent 360 bp region of *Tetrahymena* rDNA has weak *ARS* activity in yeast (1, 65, 105). There are 9 bp of pBR322 DNA separating the Hhal site and the *C4A2* region (195). The pSZ93 vector (150, 195) is a 7.4 kb plasmid that contains the *ARS1* region for autonomous replication (184) along with the *LEU2* gene (2) for selection in yeast. The ligation mix of pSZ93/Sall and pSZ222/Xhol/Hhal included the restriction enzymes Sall and Xhol, to digest religated pSZ93 or telomeric fragment concatemers produced during ligation, and thereby enhance the proportion of vector ligated with two *Tetrahymena* rDNA end fragments.

**b.** Proposed structure of the linear plasmid following resolution in yeast of circular or linear plasmids present in the ligation mixture. An inverted repeat of the end fragments was not prepared prior to ligation of the end fragments in pSZ93. Presumably one Xhol/Hhal end fragment would ligate to each Sall end of pSZ93 by intermolecular ligation, then circularization would occur by intramolecular ligation of the Hhal sites. In yeast, recognition of and inverted repeat structure of *Tetrahymena* end fragments results in conversion of the ligation products to monomeric linear molecules (194, 195).

Restriction sites: B, BamHI; H, HindIII; Hh, Hhal; K, KpnI; R, EcoRI; S, Sall; X, Xhol.
ARSI region (184) which functions as a strong origin of replication and hence provides high mitotic stability to linear plasmids in yeast (56, 65, 140). The restriction enzymes Sail and XhoI were included in the ligation mixture since this increases the percentage of ligation products with inverted Tetrahymena ends in the circular pSZ93 vector (195). Presumably, other ligation products include linear plasmid monomers and concatamers. Upon transformation of the ligation mixture into yeast, the Tetrahymena inverted repeat region is recognized and resolved to give predominantly linear plasmid molecules (Fig. 24b), (65, 194, 195). The 9 bp of pBR322 sequence which separates the 5′-C4A2-3′ repeat sequence region from the Hhal site on the Tetrahymena end fragment does not interfere with the resolution process in yeast (195).

The terminal 1.8 kb (+/- 0.2 kb) EcoRI fragment on IIL (Fig. 25a) contains a 1.2 kb type X ARS region upstream from the 0.6 kb (+/- 0.2 kb) 5′-C1-3A-3′ repeat region. The cloned telomere from IIL, referred to here as TF1, is a 1.54 kb EcoRI fragment which has 356 bp of 5′-C1-3A-3′ repeat sequence adjacent to a 1.2 kb type X ARS region (Fig. 25a). Telomeric fragments which have progressive deletions at the IIL end were prepared by ExoIII / S1 nuclease digestion of MTLB6S12 to produce clones TF2 to TF7. The TF8 deletion clone was prepared by ExoIII/S1 nuclease digestion of the reverse orientation clone MTLB6S21. The telomeric fragments were transferred initially from the MTLB6S12 deletion clones in the M13mp 18 vector to M13mp 19 (213). The resulting TF deletion clones had the IIL fragments flanked by the required restriction sites for subcloning in the yeast vector pSZ218 (Fig. 25b) to produce the linear plasmids. Vector pSZ218 (194) rather than pSZ93 was used for preparing plasmids with IIL termini, since pSZ218 does not contain a yeast ARS region. Consequently, the ARS activity of the X region on the IIL telomeric fragments is a determining factor in the mitotic stability of the linear plasmids in yeast (65). The BamHI / EcoRI fragments were isolated for each of TF1 to TF7 for cloning into the single BglII site of pSZ218. The restriction enzymes BglII and BamHI were included to select for the desired ligation products which were either circular plasmids containing inverted telomeric repeats or linear plasmids with IIL telomeric fragments at the ends (194, 195). The assumption was that upon transformation in yeast, plasmids with sufficient telomeric DNA information should be recognized, resolved, and then be maintained as
Figure 25. Construction of Plasmids to Study the Function of Fragments from the IIIIL Telomere.

a. Maps of the telomeric region isolated from chromosome IIIIL, the extent of the cloned IIIIL telomere (TF1), and the ExoIII / S1 nuclease derivatives of TF1 (TF2 - TF7) or the reverse orientation deletion clone (TF8). The IIIIL telomere contains a 0.6 kb (+/-0.2 kb) region of 5'-C1-3A-3' repeat sequence adjacent to a 1.2 kb type X ARS region. The ARS consensus sequence (33, 42, 104) maps 0.52 kb distal to the IIIIL terminal EcoRI site. Short flanking sequences from the M13 polycloning site are terminated by BamHI, Sall, HindIII, or EcoRI and this region exists on the centromere proximal and distal ends of the telomeric TF1 to TF7 fragments as indicated for TF1. TF8 is flanked by HindIII on the centromere proximal end and EcoRI on the distal end. The lengths of the deletion fragments are: TF1, 1.54 kb; TF2, 1.23 kb; TF3, 1.18 kb; TF4, 0.92 kb; TF5, 0.72 kb; TF6, 0.47 kb; TF7, 0.29 kb; TF8, 0.71 kb.

b. Cloning the fragments from the IIIIL telomere in pSZ218. Vector pSZ218, (194), was digested with BgIII and used for cloning BamHI/EcoRI fragments from the IIIIL telomeric region. TF1 to TF7 fragments were ligated to pSZ218 in the presence of BamHI and BgIII. The linear monomer is the predicted plasmid structure following transformation of the ligation mixture in yeast, if the circular and dimeric molecules are resolved in yeast (65, 194, 195). The estimated length of resultant plasmid is the combined length of the pSZ218 vector (5 kb) plus two end fragments. Transformants were named according to the telomeric fragments used in the particular plasmid construction, ie. YeTF1-1 to YeTF7-1 contained plasmids with TF1 to TF7 telomeric fragments respectively.

c. Cloning IIIIL telomeric fragments on a LEU2 fragment. The LEU2 fragment was isolated as a 2.2 kb Sall/Xhol fragment from pSZ218. It was ligated to TF1/Sall/EcoRI (IIIIL X and T regions), or to TF3/Sall/EcoRI (entire T region deleted), in the presence of Xhol. Sall was not included since Sall ligation was required to add a telomeric fragment at one end of the LEU2 fragment.

d. The effect of different telomeric fragments on either end of the LEU2 fragment was determined. The LEU2 Sall/HindIII fragment (2.5 kb) was isolated from pSZ218 and ligated with a combination of telomeric fragments, either TF3/Sall/EcoRI and TF2/HindIII/EcoRI or TF3/Sall/EcoRI and TF8/HindIII/EcoRI. The monomeric linear plasmid expected to result following transformation in yeast is sketched for each construction.

Symbols: X region ( ), ARS consensus sequence ( ), T region ( ), IIIIL terminus ( ). Restriction sites: B, BamHI; Bg, BgIII; H, HindIII; R, EcoRI; S, Sall; X, Xhol.
linear plasmids in the yeast cell. Due to the numerous subcloning steps involved in preparing the BamHI / EcoRI fragments from the cloned IIIIL telomere, there were about 30 nucleotides of M13 vector DNA sequence separating the IIIIL distal end (the 5'-C1-3A-3' repeat end) and the EcoRI site on each fragment. Studies conducted with Tetrahymena ends cloned on linear plasmids in yeast indicate that up to 54 bp of foreign DNA sequence, adjacent to the 5'-C4A2-3' repeat region, do not significantly affect the resolution of inverted telomeric repeats since the majority of transformants had linear plasmids while the rest had a mixture of linear and circular configurations (195).

Further plasmids were designed to determine whether a yeast LEU2 fragment, without the adjacent plasmid sequences, can be stabilized and subsequently be maintained in a linear form in yeast (65) with IIIIL telomeres. The LEU2 fragment, (2.2 kb Sall / Xhol), was isolated from pSZ218 (2, 194) and ligated to TF1 or TF3, to determine whether the 5'-C1-3A-3' repeat sequences are required at the ends of the plasmids to result in linear plasmid resolution and maintenance in yeast (Fig. 25c). The requirement for 5'-C1-3A-3' repeat sequence and the ARS region at one or both ends of a linear plasmid was addressed by ligating different IIIIL fragments to either end of the LEU2 fragment (Fig. 25d). In one case, the TF2 and TF3 fragments were used as ends since both have ARS regions, but TF3 lacks the 5'-C1-3A-3' repeat region, whereas TF2 contains 49 bp of the T region repeat sequence. Secondly, the TF3 and TF8 fragments were used as ends on the LEU2 fragment since TF8 lacks the ARS region, but contains 356 bp of the 5'-C1-3A-3' repeat region whereas TF3 has the type X ARS region but lacks the T region. The estimated length is given for each plasmid construction but it does not account for any addition reactions that are known to occur at the ends of linear plasmids in yeast (65, 175, 195). Instead, it represents the minimum linear plasmid length for the various classes of plasmid constructions.

O. The Cloned IIIIL Telomere Functions on Linear Plasmids in Yeast

Ligation mixes for each of the plasmid constructions were transformed into the yeast strain A281, which has a nonreverting leu- genotype, (65, 195) and prototrophic LEU+ transformants were selected. The TF1 to TF7 fragments are numbered according to the extent of the deletion from the IIIIL telomere. Telomeric fragments TF6 and TF7 (Fig. 25a) contained only 0.47 kb and 0.29 kb
respectively of the X region and did not produce transformants when cloned in pSZ218. The TF5 fragments contained 0.72 kb of the X region and gave very few transformants when cloned in pSZ218. The TF4 to TF1 fragments contained between 0.92 kb to 1.54 kb from the 1.8 kb (+/- 0.2 kb) IIL telomere. The transformation frequencies for each of these fragments cloned on pSZ218 was equivalent (between 10 and 25 transformants per ligation mixture). The molar ratio of IIL insert to pSZ218 vector was equivalent for all of the plasmid constructions (10 x excess). Apparently the extent of the deletions from the IIL telomere, which averaged between 1.1 kb and 1.5 kb in TF5, TF6, and TF7, precluded the cloning of these fragments on pSZ218 in yeast. The ARS consensus sequence (34, 42, 104, 182) was deleted in the TF6 and TF7 fragments (Fig. 25a), hence there was no functional origin of replication on the pSZ218 plasmids ligated with TF6 or TF7. The TF5 fragment extended 200 bp distal to the ARS consensus sequence (Fig. 25a). The low transformation frequency observed with the TF5 fragments ligated with pSZ218 may reflect an ARS domain that is required for optimal ARS activity and that extends beyond the TF5 deletion (42, 182). Transformants were selected to assay the mitotic stability and structure of the resident LEU2 plasmid for each of the plasmids constructed with deletion fragments that contained the type X- ARS region (TF5 - TF1).

