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Progressive Rearrangement of Telomeric Sequences Added to Both the ITR Ends of the Yeast Linear pGKL Plasmid

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ABSTRACT

Relocation into the nucleus of the yeast cytoplasmic linear plasmids was studied using a monitor plasmid pCLU1. In *Saccharomyces cerevisiae*, the nuclearly-relocated pCLU1 replicated in a linear form (termed pTLU-type plasmid) which carried the host telomeric repeats $TG_{1\text{-}3}$ of 300-350 bp at both ends. The telomere sequences mainly consisted of a major motif TGTGTGGGTGTGG which was complementary to part of the RNA template of yeast telomerase and were directly added to the very end of the pCLU1-terminal element ITR (inverted terminal repeat), suggesting that the ITR end played a role as a substrate of telomerase. The telomere sequences varied among isolated pTLU-type plasmids, but the $TG_{1\text{-}3}$ organization was symmetrically identical on both ends of any one plasmid. During cell growth under non-selective condition, the telomeric repeat sequences were progressively rearranged on one side, but not on the opposite side of pTLU plasmid ends. This indicates that the mode of telomeric DNA replication or repair differed between both ends. Clonal analysis showed that the intense rearrangement of telomeric DNA was closely associated with extreme instability of pTLU plasmids.

INTRODUCTION

The *Kluyveromyces* linear plasmids, pGKL1 and pGKL2, have specialized terminal structure: inverted terminal repeats (ITRs) whose 5'ends are covalently associated with terminal proteins (TPs). They replicate in the cytoplasm of yeast, either *Saccharomyces cerevisiae* or *Kluyveromyces lactis*, presumably using TP as a primer of DNA-replication similar to the adenovirus or bacteriophage ϕ29 genome (1-6). The pGKL plasmids have a unique transcription system which functions in the cytoplasm. All 15 genes (ORFs) on pGKL1 and pGKL2 are preceded by the specific promoter of their own, termed UCS (Upstream Consensus Sequence), with a motif ACT(A/T)ATNTGA (N, any base) (4, 6, 7, 8), which are directed by the pGKL2-encoded RNA polymerase. The mRNA-5'capping may also occur by an action of the pGKL2 gene (9). The pGKL gene expression system is different from the nuclear system. That is, the pGKL-genes are non-expressible on conventional nuclear vectors of yeast (7, 10). Conversely, the nuclear or foreign genes (e.g. Sc*LEU2*, Sc*URA3*, Km^R, etc.) do not function on the cytoplasmic pGKL while they are expressible if the 5'non-coding regions are replaced by the UCS promoter (11, 12).

The pGKL plasmids normally locate in the cytoplasm, but relocate into the nucleus at a low frequency when forcibly selected for the expression of nuclear or foreign genes integrated on pGKL (13, 14). In *S. cerevisiae*, the nuclerly relocated plasmids replicate at multiple copies in either circular or linear form. The linear form, termed pTLU-type, lacks TP but carries the host telomeric repeats $(TG)_{1\text{-}6}TG_{2\text{-}3}$ ($TG_{1\text{-}3}$ in short) of about 300-350 bp long at the plasmid ends. This event was further studied using a monitor linear plasmid pCLU1 (8.4 kb) carrying the cytoplasmic (UCS-Sc*LEU2*) and the nuclear (Sc*URA3*) markers on pGKL1 (15-17). Like pGKL1, pCLU1 normally replicates in the cytoplasm with the helper pGKL2. Thus, using a *leu2 ura3* mutant as host, the relocation of pCLU1 into the nucleus could easily be assayed by the phenotypic change from Leu^+ Ura^- to Leu^- Ura^+. Based on this rationale, a number of nuclearly relocated pCLU1-derivatives were selected from colonies growing on uracil lacking medium. This paper describes an effective use of pGKL-based linear plasmids in structural and functional study of telomeric

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sequences and includes a protocol for a simultaneous sequencing analysis of telomeres at both the plasmid ends.