Mitotic stability, as represented by the percentage of plasmid bearing cells in the population (195) was determined by growing the transformants in selection medium that lacked leucine until log phase, plating an aliquot on complete medium (YPD), incubating at 30 °C for 1-2 days, then replica plating on selection medium (SC-leu). A summary of the plasmid constructions and the mitotic stabilities determined for representative transformants are presented in Table II. Transformants with Tetrahymena rDNA ends on pSZ93 (Fig. 24b), had average stabilities of 40% (eg. YeTFSZ-1), which is slightly lower than previously reported values for linear ARS1 plasmids with Tetrahymena rDNA ends in yeast (56, 65, 139, 140). Alternatively, LEU* transformants containing the circular pSZ93 plasmid (eg. YeSZ93-1) had mitotic stabilities of 5-10%. Transformants that contained the LEU2 marker on pSZ218 ligated with the IIL telomeric fragments displayed a wide range of stabilities (15-100%) and the LEU* marker stability was inversely proportional to the extent of the telomeric deletion. Transformants that contained plasmids with TF1 fragments (average 0.3 kb deleted from the
Table II. Stability and Structure of Plasmids with Telomeric Fragments in Yeast.

All plasmids were transformed into the yeast strain A281. The plasmid constructions used for each set of genetic transformations are given in the left column. The estimated length of each plasmid is the sum of vector length plus the length of two telomeric fragments. Transformants were named according to the telomeric fragments and the vector used in the plasmid construction (first and second numbers in the name) and to the number of the particular transformant that was isolated and characterized (third number). For example, transformant YeTF1-1-1 had TF1 ends, on the vector pSZ218, and it was the first transformant isolated that contained this type of plasmid construction. The percentage of plasmid bearing cells represents the mitotic stability of the plasmid in the transformant which was determined by measuring the fraction of colonies that grow on leucine selection medium, after replica plating colonies from nonselective medium. The linear or circular nature of the plasmid in each transformant was data derived from agarose gel electrophoresis and Southern hybridization analysis of DNA from the transformants (Fig. 26 and 27).
<table>
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<th>Construction</th>
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<th>Mitotic Stability</th>
<th>Linear Plasmid</th>
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IIIIL telomere in strain AB20α XP8-10B), ligated with either pSZ218 (YeTF1-1) or the LEU2 fragment (YeTF1-3 or YeTF1-4), had the highest average plasmid stabilities (70-80%). In transformants with mitotic stabilities approaching 100% (YeTF1-3-1, YeTF3-5-1, YeTF5-1-1), the LEU2 marker may have integrated into the yeast genomic DNA. The homology of the SalI/XhoI ends of the LEU2 fragment with the leu gene in genomic DNA provides the preferred conditions for marker integration through homologous recombination with genomic DNA (149, 150, 196). The YeTF2-1 transformants had pSZ218 plasmids with only 49 bp remaining of the T region (5'-C1-3A-3' repeat sequence) compared with 356 bp of T region on the plasmids in the YeTF1-1 transformants. However, the mitotic stability of the LEU2 plasmids in the YeTF2-1 transformants was 55-75%, and equivalent to that observed for the TF1-1 transformants. The deletions in the TF3 and TF4 fragments extended past the T region of the IIIIL telomere (Fig. 25a) and this resulted in marked reductions in the mitotic stabilities of the LEU2 marker in most of the YeTF3-1 and YeTF4-1 transformants. For example, pSZ218 with TF3 or TF4 fragments had average stabilities of 20%. An exception was transformant YeTF3-1-4 which had a mitotic stability equivalent to that observed for transformants that contained pSZ218 plasmids with TF1 or TF2 fragments. Transformants such as YeTF3-4-3 that contained the LEU2 fragment ligated with TF3 ends had mitotic stabilities between 15 and 40%, and equivalent to the stabilities for TF3 fragments cloned on pSZ218 plasmids (15-25%). However, plasmids constructed with the T region or the T-X region at one end of LEU2 and the X region (TF3) at the other such as in the transformants YeTF3-5-3 and YeTF3-6-1, had mitotic stabilities of 75%. Such high mitotic stabilities were equivalent to those determined for transformants that contain plasmids with a type X ARS region and a T region at both ends of the LEU2 plasmid (eg. YeTF1-4-1).

The ability of the IIIIL end fragments to function as yeast telomeres was determined by analyzing the structure of the plasmids in the genomic yeast DNA prepared from selected transformants. A IIIIL fragment was defined as a functional telomere if it maintained the LEU2 marker as a linear molecule in yeast. The estimated length for the linear plasmid expected to result from a given plasmid construction was the combined length for the vector fragment and two telomeric fragments (Table II). The transformants obtained for a given plasmid construction were screened for
the presence of linear plasmids in two ways. First, if a linear plasmid is present in the transformant, an extrachromosomal band is observed in the DNA of the transformant which is absent in the DNA from the host strain A281. The extrachromosomal band is detected by fractionation of the genomic DNA that has not been digested with restriction enzymes by agarose gel electrophoresis and observation of the EtBr stained gel by UV-fluorescence (Fig. 26A). Since the genomic DNA from the host strain A281 is void of extrachromosomal bands such as the yeast endogenous 2μm plasmid DNA, an extra band in transformant DNA which results from multiple copies of a linear plasmid can be identified (63, 65, 139, 195). Secondly, linear plasmids can be detected by Southern hybridization using probes from the vectors pBR322 or pSZ218 which hybridize with a single band in transformants that contain linear plasmids. However, if the transformant contains a circular plasmid, the probes hybridize with multiple bands which correspond to supercoiled, monomer, dimer and concatameric plasmids (Fig. 26B), (195).

The presence or absence of linear plasmids in the selected yeast transformants (Fig. 26, A&B) is given in Table II for the various plasmid constructions. The mitotic stability of the LEU marker in a given transformant correlates with the linear or circular nature of the plasmid in the transformant such that a high mitotic stability is indicative of a linear plasmid in the transformant. The total genomic DNA, that was not digested with restriction enzymes, contained only the chromosomal DNA smear in the host strain A281. Extrachromosomal bands that had lower molecular weight than the genomic DNA band were evident in the total DNA isolated from selected transformants (Fig. 26A). An additional band was evident in transformants that contained plasmids constructed with *Tetrahymena* rDNA ends at the expected 9 kb region (Fig. 26A, lanes 18, 21, 22). Plasmid pSZ218 with TF1 or TF2 ends (Fig. 26A, lanes 4 - 11), or the LEU2 fragment with TF1 ends (Fig. 26A, lanes 24-28) were linear in some transformants since extrachromosomal bands were observed with the predicted lengths. Southern hybridization analyses with pBR322 or pSZ218 probes (Fig. 26B) demonstrated that these extrachromosomal bands corresponded to the linear plasmids. The pSZ218 probe, which contains the LEU2 fragment was used to detect plasmids constructed with the LEU2 fragment. The *leu* region in the yeast chromosomal DNA was also homologous with the LEU2 region of pSZ218, and this
Figure 26. Assay for Linear or Circular Plasmids in the Yeast Transformants.

A. Yeast DNA was isolated from selected yeast transformants, and undigested DNA was fractionated by electrophoresis on 0.65% agarose gels, alongside size markers. Ethidium bromide stained gels were observed by UV fluorescence. Samples lanes 1-18 and 19-38 represent two separate agarose gels. Positions of extra low MW bands in some transformants are indicated by at left of gels in 6 kb -10 kb region. Sample lanes: (1) λ/H/R, (2) λ/H, (3) A281, (4) YeTF1-1-1, (5) YeTF1-1-2, (6) YeTF1-1-10, (7) YeTF1-1-11, (8) YeTF2-1-1, (9) YeTF2-1-2, (10) YeTF2-1-26, (11) YeTF2-1-27, (12) YeTF3-1-1, (13) YeTF3-1-33, (14) YeTF3-1-37, (15) YeTF4-1-1, (16) YeTF4-1-2, (17) YeTF5-1-1, (18) YeTFSZ-1.


B. DNA was transferred bidirectionally from the agarose gels to gene screen plus filters for Southern hybridization analysis. The bottom filters (contain less DNA than top filters) were hybridized with nick-translated pBR322 for samples 1-18 and with nick-translated pSZ218 for samples 19-38. Linear plasmids are identified in the transformant DNA as single or double bands that are a minimum length of 6 kb. Circular plasmids exist as multiple bands in the transformants, ranging in length from <4 kb (covalently closed circles) to >24 kb (multimeric circles). Transformants which contain linear plasmid bands (6-10 kb region) are indicated by arrows at the bottom of the lanes. Size standards are λ/HindIII/EcoRI fragment positions.

C. The filters taken from the top of the agarose gels during bidirectional transfer of the DNA samples were hybridized with a nick-translated Y' region probe, the 1.7 kb BglII fragment from Yrp131B. There are no obvious differences between linear plasmid and circular plasmid containing transformants.
resulted in bands with relatively weak hybridization intensities in the Southern blots (Fig. 26B, lanes 20 - 37). The hybridization intensity of the single copy leu gene in the yeast genome provided a useful internal control, in Southern blots with a LEU probe, for estimating the copy number of the LEU plasmids in the transformants.

In Southern analysis with the pBR322 or pSZ218 probes, a 9.0–9.5 kb band hybridized in transformants YeTFSZ-1 and YeTFSZ-2 that contained the pSZ93 plasmid with Tetrahymena rDNA ends, (Fig. 26B, lanes 18, 21, 22). The estimated length for a linear plasmid in the YeTFSZ transformants was 8.8 kb but the addition of 5'-C1-3A-3' repeat sequence may account for the extra 0.7 kb in plasmid length observed in the transformants (40, 65, 175, 206). Plasmids constructed with TF1 or TF2 ends were present in transformants as linear molecules and in most cases, the length of the plasmids agreed with that estimated for linear monomers (Fig. 26B, lanes 4 - 11, and 24 - 28). In Southern analysis, the pBR322 or pSZ218 probes hybridized with other bands than the monomeric linear plasmids for some transformants, and the length of these bands suggested that they were dimeric linear plasmids (Fig. 26B, lanes 10, 11, 24 - 28). Transformant YeTF1-3-1 (Fig. 26B, lane 24) was an exception since it had predominantly integrated or multimeric plasmid, and a minor DNA band which migrated ahead of the position expected for linear monomeric plasmids (5.5 kb). In all transformants, the lengths of the linear plasmids exceeded the estimated lengths calculated from the combined lengths of the vector and telomeric fragments. Possibly addition of 5'-C1-3A-3' repeat units occurred at the IIIIL ends on the linear plasmids, as described for linear plasmids constructed with Tetrahymena ends (65, 175) or for yeast ends (40, 206).

The transformants that contained the plasmids constructed with pSZ218 and TF3 or TF4 ends, YeTF3-1 and YeTF4-1 transformants respectively (Fig. 26B, lanes 12-16), had multiple plasmid bands that hybridized with the pBR322 probe. This reflected the circular structure of the plasmids, and the multiple bands were the closed circular, open circular, and concatameric forms of the plasmid DNA. Similarly, the plasmids constructed with the LEU2 fragment from pSZ218 and the TF3 end fragments were maintained as circular plasmids in the YeTF3-4 transformants (Fig. 26B, lanes 29 - 31). However, if the plasmid was constructed with the 5'-C1-3A-3' repeat region at only one end of
the LEU2 fragment and the ARS region from the IIIIL telomere (TF3) at the opposite end, some transformants maintained the plasmids as linear molecules (Fig. 26B, lanes 33,35,37). Linear plasmids were observed in some of the YeTF3-5 and YeTF3-6 transformants but these linear molecules were longer than the structures predicted for the plasmids constructions (Table II). The increase in length was presumably due to terminal T region addition at the TF3 end which lacked the terminal 5'-C_3A-3' repeat region. There were also plasmid bands in the YeTF3-5-3, YeTF3-6-1 and YeTF3-6-5 transformants that were longer than the 9.5 kb linear monomer fragments, and these may be due to inefficient resolution of the partial IIIIL telomeres.

The single transformant that was isolated for the plasmid constructed with pSZ218 and TF5 telomeric fragments had the vector integrated in the yeast chromosomal DNA. This was concluded from the high mitotic stability of the LEU in YeTF5-1-1 (Table II), and the Southern hybridization with pBR322 which showed the vector sequences were present in low copy number and the vector had the same mobility as yeast genomic DNA in YeTF5-1-1 (Fig. 26B, lane 17). In Southern hybridization, the DNA from transformants with circular plasmids such as pSZ93 had weak hybridization signals compared to the DNA from transformants with linear plasmids, reflecting the relatively low copy number of circular plasmids (Fig. 26B, lane 23). In previous reports, the linear ARS1 plasmid copy number was estimated as 25 to 50 copies per plasmid bearing cell (140). Differences in the intensity of hybridization signals between the genomic leu2 and the linear plasmid LEU2 region (Fig. 26B, lanes 20 - 37) reflect a similar copy number (25 - 50 copies) for linear plasmids having IIIIL ends. For all of the transformants examined, high mitotic stabilities correspond to linear plasmids (Table II); linear plasmids with IIIIL ends displayed high mitotic stabilities (average 75%) while transformants with circular plasmids containing the IIIIL end region had moderate to low mitotic stabilities (average 20%).
P. Linear Plasmids With Partial III1 Telomeres are Not Stabilized with Y’ Regions

Short linear plasmids that lack an ARS region and have Tetrahymena rDNA ends, or similar linear plasmids that contain a portion of the Y’ ARS region at one end, are stabilized by the addition of Y’ ARS elements to one end of the plasmid in RAD+ yeast strains (65). I asked whether Y’ addition events occurred on the linear plasmids that were constructed with either a complete or partial X ARS region from the III1 telomere in the RAD+ yeast strain A281. Duplicate filters of those hybridized with pBR322 or pSZ218 (Fig. 26B), were probed with a Y’ ARS region probe, the 1.7 kb BglII fragment from YRp131B (48, 49), (Fig. 26C). This Y’ fragment hybridizes with between 20 and 30 regions in the haploid yeast genome (49, 93). In the yeast transformants that contained plasmids constructed with the deletion fragments from the III1 telomere, linear plasmids existed in multiple copies and migrated as extrachromosomal bands in undigested DNA samples (Table II, Fig. 26B). Therefore, healing events that involved Y’ ARS region addition on the ends of the linear plasmids should be obvious in Southern hybridization analysis with a Y’ probe (Fig. 26C). If the linear plasmids were stabilized with Y’ regions at the ends, the Y’ probe should hybridize intensely with the linear extrachromosomal plasmids, but should not hybridize with the circular extrachromosomal plasmids, that were detected with the pBR322 or pSZ218 probes (Fig. 26B). In Southern analysis, the Y’ probe hybridized with only the high molecular weight chromosomal DNA band in the transformants, and this hybridization pattern was similar for the transformants with circular or linear plasmids (Fig. 26C). Variation in hybridization intensities with the Y’ probe for the transformants reflects variation in the amount of DNA in each sample lane rather than varying amounts of Y’ regions in the transformants, since the intensity of UV fluorescence is not uniform for the EtBr stained DNA samples (Fig. 26A). As with the structure of the T-X telomere on chromosome III1, the telomeric fragments isolated from III1 did not require the presence of the conserved Y’ regions for maintaining stable linear plasmids in the yeast transformants.
Q. Structures of the Plasmids with IIIL End Fragments in Yeast

To elucidate the structure of plasmids containing the TF fragments from the IIIL telomere following their replication in yeast, genomic DNA was isolated from selected transformants, DNAs were digested with restriction enzymes and subjected to Southern analysis using pSZ218 as hybridization probe (Fig. 27). The restriction maps for the linear or circular plasmids were deduced from the Southern blots and are represented schematically (Fig. 28, A-G). The plasmids constructed with the vector pSZ218 and the TF1 or TF2 fragments which contain the ARS and 5'-C_1-3A-3' repeat regions from the IIIL end, were maintained as linear monomers or dimers in yeast (Fig. 27A, lanes 3-18, Fig. 28, A&B). The pSZ218 probe hybridized with a single linear plasmid with the expected length for a linear monomer in transformants YeTF1-1-1 and YeTF2-1-1, however YeTF1-1-10 and YeTF2-1-26 contained linear plasmids with the expected lengths for both monomers and dimers. The plasmid constructed with pSZ218 and TF1 or TF2 ends was predicted to exist as a linear monomer, about 8 kb in length (Fig. 25b) which corresponds to the plasmid band at about 8-9 kb in the undigested DNA samples (Fig. 27, lanes 3, 7, 11, 15). For the linear plasmid that exists as a monomer, XhoI digestion results in the removal of one end and Sall digestion cuts the linear plasmid in the central region (Fig. 25b). The 7 kb XhoI fragment and the 4.3 kb to 4.7 kb Sall fragments (Fig. 27) agreed with the predicted structure for a linear monomer, if the length of the TF1 and TF2 fragments was about 1.7 kb (Fig. 28A). Since the TF1 fragment was initially 1.54 kb and the TF2 fragment was 1.23 kb, different extents of T region addition must have occurred at these ends. Such addition reactions would result in equivalent lengths of T region on the linear plasmids and on the end of chromosome IIIL in the yeast genome. This agrees with recent reports showing that the length of the yeast telomeric repeat (T region) is under genetic control (40) and it is similar for all chromosome ends in a given yeast strain (206). The undigested DNA from transformants YeTF1-1-10 and YeTF2-1-26 had plasmid bands that were about twice the length of the linear monomeric plasmids (Fig. 27, lanes 7, 15). This together with the extra Sall fragment that hybridized with pSZ218 in these transformants, indicated that YeTF1-1-10 and YeTF2-1-26 contained dimeric as well as the monomeric linear plasmids. The extra Sall fragment was about 7 kb in length (Fig. 27, lanes 9 & 17).
Figure 27. Determination of the Restriction Maps for the Linear or Circular Plasmids in the Yeast Transformants.

Yeast transformant DNA (2 μg) was digested with restriction enzymes and fractionated on 0.65% agarose gels. After unidirectional transfer to gene screen plus filters, Southern hybridization was with nick translated pSZ218 probe (10^7 cpm/filter).

Sample lanes: (1) pSZ218/SaiI (0.1 μg), (2) pSZ218/EcoRI (0.1 μg), (3) YeTF1-1-1/undigested, (4) YeTF1-1-1/Xhol, (5) YeTF1-1-1/SaiI, (6) YeTF1-1-1/EcoRI, (7) YeTF1-1-10/undigested, (8) YeTF1-1-10/Xhol, (9) YeTF1-1-10/SaiI, (10) YeTF1-1-10/EcoRI, (11) YeTF2-1-1/undigested, (12) YeTF2-1-1/Xhol, (13) YeTF2-1-1/SaiI, (14) YeTF2-1-1/EcoRI, (15) YeTF2-1-26/undigested, (16) YeTF2-1-26/Xhol, (17) YeTF2-1-26/SaiI, (18) YeTF2-1-26/EcoRI.


(35) YeTF1-4-1/undigested, (36) YeTF1-4-1/Xhol, (37) YeTF1-4-1/SaiI, (38) YeTF1-4-1/EcoRI, (39) YeTF3-4-1/undigested, (40) YeTF3-4-1/SaiI, (41) YeTF3-4-1/EcoRI.


Sample lanes 1-18, 19-34, 35-40, and 41-49 are separate agarose gels and Southern hybridization analyses. The positions of λ/HindIII/EcoRI fragment size markers are indicated for each.
Figure 28. Restriction Maps of the Plasmids with Fragments from the IIIL Telomere following Replication in Yeast.

A. Linear monomers were resolved in the transformants that contained plasmids constructed from pSZ218/BgIII and the IIIL telomeric fragments, TF1, TF2, or TF3 (BamHI/EcoRI fragments). Transformants YeTF1-1-1, YeTF2-1-1, and YeTF3-1-4 contain exclusively linear monomeric plasmids.

B. Linear dimers were resolved from the constructions with pSZ218/BgIII and the TF1 or TF2 fragments from the IIIL telomere. The 1.5 - 2.0 kb region at the center of symmetry in the linear dimers must be a partially deleted inverted telomeric dimer as judged from the restriction digestion patterns in Southern analyses (Fig. 27, lanes 7-10, 15-18). Plasmids in transformants YeTF1-1-10 and YeTF2-1-26 exist as both linear monomers and dimers.

C. Circular pSZ218 with inverted telomeric repeat fragments inserted at BgIII was maintained in the transformants indicated, that resulted from plasmid constructions with TF3 or TF4 fragments. Dimeric circles must also be present as determined from the doublet pattern observed with restriction enzyme digestion in the Southern analyses (Fig. 27, lanes 19-22, 27-34).