MATERIALS AND METHODS

Strain

K12-PC (*MAT*α *leu2 ura3* ρ^+ pCLU1 pGKL2) of *S. cerevisiae* was used.

Media

Yeast cells were grown in a complete synthetic medium SC (0.67% Difco nitrogen base without amino acids, 2% glucose, supplemented with 30 mg/l leucine and 50 mg/l uracil), or a selective medium SC-Ura (SC without uracil). Difco agar (2%) was added for solid medium.

Plasmids

The linear plasmid pCLU1 was constructed by integrating the cytoplasmic promoter(UCS)-fused *LEU2* and the native *URA3* genes into the non-essential ORF3-region of pGKL1 (15). pCLU1 carried terminal-protein (TP) at both ends and were normally maintained in the cytoplasm with the helper pGKL2. When relocated into the nucleus, however, pCLU1 replicated in a circular form (termed pRLU) or telomere-associated linear form (termed pTLU) independent of pGKL2 (18).

Plasmid stability

Cells harboring pTLU were suspended in sterile water, and appropriate dilutions were spread on SC-Ura and SC. Plasmid stability was estimated based on the ratio of the numbers of colonies produced on the two media.

PCR amplification and telomere sequencing

PCR and DNA sequencing of telomeric repeats at the plasmid ends are described in detail in the attached protocol. Briefly, the left and right end DNAs of pTLU were amplified by PCR, respectively, using primers #1 (5'CACACACCACACCCACACAC3') and #2 (5'GAATCAAAGTCGGCATATAC3'), and primers #1 and #3 (5'GGACTTAATGATATACCTAGAG3'), as shown in Fig. 1. The primer #1 was complementary to a major telomeric motif 5'TGTGTGGGTGTGG3' while the primers #2 and #3 were complementary to nucleotide sequences between the positions 276 to 295 inward from the left ITR end and between the positions 471 to 492 inward from the right ITR end, respectively. Telomere sequencing was done by the chain termination method (19) with primer #4 (5'GTATAATAAAATGACTTATAGG3') complementary to nucleotide sequence between the positions 171 to 192 inward from ITR both ends, using an automated sequencer (ABI373S, PE, Applied Biosystems) and a Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems).

RESULTS AND DISCUSSION

Results

Isolation of nuclearly relocated linear plasmids

The *S. cerevisiae* strain K12-PC was initially unable to grow on SC-Ura, because the *URA3* gene on the endogeneous pCLU1 did not function in the cytoplasm. After prolonged (5-6 days) culture at 30°C, however, a small number of colonies appeared, indicating the relocation of pCLU1 into the nucleus at a frequency of about 10^{-3}. Thus, we isolated a number of Ura^+ clones, which carry nuclearly relocated plasmids. Since most of these Ura^+ clones could still contain pCLU1 and pGKL2 in the cytoplasm, cells were subjected to UV irradiation to remove them (a low-dose of UV-light efficiently eliminates the cytoplasmic plasmids without causing impairment of host cells (20)). Plasmid DNAs were extracted and analyzed to differentiate the pTLU-type (telomere-associated linear) from the pRLU1-type (circular), based on restriction analysis and Southern blotting with a telomere probe. The telomere-association at the plasmid ends was observed in *rad52* mutant of *S. cerevisiae* (16, 17), but not in telomerase-subunit mutants of *TLC1* encoding RNA template (21) and *EST2* encoding reverse-transcriptase (22), as shown in Table 1. Only circularized plasmids were found in these mutants while the frequency of the plasmid relocation into the nucleus was not affected, supporting the view that the event was due to the action of host telomerase rather than *RAD52*-dependent recombination with chromosomal telomeric repeats.