D. Ligation of TF1 ends on the LEU2 fragment resulted in monomers and dimers of linear plasmids following replication in yeast.

E. Plasmids constructed with TF3 ends on the LEU2 fragment were not resolved into linear molecules in the yeast transformants, as in YeTF3-4-1. The TF3 fragments were maintained as an inverted dimer in a circular plasmid.

F. Resolution from a circular to a linear plasmid occurred for the plasmid constructed with a TF2 fragment on one end of the TF3-LEU2 plasmid in the transformant YeTF3-5-3.

G. Linear plasmid resulted from resolution of TF8 fragment on one end of the TF3-LEU2 fragment in transformant YeTF3-6-5.

Restriction sites: B, BamHI; Bg, BgIII; H, HindIII; R, EcoRI; S, SalI; X, Xhol. Symbols: T region repeat sequence, ([ ]; type X ARS region, (-- --); ARS consensus sequence (•); pSZ218, (-----) LEU2, (-----). Lengths determined for the X and T regions are indicated for each type of plasmid construction in the yeast transformants. Telomeric fragments on linear plasmids were elongated to approximately 1.7 kb except for TF8 which was 0.9 kb, presumably by T region addition to render equivalent T region length on all linear plasmids.
and it must map to the central region of the dimeric linear plasmids in YeTF1-1-10 and YeTF2-1-26 (Fig. 28B). Presumably two linear monomers were ligated together in a head to tail arrangement, and the inverted repeats of the TF1 or TF2 fragments in the central region of the molecule were not resolved to yield two linear monomers upon replication in yeast. Instead, part of the inverted repeat region was deleted, leaving about 2 kb for the 3 kb TF1 inverted repeats in YeTF1-1-10 or for the 2.4 kb TF2 inverted repeats in YeTF2-1-26. The other 5 kb in the 7 kb Sall fragment is the pSZ218 vector (Fig. 28B). A deletion in the inverted repeats of the telomeric fragments would explain the incomplete resolution of the plasmids in transformants YeTF1-1-10 and YeTF2-1-26 that resulted in both linear monomers and dimers.

The TF3 fragments had the entire ARS region from the IIIIL end but none of the 5'-C1-3A-3' repeat region (Fig. 25a), and when ligated to pSZ218 resulted in plasmids that were replicated as linear as well as circular molecules in yeast (Fig. 27, lanes 19-26 and Fig. 28, A & C). In transformant YeTF3-1-1 (Fig. 27, lanes 19-22), circular monomers were present as indicated by the 7.5 kb XhoI fragment and the 7.5 kb Sall fragment which are the expected size for a plasmid containing pSZ218 (5 kb) and two TF3 fragments (2.36 kb), (Fig. 25a,b). Apparently, there was also a population of circular plasmids that contained a 1 kb deletion, likely in the inverted repeat region of the TF3 fragments, to result in the 6.5 kb XhoI fragment and the 6.5 kb Sall fragment in YeTF3-1-1. The 4.4 kb Sall fragment that is heterodisperse may result from the generation of a Sall site during the proposed deletion event in the TF3 region or alternatively the 4.4 kb Sall fragment may reflect the resolution from circular to linear plasmids in some YeTF3-1-1 transformants (Fig. 27, lane 21). Transformant YeTF3-1-4 also contained plasmids constructed with pSZ218 and TF3 end fragments, however only plasmids maintained as linear monomers were observed in YeTF3-1-4 (Fig. 27, lanes 23-26). As described for transformants YeTF1-1-1 and YeTF2-1-1, a single 8-9 kb plasmid band was present in the undigested sample and a 7 kb XhoI fragment and the 4.3-4.7 kb Sall fragments agreed with the predicted map for the linear plasmids (Fig. 25b, 28A). The lengths of the XhoI and Sall fragments indicate that the TF3 fragments were increased from 1.18 kb to the 1.7 kb ends on the linear plasmid, similar to the addition events described for the TF1 and TF2 fragments.
Presumably, the extra 0.5 kb on the TF3 fragments is T repeat sequence, which resulted in the stabilization of the linear plasmid in YeTF3-1-4. This speculation, however, has not been tested by Southern hybridization with a T region probe. If the addition is T region, it may be required for the efficient resolution of circular plasmids and maintenance of linear plasmids in yeast since the telomeric fragments which lacked the T region (TF3) existed as circular molecules in YeTF3-1-1 but as linear molecules with additions to the ends in YeTF3-1-4. Similarly, the telomeric fragments that contained a portion of the T region, such as TF1 (356 bp of T region) or TF2 (49 bp of T region) were present exclusively on linear plasmids. Plasmids constructed with pSZ218 and the TF4 fragments, which had only 919 nt of the X region and no T region (Fig. 25a), were maintained exclusively as circular molecules in the transformants (Fig. 27, lanes 27-34, Fig. 28C). The 7 kb Sall and Xhol fragments are expected for circular plasmids constructed with pSZ218 (5 kb) and inverted TF4 repeats (1.84 kb), (Fig. 28C). The 6 kb plasmid fragments in Sall or Xhol digestions may be circular plasmids that contain a deletion in the TF4 Inverted repeat region (Fig. 27, lanes 28, 29, 32, 33). For each of the constructions with pSZ218 and the TF fragments, a deletion event in the inverted repeat region of the telomeric fragments has been proposed to explain the extra bands observed in the Southern blot. Presumably, an Inverted repeat structure is unstable for telomeric repeats in yeast and a deletion event rather than a resolution event may occur in this region especially if there is no T repeat region in the telomeric fragments.

Plasmids were constructed with only the yeast LEU2 gene fragment from pSZ218 and the IIII end fragments (Fig. 25c, d) and these were replicated as linear molecules in yeast if at least one end of the plasmid contained the 5'-C\_3A-3' repeat region and one had an ARS region. The LEU2 fragment with TF1 end fragments was maintained as linear monomers and dimers in transformant YeTF1-4-1 (Fig. 27, lanes 35-37 and Fig. 28D), and the TF1 ends were lengthened from 1.54 kb to 1.7 kb on the linear plasmids, as observed for pSZ218 with TF1 end fragments in YeTF1-1-10. The length of the TF1 end was heterodisperse following replication in yeast, as evidenced by the smeared 5-6 kb band in the undigested DNA which was the linear plasmid monomer (Fig. 27, lane 35). Sall digestion removes one TF1 end from the linear monomer and bisects the linear dimer (Fig. 28D), resulting in a
heterodisperse 3.6-4.1 kb fragment (Fig. 27, lane 36) that includes the 2.2 kb LEU2 fragment plus a TF1 fragment. Presumably heterogeneity of the TF1 fragment length 1.7 kb (+/-0.2 kb), equivalent to that on the end of chromosome III, is the explanation for the heterodisperse lengths observed for the plasmid in YeTF1-4-1. The plasmid constructed with the LEU2 fragment and the TF3 telomeric fragment was maintained as a circular monomer which contained the TF3 inverted repeat in transformant YeTF3-4-1 (Fig. 27, lanes 38-40, and Fig. 28E). The low copy number for circular plasmids versus linear plasmids in yeast is evident in the reduced intensity of pSZ218 hybridization for YeTF3-4-1 compared to YeTF1-4-1 (Fig. 27, lanes 35-40). If the plasmid was constructed with a 5'-C1-3A-3' repeat region at only one end of the LEU2 plasmid, such as with the TF2 or TF8 fragments (Fig. 25a, d), plasmids were maintained as linear monomers and dimers in transformants YeTF3-5-3 and YeTF3-6-5 (Fig. 27, lanes 43-49 and Fig. 28, F&G) however circular plasmids remained in transformants YeTF3-5-1 and YeTF3-6-1 (Fig. 27, lane 42). To establish whether addition to ends of the linear plasmids had occurred in YeTF3-5-3 and YeTF3-6-5, the lengths of the terminal fragments on the linear plasmids were estimated through restriction enzyme digestions which remove one of the two ends. The plasmid in YeTF3-5-3 (Fig. 28F) was constructed using the LEU2 fragment (2.5 kb), a TF3 end (1.18 kb) and a TF2 end (1.23 kb). Sall digestion removes both ends from the 5.5-6.0 kb linear plasmid leaving the 2.5 kb vector fragment (Fig. 27, lane 44) and digestion with HindIII removes only the TF2 end, leaving a 4.2-4.4 kb fragment containing the LEU2 fragment plus a TF3 fragment (Fig. 27, lane 45). Consequently, both TF3 and TF2 ends must have been increased to an average length of 1.7 kb, and addition reactions at the ends presumably resulted in the heterodispersity observed for the length of the linear monomeric plasmid (Fig. 27, lanes 43). Similar characterization of YeTF3-6-5, which contained the LEU2 fragment with TF3 and TF8 end fragments, indicated that addition had occurred at the ends of the linear plasmid resulting in a 1.7 kb TF3 end and a 0.85 kb TF8 end (Fig. 27, lanes 47-49, Fig. 28G). It is interesting that the TF1 and TF8 fragments which initially had equivalent T region lengths (0.36 kb) were both lengthened by about 0.15 kb on the linear plasmids in yeast, regardless of the X region differences for the TF1 and TF8 fragments (Fig. 25A). The similar addition of DNA to fragments that initially had equal T region
but contrasting X region extents, provides evidence for the assumption that additions to the TF1 and TF8 end were T region and not X ARS region sequences.

The length of T repeat region was maintained at 0.5 kb (+/- 0.2 kb) at both ends of the various linear plasmids in the transformants that were characterized. Therefore, the addition of T region to the linear plasmids was regulated to maintain the same average length on all ends which supports the idea that the telomeric repeat length at chromosome ends is regulated genetically (40, 206). It was not determined whether the 30 bp of M13 sequence present initially on the end of each telomeric fragment had been removed during this process of telomere addition. Its presence did not appear to hinder the resolution of circular to linear plasmids, since plasmids with TF1 or TF2 ends were maintained exclusively as linear molecules. The extra 60 bp at the center of the inverted repeat may have been one reason for the inappropriate resolution of linear dimers to monomers in some transformants (YeTF1-1-10, YeTF2-1-26, YeTF1-4-1).
DISCUSSION

A. Chromosome Walking

In this study the telomeric region from the left end of yeast chromosome III was isolated by chromosome walking from the distal genetic marker, HMLα, to the chromosome III L end. This telomeric region was functional in replicating a plasmid as a linear minichromosome in yeast. This approach for isolating the III L telomere is analogous to that used in cloning the first yeast centromeric region, which was isolated from chromosome III (51, 53). The first yeast telomere that was molecularly characterized was isolated by the alternate approach, in which selection was for the function of the telomere in stabilizing a linear plasmid in yeast (193). The fragments that exhibited the functional characteristics of telomeres, were mapped to the ends of chromosomes by Southern hybridization, however the chromosomal origin of each telomeric fragment remains unknown.

Chromosome walking offered the advantage of identifying a given telomeric region with a specific chromosome arm. In addition, the isolation of a telomere by this method allowed the molecular characterization of a specific end structure and comparative studies with other telomeric regions in the yeast genome. The problem with this method was the unknown distance separating the telomere from HMLα on chromosome III L. The genetic map distance on chromosome III is about 130 cM as estimated from the yeast genetic linkage map (107, 136, 146). Equating physical and genetic map distances on chromosome III at 2.7 kb/cM (186), the physical length of chromosome III was calculated as 350 kb between the distal genetic markers HMLα and MAL2, on III L and III R respectively. Yeast karyotype analysis, which could estimate the physical length of chromosome III, was necessary to establish the amount of DNA on chromosome III that is unaccounted for in the genetic map which presumably consists of the regions distal to HMLα and MAL2. Due to the lack of condensation of yeast chromosomes, (69, 78) a useful karyotype was not available until recently when an electrophoretic karyotype for yeast was established with the physical lengths of yeast chromosomes being estimated using orthogonal-field-alternation gel electrophoresis, (OFAGE), (36, 37, 173). Chromosome III was estimated to be about 370 kb in length on OFAGE gels (36, 37). Since the genetic map accounts for about 350 kb, about 20 kb must include both the distance from HMLα to the III L terminus and the
distance from \( \text{MAL2} \) to the \( \text{IIIR} \) terminus. Had the relatively small length between \( \text{HML0} \) and the telomeric region on \( \text{IIIL} \) been known at the outset, the negative results obtained for chromosome walking experiments could have been interpreted.

The first problem was that the screening of many yeast genome equivalents in the phage DNA library failed to yield clones from the chromosome \( \text{IIIL} \) region that were more than 10 kb distal to \( \text{HML0} \). The lack of clones from the \( \text{IIIL} \) distal region in yeast genomic libraries could be attributed to a nonrandom distribution of insert fragments in the \( \lambda \text{Ch4A-yeast} \) or \( \lambda \text{MG14-yeast} \) DNA libraries, or to the proximity of a region on chromosome \( \text{IIIL} \), possibly the telomere, that cannot be cloned in phage or circular plasmid vectors. The latter of these two possibilities was shown to be correct. The distance from the \( \text{IIIL} \) telomere to the most distal probe on \( \text{IIIL} \) that was isolated from the phage clones was only 2.3 kb (\( +/−0.2 \) kb) and was determined through \( \text{Bal31} \) nuclease digestion of chromosomal DNA. \( \text{Bal31} \) nuclease is specific for the ends of chromosomal DNA molecules in the genome, hence telomeres yield shorter restriction fragments when obtained from chromosomes that have been digested progressively with \( \text{Bal31} \) nuclease while fragments from the internal regions on these chromosomes are of constant length (18, 24, 60, 175, 203, 206, 209). The 1 kb EcoRI/Sall fragment on \( \text{IIIL} \) which was 8.6 kb distal to \( \text{HML0} \) (probe 2*) was unique in the yeast genome and hybridized to \( \text{Bal31} \) nuclease sensitive \( \text{IIIL} \) fragments. Further evidence that this \( \text{IIIL} \) distal probe (probe 2*) was identifying restriction fragments which contained the \( \text{IIIL} \) telomere was the heterodisperse length of fragments that hybridized with probe 2* for some of the restriction enzyme digestions. Previous studies using non-unique telomeric probes from yeast chromosomes demonstrated the length heterogeneity of telomeric fragments, attributed to variable amounts of simple repeat sequences that are present at chromosome ends (18, 24, 40, 49, 60, 93, 175, 193, 203, 206, 209). However, length variability in individual chromosome ends could not be distinguished in those reports since the telomeric probes were not chromosome specific. In defining the role of telomere elongation in replicating yeast chromosomes, it is essential to determine events at a defined telomere, as in the case of trypanosome telomeres (18, 60, 209). The unique probe on chromosome \( \text{IIIL} \) (probe 2*) that hybridizes with the \( \text{IIIL} \) telomere demonstrated that this chromosome end varies in length in an
asynchronous cell population, since it hybridized to IIIIL terminal fragments that had a size distribution of $+/-0.2$ kb. Telomere addition and elimination reactions with the terminal 5'-C$_1$-3A-3' repeat sequences during DNA replication are presumably the cause of the length variability on chromosome ends in yeast (40, 65, 175, 193, 195, 205, 206). The IIIIL telomeric region that was isolated in the MTLB6S12 clone contained 356 bp of 5'-C$_1$-3A-3' simple repeat sequence. The length of this T region on the end of chromosome IIIIL in the AB20 strain XP8-10 genome was estimated by Southern hybridization with restriction enzyme digests of chromosomes to be 0.6 kb ($+/-0.2$ kb). Therefore between 50 bp to 400 bp of repeat sequence was deleted from the IIIIL end with Bal31 nuclease during cloning. If the length of the T region at yeast telomeres is in a constant state of flux with addition and elimination reactions during DNA replication, the deletion of 50 to 400 bp from the T region on IIIIL leaving 356 bp of T region, should not have a detrimental effect on telomere function. This shown to be the case since the telomeric region from IIIIL in MTLB6S12 provided a functional telomere on linear plasmids in yeast. This was also indirect proof that the deleted 50-400 bp region from the IIIIL end consisted of additional T region sequence (5'-C$_1$-3A-3'), which is predicted to extend to the terminus of yeast chromosomes (40, 49, 65, 175, 206).

B. Chromosome IIIIL End Is a T-X Class Telomere

Yeast chromosome ends are distinguished by the complex repeat regions referred to as X and Y' that are proximal to the T region which consists of simple 5'-C$_1$-3A-3' repeat units at the terminus of the chromosome. The telomere associated X and Y' regions represent a family of ARS regions that are thought to be origins of replication in yeast (47, 48, 49). The majority of yeast telomeres have a type X and between one to four type Y' ARS elements and are referred to as the T-Y'-X class. The second class has a single type X ARS region and is referred to as T-X telomeres. In T-Y'-X class, the T region separates the X and Y' regions as well as terminates the chromosome (49, 93, 205, 206). Yeast telomeres that were previously isolated represent the T-Y'-X class (49, 193). The T-X class has been postulated to exist because of the results obtained from Southern hybridization with restriction enzyme digestions of yeast chromosomes (93, 175, 205, 206), but no example has been isolated until this report.
The proximity of the IIIL terminus with the unique region on the end of chromosome IIIL (probe 2*) was the first indication that the IIIL telomere represented the T-X class. Telomeric regions of the T-Y'-X class are apparently longer than 7.5 kb (49) whereas the IIIL telomeric region was confined to a region that was less than 2.3 kb. Further characterization by Southern hybridization with previously isolated X and Y' region clones (48, 49) showed that the cloned telomeric region from chromosome IIIL contained homology with the X ARS region, but not the Y' ARS region. The X region on IIIL was localized to the 1.2 kb region adjacent to the telomeric 5'-C1-zA-3' repeats, with an ARS consensus sequence in the center of the X region. Although the type X ARS regions are heterogeneous in restriction enzyme sites and in length, they are defined as X regions by their homology with one another (48, 49). It is assumed that all yeast telomeres contain an X region (49, 205), hence the X region from the end of chromosome IIIL should hybridize to each of the 34 chromosome ends in the haploid yeast genome. In Southern analysis of chromosomal DNA, numerous fragments were homologous with the X region probe and no additional fragments were detected with the T-X region probe from chromosome IIIL. Thus, all telomeres that contain a T region must also contain an X region. It was not possible to distinguish X region fragments corresponding to each chromosome end in the haploid yeast genome. Perhaps the telomeric fragments from some of the chromosomes had identical lengths or at least sizes that were too close to be separated by the conditions used for fractionation by electrophoresis. Bal31 nuclease sensitivity assays confirm that all fragments homologous to the X region on chromosome IIIL are telomere proximal, and at least 30% of the chromosome ends in the yeast genome are of the T-X class (49, 205, 206).

C. Heterogeneity of the Length of the IIIL Telomere Among Yeast Strains

Restriction enzyme site and length heterogeneity of the telomere associated Y' region and length heterogeneity of the terminal T region has been observed in Southern hybridization analysis of the telomeres in various yeast strains (40, 92, 93, 206). The length of the terminal fragments from T-X class telomeres was heterogeneous among yeast strains, as observed by Southern hybridization with the IIIL telomeric probe. The differences observed in the fragment lengths for the T-X telomeres were attributed to variation in the length of the T region and not in the type X ARS region. For the T-X
that were identified using the probe from the III L end, heterogeneity among strains was obvious in the length of the terminal restriction fragment. In any given yeast strain, the length heterodispersity of the terminal fragment on a T-X telomere was limited in size range (+/- 0.2 kb), but the average length of this terminal fragment also varied by (+/-0.2 kb) among the strains examined. Recently, the length of the terminal T region was shown to be genetically controlled and it appears that regulation is by more than one genetic locus (40, 206). Possibly the variation observed for the average length of the terminal fragment on a given chromosome end in different yeast strains is a consequence of the genetic cloning of a particular T region length for a given chromosome end in one strain. This T region length then determines the average length for the telomere, with addition and elimination reactions at this end resulting in variation for the T region length within the particular strain. Genetic regulation of the addition and elimination reactions would explain the conservation of the average length, and the limited variability (+/- 0.2 kb) of the average length.

Among different yeast strains, there are some examples of restriction-fragment-length-polymorphisms on chromosomes that are homologous with the X region probe from the III L end. However, these variations occurred with fragments that mapped to the T-Y'-X class of telomeres. Alternatively, the fragments that mapped to T-X telomeres had variation in the length of the T region and conservation of the length of the X region. This may reflect the requirement for a conserved X region at T-X telomeres, since it provides the ARS function, while the X region at T-Y'-X telomeres may vary since the ARS function may be from the Y' region. Furthermore, Southern hybridization with the T-X probe from the III L telomere indicated that the ratio of T-Y'-X to T-X class telomeres is maintained in the genomes of the different yeast strains. Perhaps this ratio is essential for proper telomeric associations and chromosome segregation. The reported changes in the number of Y' regions in different strains and in the meiotic segregants of a given strain (93) possibly reflect variations at the T-Y'-X class of telomeres rather than conversion events between T-X and T-Y'-X telomere classes.