Sequence analysis of telomeric DNA

Restriction and Southern blot analyses showed that the pTLU plasmids from Ura^+ clones were structurally identical to pCLU1 except the addition of telomeric DNA (300-350 bp) at both ends, as shown in Fig. 1 (15, 16). To clarify the mechanism of terminal telomeric addition, the pTLU plasmids (pTLU1 to pTLU4) from four independent Ura^+ clones were subjected to sequence analysis for about 100 bp of the telomeric DNA adjacent to the left and the right ITRs of plasmids. The results are given in Fig. 2, providing the following information. (I) In all cases, the terminally added telomere sequences basically consisted of $(TG)_{1\text{-}6}TG_{2\text{-}3}$ (or $TG_{1\text{-}3}$) in accordance with the host telomere DNA (23, 24), showing repeats of a major motif 5'TGTGTGGGTGTGG3'(13 bp) or 5'TGTGGGTGTGG3'(11 bp) which is complementary to part of nucleotides within the *TLC1*-encoded RNA template of host telomerase (21). (II) The telomeric DNAs were directly added to the very ends of the left and the right ITRs. (III) The TG organization in the added $TG_{1\text{-}3}$ sequence varied among pTLU plasmids from different Ura^+ clones, but surprisingly,

(IV) it was symmetrically identical on both ends of any one plasmid.

The variation in the TG organization among pTLUs in (III) probably comes from the nucleotide heterogeneity of RNA-template (21, 23) or due to a primer-template slippage or nonprocessive telomere synthesis (25, 26). The ITR-end telomere-addition in (II) and the symmetrical telomeric sequences at both ends in (IV) may be explained as in Fig. 3: that is, a) the juxtaposition of the termianlly separated ITR regions according to the racket frame-shape model of invertron elements (27), b) the 3' single-stranded overhanging of ITR-end by an action of a 5'-3' exonuclease, following loss of TP, c) the telomeric addition by telomerase to one of both ITR ends using the 3' ITR-end overhang (5'...TGTGT3') as substrate, d) the formation of complementary strand by DNA polymerase, and e) the single-strand invasion and copy-transfer of the telomere added to one plasmid end into another ITR end (16). The ITR-end association of telomeric repeats was constantly observed in a variety of strains of *S. cerevisiae*, either wild-type or telomere-related mutants such as *tel1, mre1, exo* (data not shown), emphasizing that the ITR-end sequence TGTGT played a crucial role in the telomere-addition, probably as a primer for telomere elongation.

Clonal analysis of Ura^+ clones carrying pTLU

Like the ends of eukaryote chromosomes, pTLU-telomeres would undergo the terminal shortening and DNA repair during cell division. Thus, after some cell proliferation, a sequence rearrangement is expected to occur in the pTLU-telomeric repeats. Hereupon, a drastic rearrangement would afffect the telomere function and may result in instability or a total loss of the pTLU plasmid. In fact, when cells of K12-PT2 (K12-PC derived strain harboring a telomere plasmid pTLU2, Fig. 2) were grown on SC and plated on selective medium SC-Ura, a mixture of large and small (slow-growing) colonies was produced while the colonies were uniformaly large when plated on SC. Clonal analysis showed that the slow growing trait of small colonies resulted from low expression of the *URA3* gene due to instability of the endogeneous pTLU siblings whereas large colonies on SC-Ura constantly contained cells with relatively stable pTLU. A possibility that the low expression of the *URA3* gene may be due to a position effect of the nearby telomeric sequence (28, 29) was ruled out by the fact that Ura^- clones growing on SC with 5-fluoroorotic acid (30) were all devoid of pTLU plasmids (17).

Progressive rearrangement of telomeric sequence

To check the above problem, six large and eight small colonies of K12-PT2 growing on SC-Ura were picked up, and the endogeneous pTLU plasmids were extracted to analyze the telomeric sequences at both ends. Knowing that the telomeric size of pTLU from these clones was about 300-350 bp long, similar to that of the original pTLU2, the ITR-adjacent telomeric nucleotides of about 100 bp long were sequenced. As a result, all pTLU siblings from six large colonies were found to have telomeric repeats identical to those of the original pTLU2 (data not shown). Of the eight pTLU plasmids from small colonies, three had the same telomere sequence as pTLU2 (data not shown). However, the remaining five (pTLU2/S1 to pTLU2/S5) were deviated from pTLU2, as shown in Fig. 4. In fact, they differed in the TG_{1-3} organization over a wide range, extending from the telomeric end to various internal sites (positions 70-52) and varying in the number and order of the TG tract flanked by the TGG and/or TGGG array.