Recently, Surosky and Tye constructed a telocentric chromosome III in a diploid yeast strain by deleting 80-100 kb from the left end of chromosome III (190). The deletion on chromosome III L was thought to result from a double reciprocal recombination event between chromosome III and a
short fragment, containing both a portion of the Y' ARS region and a LEU2 or CEN3 region, that was introduced into the yeast strain 3285 by transformation. It was assumed that deletion of the region between LEU2 or CEN3 and end of IIIIL occurred by replacement recombination with the short fragment. However, there is no Y' region on the end of chromosome IIIIL if yeast strain 3285 is like those examined by Southern hybridization analyses with the telomere proximal fragment (probe 2*) that is unique to IIIIL. Instead, the alternate explanation for the production of the telocentric chromosome III was more probable (190); deletions were generated by a single recombination event between LEU2 or CEN3 on the introduced fragment and LEU2 or CEN3 on chromosome IIIIL. This event would result in the replacement of the region distal to LEU2 or CEN3 on IIIIL with the partial Y' region on the introduced fragment. Presumably, this partial Y' region on IIIIL was functionally healed with a Y' region from a T-Y'-X chromosome end. This explanation also agrees with the proposed function of Y' regions in providing stability to broken chromosome ends (65). The alteration of the telomere associated region from X to Y' on IIIIL in the telocentric chromosome III strain does not significantly affect the mitotic stability of chromosome III but it does result in slight defects in meiotic pairing and segregation (190). This suggests that the telomere associated regions, X and Y', are at least partly responsible for telomeric associations and the proper segregation of chromosomes in meiosis (49).

D. Requirements for a Functional Telomere in Yeast

The termini of Tetrahymena rDNA function as telomeres in yeast as shown by cloning the rDNA ends on linear plasmids, then selecting for the plasmid marker in yeast (193). A yeast telomere was isolated by the replacement of one Tetrahymena end on the linear plasmid with a yeast fragment that displays telomeric function (193). Characterization of replication intermediates for yeast chromosomes (195), studies on yeast chromosome mechanics and stability (56, 88, 139, 140), as well as the healing events that occur at chromosome ends in yeast (65, 215) were conducted in yeast using linear plasmids which had termini from Tetrahymena, Oxytricha or S. cerevisiae. Similarly, to establish that the cloned chromosome IIIIL telomere contained the essential information for telomere function, the IIIIL end fragment was cloned on linear plasmids in yeast.
Plasmids constructed with the 1.2 kb type X ARS region from IIII along with 49 bp or 356 bp of T region (5'-C1-3A-3' repeats) at the termini of the plasmid, were replicated exclusively as linear molecules in yeast. The ligation mixture of circular and linear plasmids was introduced into yeast by genetic transformation, and the plasmids were resolved into linear molecules in the yeast, whether the vector portion of the plasmid was the entire pSZ218 plasmid (194) or a LEU2 fragment from pSZ218 (2, 65). The mitotic stability of linear plasmids with IIII termini was about 75% plasmid bearing cells while the stability for linear plasmids which contained the ARS1 region, which is a strong origin of replication in yeast (184), and termini from Tetrahymena rDNA (194, 195) was only 40% plasmid bearing cells. The doubling of the mitotic stability for linear plasmids with yeast telomeres rather than Tetrahymena ends may reflect the cellular recognition of yeast ends, the preferred association with yeast telomeres for the linear plasmids with yeast chromosome ends, or to the efficient ARS activity in the X region of the yeast IIII ends. In addition, the ARS regions at yeast ends (X or Y regions) may stabilize these minichromosomes due to their interaction with a cellular factor (124). Since the isolated T-X region from chromosome IIII allowed the resolution and replication of linear plasmids in yeast that had high mitotic stabilities for the LEU plasmid marker, the terminal region on the IIII end that was deleted with Bal31 nuclease for cloning in a circular vector, was not essential for a functional telomere. Presumably this 50 bp to 400 bp terminal region on IIII consisted of additional T region sequences (5'-C1-3A-3' repeats) that extended the 356 bp of T region that was present on the isolated IIII end.

At least a portion of the T region is required for the recognition of telomeric fragments in circular plasmids and the resolution of circular plasmids to linear molecules in yeast. Deletion of the entire 5'-C1-3A-3' repeat region from the T-X fragment, leaving the 1.2 kb X region from IIII on the telomeric fragments, resulted in the replication of circular plasmids in yeast for most transformants. However, one yeast transformant did contain linear plasmid molecules for the plasmid that was constructed with X region termini. Presumably, the stabilization of this plasmid was the result of a recombination event in yeast between X regions on the plasmid and on a chromosome that resulted in T region addition at the termini of the plasmid. Deletion of the T region plus the adjacent 200 bp from
the X region on the III1 end resulted in a fragment that was not recognized as telomeric and hence only circular plasmids containing this deletion were replicated in yeast. The distal portion of the X region (i.e., X-T border region) appeared largely conserved among X regions as determined by Southern hybridization analysis with deletion probes from the X region or by comparison of the restriction maps for different X regions (48, 49). Perhaps the elimination of the distal portion of the X region removed a region that was essential for homologous recombination with chromosomal X regions and prevented the subsequent addition of the T region required to stabilize linear plasmids. The transformation frequency of circular plasmids that were constructed with X regions that had the distal 0.2 kb portion deleted was equivalent to that observed for linear plasmids with the full length X region from the III1 end. Consequently, it was estimated that the distal domain of the ARS region on the III1 end (42, 182) was in the distal 0.2 - 0.4 kb of the X region. Deletion of more than 400 bp from the distal end of the X region produced few or no transformants, presumably since the deletion interfered with the ARS function. Plasmids constructed with the T region on just one end of the plasmid and the III1 ARS region on either one or both ends were replicated and stably maintained as linear molecules in yeast, for some transformants. These linear plasmids were constructed with the T region at only one end of the plasmid, however T region addition apparently occurred at both ends of the linear molecule during replication. The plasmids constructed with an X region at only one end of the plasmid did not acquire additional X or Y' regions during replication in yeast. On these short linear plasmids that are replicated in high copy number, there is no requirement for proper segregation like there is for natural chromosomes in the yeast cell. Therefore, a telomere associated region may not be needed at both ends of the linear plasmids, whereas all evidence to date indicates that all chromosome ends in yeast have telomere associated regions. The T region was present at the ends of all linear plasmids, which reflects its essential role in replication and in stabilization of the ends of linear molecules.

E. The III1 End Is Stabilized with T Region but without Y' Region Addition

Linear plasmids that had III1 telomeric fragments at the termini had the T regions lengthened in yeast by a yet uncharacterized addition reaction (40, 49, 65, 175, 206) such that the average length of the T region on the linear plasmids was equivalent to that on the natural III1 end. This may
reflect the genetic control of the length of terminal fragments on chromosome ends which agrees with previous reports showing the genetic variation of T region lengths between parental strains and their meiotic segregants or among various yeast strains (40, 206). A linear plasmid that was constructed with a defined length of the IIIIL telomeric fragment had a heterodisperse length for the terminal fragment following its propagation in yeast which provides further evidence for telomere addition and elimination events at yeast telomeres (18, 175).

Telomeres are stabilized or “healed” through the addition of Y' ARS regions and T regions at the ends of linear plasmids in yeast if the plasmid contains only weak ARS regions, such as those on *Tetrahymena* termini (1, 65, 105, 195, 215) or if the plasmid has only a partial Y' region (65, 190). There was no evidence for the addition of Y' regions on the ends of the linear plasmids constructed with IIIIL fragments, using either complete or partial X ARS regions as telomeres. If interaction or recombination did occur between the X region from the IIIIL telomere that was on the linear plasmids and the telomeres on yeast chromosomes, it must have been with the T-X class and not the T-Y'-X class of yeast telomeres. The ARS region on the end of chromosome IIIIL must be a highly active replication origin on linear plasmids, and consequently Y' ARS regions were not added to the IIIIL termini since this would not enhance the stability of the linear plasmids (65). The IIIIL end fragments which had deletions extending into the X ARS region resulted in few viable transformants and these were not healed with Y' or X ARS regions. This lack of recombination events at the partial IIIIL ends is reasonable since recombination with genomic DNA in yeast is enhanced only when broken ends, highly homologous with chromosomal DNA, are introduced into yeast on a linear fragment (149, 150, 196). However, the plasmids with X region deletions did not conform to this arrangement since: (1) The majority of the plasmid DNA was circular in the ligation mixes used for the genetic transformations, and these remained as circular plasmids in yeast since the absence of a T region precluded the resolution of circular to linear molecules; (2) Sequence heterogeneity exists among X regions, especially on the centromere proximal side of the ARS region (48, 49); (3) X and Y' regions share relatively no homologous regions for recombination events to occur between them (48, 49).
F. Why are There Two Classes of Telomeres Maintained in Yeast?

If an X ARS region provides the requirement for a telomere associated region without the Y' region, then for the simplest arrangement of chromosome ends in yeast, one would expect all telomeres to belong to the T-X class. The existence of two classes of telomeres in yeast may be due to the requirement for a strong origin of replication at the telomere to initiate replication at the end of the chromosome (49). The type of telomeric arrangement would therefore depend on the ARS activity in the telomere associated region. If the ARS in the X region provides the necessary function, then no additional Y' region sequences are required. However, should the ARS in the X region suffer mutations, deletions, rearrangements, or other damaging events, the Y' ARS regions would provide the ARS activity at the telomere, as observed by their role in healing telomeric regions (65). Addition of Y' regions to inactivated T-X class telomeres would possibly occur by recombination between the distal T region on the T-X telomere and the internal T region (between Y' and X regions) on the T-Y'-X class telomere (65, 205). The telomere healing experiments conducted by Dunn et al. (65) support these ideas. In these studies, chromosome ends were healed by recombination with Y' regions if a partial Y' region (no X region) was at the end of a linear plasmid or if the Tetrahymena rDNA ends (weak ARS regions) were used as termini. Addition of Y' regions to Tetrahymena rDNA termini occurred by recombination with the T region that separates the X-Y' regions on T-X-Y' telomeres, similar to the mechanism proposed for the healing of T-X telomeres with Y' regions. Alternatively, a strong origin of replication (ARS1) at the plasmid end precluded Y' region addition. In the same manner, the T-X telomeres may have sufficiently strong ARS activity in the X region such that further Y' region activity is not essential.

The ability of yeast ARS regions to mediate high frequency transformation in yeast is affected by domains surrounding the ARS consensus sequence (42, 104, 182). Different sequences surround the various yeast ARS regions that have been isolated (34, 42, 104, 184, 202), hence these domains cannot be defined in a general manner with respect to DNA sequence requirements. Because point mutations within the ARS consensus sequence destroy ARS activity (42, 104, 182), this sequence must be conserved in Y' or X regions for a functional origin of replication. The X regions isolated
previously (48, 49) belong to the T-Y'-X telomere class since all clones contain the 131 region which forms part of the Y' region (49). Conversely, the X region on the IIII end, the only T-X telomere isolated to date, had no homology with the 131 region. Comparison of the restriction enzyme maps for the X regions indicates they are heterogeneous in length, restriction map, and extent of the ARS region. Perhaps the ARS activity in these X regions also displays such variability. Variation in the stability of the ARS for X regions from T-Y'-X telomeres was demonstrated in mutant strains defective in minichromosome maintenance, whereas the stability of the Y' ARS region was conserved for different Y' regions on the minichromosomes in these mutant strains (124). Different yeast strains were apparently polymorphic at X regions belonging to T-Y'-X telomeres and conserved at T-X telomere associated regions. This may reflect the requirement for ARS function in the X region of T-X telomeres. The Y' region may provide ARS activity at T-Y'-X telomeres and hence there is less selection pressure for X region maintenance at such telomeres.

The repetition of Y' regions on some telomeres (maximum of 4), (49) may be the result of gene conversion or other recombination events between the homologous Y' regions of different telomeres. The complex direct repeat sequences that are conserved in the Y' region (92) are thought to be involved in the interaction of different Y' regions. Perhaps the variable number of Y' regions on yeast telomeres has a regulatory role in replication, such as control of replication initiation points in the cell cycle (49, 65, 70), or in the telomeric associations important in meiotic pairing and segregation events (25, 35, 49, 55, 91). Alternatively, the multiple nature of Y' regions may be a consequence of rather than a cause for telomeric associations. Possibly conversion events between Y' regions resulting in the addition of Y' regions at the ends of chromosomes by a RAD52+ dependent mechanism, (65) maintains the DNA sequence of the Y' ARS region which ensures that a functional ARS region is present at the end of the chromosome. The finding that Y' regions vary substantially in number among yeast strains for T-Y'-X telomeres (93) suggests that there is no control mechanism for the number of Y' regions at a given telomere, and the only requirement may be the presence of at least one functional ARS region. Further support for the idea that a functional origin of replication is essential at the telomeric region is obtained from the studies conducted on heterologous telomeres
which also stabilize linear plasmids in yeast. These heterologous telomeres may also contain ARS regions that are functional in yeast, as described for Tetrahymena rDNA termini (1, 25, 65, 105, 156, 193). To establish that a region with strong ARS activity is required at chromosome ends, linear plasmids could be constructed with T-X termini, using the X region derived from a T-X telomere for one plasmid and the X region from a T-Y'-X telomere for a second plasmid construction. After replication of these plasmids in yeast, transformants would be screened by Southern hybridization to discern whether Y' regions are used to repair the T-X telomeres that were constructed with X regions from T-Y'-X telomeres. The linear plasmids constructed with T-X termini from T-X telomeres should not have Y' addition at the ends, if these are like the linear plasmids constructed with the T-X telomere from chromosome III. Differences in the mitotic stability of linear plasmids with different X ARS regions could be detected using the colony color sector assay test (88) which can distinguish chromosome nondisjunction and chromosome loss events. Similarly, the effect that additional Y' regions on the ends of the linear plasmids has on plasmid stabilities could be assayed in this manner. Cloning an X region from either a T-X or T-Y'-X telomere on a linear plasmid in yeast, followed by comparison of the replication patterns, mitotic stabilities, and telomere modifications of the T-X termini would demonstrate any differences in the ARS activities for X regions of the two telomere classes. These results would indicate whether conversion between the T-X and T-Y'-X telomere classes can occur on specific chromosome ends in yeast.

6. How are Yeast Telomeres Replicated?

Simple repeat sequences exist at all eukaryotic chromosome ends that have been sequenced (20, 24, 67, 98, 102, 113, 147, 175, 203, 212) and these adhere to the general formula 5'-[C\textsubscript{1-6}(A/T)\textsubscript{1-4}]-3' (23). Although the sequence of the repeat units is divergent in heterologous systems, the function of this T region is maintained. Both Tetrahymena (5'-C\textsubscript{4}A\textsubscript{2}-3') repeat units, (56, 65, 139, 140, 193, 194, 195) and Oxytricha (5'-C\textsubscript{4}A\textsubscript{4}-3') repeat units, (156) are functional as telomeres on linear plasmid vectors in yeast, although yeast chromosomes have 5'-C\textsubscript{1-3}A-3' repeat units at the T region of the telomeres (175). In forming the macronuclear genome from micronuclear fragments in ciliates such as Tetrahymena or Oxytricha, the repeat
sequence must be added to the ends of the linear fragments (22), but the addition mechanism is not yet defined. Similarly, addition of T region sequences occurs on chromosome ends in yeast. The yeast 5'-C1-3A-3' repeat units are added to both Tetrahymena and Oxytricha termini (156, 175) and to the deleted IIII telomeric fragments on linear plasmids in yeast. Further evidence for terminal addition and elimination events at telomeres in yeast is the heterogeneous length of the terminal fragment from chromosome IIII observed on the chromosome or on linear plasmids in yeast. Presumably, simple repeat sequences are routinely added to or deleted from telomeres in replication (18, 65, 175, 203). Heterogeneity of the length of the terminal restriction fragment on yeast chromosomes suggests that a solution for the problem of completing chromosome ends in DNA replication (208) must only provide a mechanism for maintaining an average length for the telomere rather than the specific length of the parental DNA strand. Consequently, the gap resulting from removal of the 5' terminal primer in DNA replication may remain incomplete and produce a shorter telomeric repeat length on the progeny chromosome. Simple telomeric repeat sequences presumably conserve the average length through an undefined addition reaction. Models proposed for the addition of repeat region sequences at chromosome ends in DNA replication involve either a recombination mechanism (17, 77, 87, 91, 203, 205) or a novel terminal transferase-like enzyme activity (80a, 175, 195). However, neither alternative fully explains the addition events at chromosome ends since the model must accommodate the following: (1) heterodispersity of terminal fragment length, (2) addition of T region repeats but maintenance of the telomeric length within a given range, (3) terminal single-stranded breaks, (4) inability to label or ligate the terminus, (5) addition of the T region to heterologous telomeric repeats, (6) a RAD52 independent mechanism is responsible for addition of the 5'-C1-3A-3' repeat sequences to the linear plasmid ends (40, 65, 215).

If the average length of the T region at the chromosomal end is maintained via recombination between the simple repeat units, then recombination must occur by intrachromosomal or interchromosomal events that are RAD52 independent (65, 215). Both an intrachromosomal loop-back model (203) and a model that involves recombination between the T region on all chromosomes (205) have been proposed. In addition, unequal sister chromatid exchange which occurs by a RAD52
independent mechanism (157, 192, 196, 203, 216) may contribute to the observed length heterogeneity of telomeres. Deletion and elongation events that occur at the terminal simple repeat units are equivalent to the sister chromatid exchange events that occur within the repeat units of yeast ribosomal DNA (192). The length variation of the terminal fragment on the yeast chromosomes is within a given range (+/- 0.2 kb) which could indicate that chromatid pairing occurs in an ordered manner, and the exchange processes are regulated to maintain an average terminal length. Cross-over events in the loop-back model (203), interchromosomal recombination model (205), or unequal sister chromatid exchange model may be initiated at the single-stranded gaps near the terminus of the chromosome. Possibly, the regulation of a telomere specific endonuclease may simultaneously regulate the extent of cross-over events between chromatids. Activity of the CDC17 gene product may fulfill this role since it prevents the telomeric region from continually elongating (40). Reduced amounts of CDC17 gene product results in the length of the average telomere being increased every generation. Perhaps fewer single-stranded gaps are present at the termini with reduced amounts of CDC17 protein, and recombination at more internal sites would produce longer extensions on some chromosomes (40). If unequal sister chromatid exchange is responsible for telomere elongation, then longer telomeric regions may have a selective advantage for chromosomal stability and hence longer average lengths for the telomeric repeats are maintained in the CDC17 mutants (40).

According to this mechanism, the X and Y' regions at yeast telomeres play an integral role in the replication of chromosomes by mediating the telomeric associations that are required for the interactions and recombination between T regions. Recombination events occur among telomeric regions during the healing of incomplete telomeres in yeast, resulting in the addition of Y' regions to chromosome ends by a RAD52 dependent mechanism (65). In a similar manner, telomeric associations may result in recombination at the T regions to maintain the average length of the T region in a given yeast strain (206). This model for completion of chromosome ends by recombinational mechanisms can accommodate protected chromosomal termini, either by DNA structure or protein blockage. Addition of the yeast T region to heterologous telomeres may result from interaction between the terminal repeat regions on the yeast chromosomes and the linear plasmids with Tetrahymena or
Oxytricha ends, resulting from a similar secondary structure of the terminal repeat units in the cell followed by recombination at the single-stranded nicks in the repeat sequences.

Completion of chromosome ends by recombinational mechanisms does not require any novel molecular mechanisms unlike those that invoke a terminal transferase-like activity for the addition of terminal repeats (80a, 175). The terminal transferase mechanism is based on the addition reactions that have been observed for most telomeres (Reviews 22, 25) and the addition of yeast repeat units to heterologous telomeres in yeast (156, 175). The transferase is thought to add specific repeat units to the incomplete termini in DNA replication, and these eventually loop back to prime the synthesis of the opposite strand (80a, 175). While terminal transferase enzymes have been characterized (31, 159), none of these add specific repeat units with the possible exception of the tRNA nucleotidyltransferase that adds a single CCA unit to the 3' end of tRNA (62). However, support for this model was recently described; a terminal transferase activity in Tetrahymena whole cell extracts has been identified that adds specific 5'-T2G4-3' repeat units to telomeric DNA sequences in vitro (80a). Perhaps a terminal transferase-like enzyme in yeast synthesizes 5'-(C2-3A(CA)1-4)-3' repeat units at chromosome ends and regulates the extent of terminal addition as observed for the IIII termini on linear plasmids in yeast. One must also consider that both mechanisms may be involved in replicating the yeast telomeres. A terminal transferase-like enzyme could result in T region addition at the chromosome ends, and the recombination of the T regions on the chromosome ends with the linear plasmid ends could explain the equivalent T region sequence and length on the natural chromosomes and on linear plasmids in yeast.

H. IIIL Distal versus IIII Alternate Region In the Yeast Genome

The 8.6 kb region distal to HMLε on chromosome IIII shared homology with an alternate and yet unidentified region in the haploid yeast genome referred to as the IIII alternate region. Dual homology of a HMLε distal fragment in the linear III yeast strain K45 and the deletion of the IIII distal fragments in the ring III yeast strains K191 and K192, was detected by Southern hybridization (109). Comparative Southern hybridization studies using probes from the HMLε distal region with linear III or circular III yeast strains distinguished IIII distal and IIII alternate region fragments that...
were maintained in the circular III strains. However, only ring III strain K192 had the IIIL distal region completely deleted, the expected consequence of the \textit{HMLc} - \textit{HMRa} fusion event that produced ring III strains (109). Other ring III strains studied (K191, K193, K195, and K196) had retained most of the IIIL distal region fragments and these replaced the IIIL alternate region fragments presumably by a recombination or gene conversion-like event, perhaps analogous to mating-type switching in yeast (110, 143). As illustrated in Figure 29, the YZ endonuclease produces a double-stranded cleavage at \textit{HMLc} in the \textit{mat1} parental strains (106, 108, 109, 111, 115, 116, 188) which results in two broken ends that are recombinogenic (82, 83, 123, 149, 150, 196). Homology between \textit{HMLc} and \textit{HMRa} resulted in recombination and fusion to form the ring III chromosome, while the \textit{HMLc} distal region invaded the IIIL alternate region and replaced it. This scenario was considered by Klar et al. (109), although they proposed that gene conversion occurred at \textit{HMLc} with the \textit{HMRa} region by recombination of flanking markers to produce a ring chromosome III plus an acentric fragment containing the chromosome ends which was subsequently lost in mitosis. However, my Southern hybridization results indicate gene conversion-like events without exchange of flanking markers as seen for other intrachromosomal gene conversion events such as \textit{MAT} switching (109, 110, 112), or like the healing events observed for broken ends on yeast chromosomes (82, 83, 123). The IIIL distal region was retained in most ring III strains, indicating it was not lost on a fragment formed from the telomeric ends from the \textit{HMLc} and \textit{HMRa} distal regions. Instead the IIIL distal region was attached to another chromosome, but it was exonucleolytically shortened at the broken end prior to this because about 2.5 kb of the region adjacent to \textit{HMLc} was absent from all ring III strains examined. The remaining IIIL distal region, including the telomere, was introduced to and replaced a homologous region on a different chromosome in most ring III strains (K191, K193, K195, K196) presumably by an interchromosomal gene conversion event (97, 110, 172). Ring III strain K192 was the exception where the entire IIIL distal region was deleted during the fusion event. Perhaps the mechanism proposed by Klar et al. (109) was operative in this case; the broken IIIL and
Figure 29. Model for the Retention of the IIIL End in a Ring III Yeast Strain.

a. Mating type interconversion at HMRα in K45 occurs due to the mar1 mutation (108), and is initiated by the YZ endonuclease (115, 116).

b. Regions flanking the YZ cut site are subjected to exonuclease digestion at the unstable ends as indicated by the arrows. Exposure of the HMRα cassette X region results in homologous recombination at HMLα, with strand invasion of the broken HMRα end.

c. Recombination results in a circular chromosome III and the terminal acentric IIIL fragment. The unstable IIIL broken is exonuclease digested in K191.

d. The fragment with the recombinogenic broken end from the HMLα distal region bears strong homology with an alternate region, which is also near a telomere, elsewhere in the genome. This results in the IIIL distal fragment replacing the alternate region by a gene conversion-like or a "telomere conversion" event.
a. K45

b. 

c. K191

d. 
IIR ends fused in this strain to form an acentric fragment with stable telomeric ends, that was subsequently lost in mitosis. This fusion event for the broken end from IIIIL has precedence in the breakage fusion bridge cycle described by McClintock for broken chromosome ends in maize (120, 121, 122). The HMRa distal region shares homology with regions elsewhere in the yeast genome (109). It was not determined if portions of the IIR distal region were retained in ring III strains, due to the lack of appropriate probes.

The IIIIL alternate region remains unmapped in the haploid yeast genome. Bal31 nuclease studies indicated that it is telomere proximal, like the IIIIL distal region. The rate of Bal31 nuclease digestion indicated that the fragment from the IIIIL distal region hybridized to a region that was closer to the terminus of the chromosome terminus at the IIIIL alternate region compared with the IIIIL distal region. This indicates that length of the telomere at the IIIIL alternate region is less than 2.3 kb, and that it belongs to the T-X class of yeast telomeres similar to the IIIIL telomere. The chromosomal origin of the IIIIL alternate region could conceivably be determined through Southern hybridization analysis of the chromosomes from linear III and ring III strains using IIIIL distal probes and OFAGE gels such as those used in yeast electrophoretic karyotyping (36, 37, 173).

Since ring III strains are viable as haploids, Klar et al. (109) proposed that there are no essential genes in the regions distal to HMLα or HMRα. However, the possibility was also considered that there are essential but functionally duplicate loci since IIIIL and IIR distal probes are homologous to other regions in the yeast genome. An essential gene in the HMLα distal region that was present but was less transcriptionally active at the IIIIL alternate region would provide an explanation for the retention of the IIIIL distal region in most ring III yeast strains. It would also explain the sequence conservation between the IIIIL distal and IIIIL alternate regions within a given yeast strain and among the different strains that were used for Southern hybridization studies in this report (AB20α, XP8-10B, AB972, and K45). The idea that the telomere proximal IIIIL distal or IIIIL alternate regions may be transcribed is supported by the finding that some gene families, such as yeast invertase (SUC) genes, and maltose fermentation (MAL) genes, map to the ends of different chromosomes (38, 39, 133, 136). In all yeast strains examined in this study, RNA hybridization analyses indicated that a
fragment from the III1 distal region was homologous with a RNA transcript. Since the RNA transcript was observed in ring III strain K192 which has the entire III1 distal region deleted, it appears that the III1 alternate region was transcribed. Preliminary searches for transcription units in the III1 region with RNA Northern analysis indicated that there is transcript with an approximate length of 1.4 kb that is homologous with the region at least 4 kb distal to HMLα. Further transcript mapping studies have yet to be conducted to accurately determine the transcript length, to determine whether both the III1 distal and III1 alternate regions are transcribed, and to define the region of transcription. Since the entire III1 distal region has been cloned, the proposed gene could easily be sequenced and compared to other yeast characterized genes as a first step in establishing the function of the gene product.

I. Future Prospects

Our understanding of the structure of yeast telomeric regions has increased considerably over the past five years. Most progress has been made in characterizing the elements at yeast telomeres that may function in chromosome replication and stability. Isolation and characterization of the telomeric region from chromosome III1 region provided answers for questions concerning the structure of a specific chromosome end that could not be determined from the characterization of the general structure for a yeast telomere. The mechanism whereby telomeres are replicated remains to be determined. Is there a terminal transferase-like enzyme, or is a recombination mechanism responsible for completion of the termini of chromosomes in DNA replication? Is there a particular DNA secondary structure or chromatin conformation at chromosome ends resulting from the simple repeat sequences? Will yeast telomeres function in heterologous systems or higher eukaryotic systems? Why are there two classes of yeast telomeres? What are the regulatory factors involved in maintaining the average length of a telomere? The isolation of defined chromosome ends, such as the III1 region, may be useful in answering some of these questions.

The isolation and characterization of the III1 telomere may be useful in the construction of artificial chromosomes in yeast. Chromosomes constructed previously had the ends of Tetrahymena rDNA as the telomeric regions (26, 56, 88, 139, 140, 141). In all constructions, these minichromosomes were less stable than natural yeast chromosomes, although the mitotic stability does
Increase with longer artificial chromosomes. If interactions between telomere associated regions and strong ARS activity are required at chromosome ends, then an authentic yeast telomere may improve the mitotic stability of artificial yeast chromosomes. The acentric linear plasmids that were constructed with the telomeric region from chromosome IIIl are more mitotically stable than similar plasmids containing Tetrahymena ends and the ARS1 region. Perhaps homology with yeast chromosomes is required at the ends of the linear plasmids to stabilize them. Interaction and recombination between artificial chromosome ends and yeast chromosome ends does occur when yeast sequences are present on the ends of the linear plasmids, as shown by the addition of Y' regions to linear plasmids in yeast (65).

Artificial yeast chromosomes that behave like authentic yeast chromosomes in their stabilities could be invaluable for a variety of investigations (194):

(1) Introduction of the artificial chromosomes into other organisms to study the function of yeast elements in higher eukaryotic systems and the functional similarity of eukaryotic chromosomal components.

(2) Artificial minichromosomes in yeast may provide a simple system to study chromosome mechanics and recombination.

(3) Cloning of long regions on artificial chromosomes may be possible since length is not a confining factor for linear plasmids while it is for circular plasmids. Perhaps regions that are unstable in circular plasmids due to DNA secondary structure may be cloned in linear minichromosome vectors. Cloning of an extensive chromosomal region would allow the study of copy number effect, polarity effects, and regulatory domains around a given gene.

(4) Determination of the potential to form Z-DNA with the 5'-C1-3A-3' repeats in the T region may be feasible with the IIIl telomeric clones. The remarkable stability afforded to yeast chromosome ends by the 5'-C1-3A-3' may be due to the secondary structure it adopts (23). Left-handed Z-DNA has an inherent tendency to self-aggregate to form networks referred to as Z*-DNA (4) and Z* DNA may form the basis for a stable telomeric structure. Moreover, Z-DNA has a high potential for recombination (145, 162) which may explain the associative nature of yeast telomeres, and the
lengthening of T regions with 5'-C1-3A-3' repeats to chromosome ends. The presence of Z-DNA at telomeric regions, detected through binding of anti-Z-DNA antibodies, has been described for diptera Chironomus thummi and Drosophila melanogaster (4) and for the Secale species (99). The alternating purine-pyrimidine sequence poly d(C-A) * poly d(G-T) adopts Z-DNA conformation under physiological conditions (145, 162). Furthermore, DNA regions containing slight deviations from the alternating Pu-Py sequence also display Z-DNA characteristics (144) shown by binding anti-Z-DNA antibodies to negatively supercoiled plasmids. The capacity for Z-DNA formation at 5'-C1-3A-3' regions could be determined in an analogous manner. The antigenicity of cloned T regions, such as the III telomeric region, to anti Z-DNA antibodies would indicate whether a similar Z-DNA structure might exist at yeast telomeres.
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