Unexpectedly, the telomere rearrangement in the above five pTLU siblings was observed only on one side of the plasmid ends (the left in pTLU2/S1 to pTLU2/S3, and the right in pTLU2/S4 and pTLU2/S5), while the opposite sides remained identical to that of pTLU2. Given that the telomeric rearrangement reflects the 5'terminal shortening and gap-repair by telomerase, the TG_{1-3} variation in the above (also in Figs. 5 and 6) ought to extend from the indicated internal rearrangement sites to the far end of added telomeres of 300-350 bp long.

A variety of telomeric rearrangement

It was suggested from the above results that pTLU2 of the parental strain underwent a variety of telomeric rearrangements during cell growth on non-selective medium, yielding slow-growing clones on SC-Ura. To reconfirm this observation, one of large colonies on SC-Ura, carrying the original pTLU2, was plated on a complete medium (SC) for single colonies. The size of colonies formed was uniformly large. Nine clones (K12-PT2/C1 to /C9) were isolated at random and streak-cultured on SC for 2 days. Then, the cells were subjected to the assay of the stability of the endogeneous pTLU2-derived siblings and their telomeric DNA sequencing. It was found that the pTLU2-siblings (pTLU2/C2, /C3 and /C7) from the three clones (K12-PT2/C2, /C3 and /C7) were highly unstable (0.16, 0.25, 0.6%, respectively) and had telomeric TG_{1-3} organization distinct from pTLU2 and from each other (Fig. 5A). The rearranged sequences ranged from the various ITR proximal positions (60, 47 and 77) toward the telomeric ends, respectively. Here again, the telomeric rearrangements were observed only on one side of the plasmids: the left in pTLU2/C2 and the right in pTLU2/C3 and /C7. In contrast, the pTLU2-siblings from the remaining six clones (K12-PT2/C1, /C4 to /C6, /C8, /C9) were more stable (34-9%, 22% on the average) and contained the same telomeric repeats as the original pTLU2 as far as telomeric nucleotides of about 100 bp were examined (data not shown).

To study more of the relationship between the stability and telomeric rearrangement, the second round of assays was carried out with pTLU2-siblings from each of one stable clone K12-PT2/C1 and three unstable clones K12-PT2/C2, /C3, and /C7. To this end, cells grown on SC-Ura were plated on SC for single colonies, and the resulting eight subclones were examined each. The telomeric assay of about 100 bp adjacent

to ITRs showed that, of the eight K12-PT2/C1-subclones, six had pTLU identical to pTLU2 (data not shown) with stability of 31-34%, close to that of pTLU2 from K12-PT2/C1. However, pTLU (pTLU2/C1-2 and pTLU2/C1-3) from the remaining two subclones differed from pTLU2 on the left side, both ranging from the identical ITR proximal position (60) toward the ends (Fig. 5B). Their stability was 17 and 9%, respectively, lower than that of the above six related subclones, but significantly higher than the stability (0.16, 0.25, 0.6%) of pTLU2 siblings from K12-PT2/C2, /C3 and /C7, described above. We presume that this extreme instability of the latter three pTLU2-siblings in the first round assay was due to the conditions of cell growth prior to the stability assay: that is, pTLU2 siblings from K12-PT2/C1 to /C9 were generally unstable, because their stability was estimated after repeated cell growth on SC, which allowed an accumulation of pTLU-cured cells or cells with pTLU of low stability.

On the other hand, of eight subclones of unstable K12-PT2/C2, /C3, and /C7 each, five carried the rearranged telomeric sequence on one side (Fig. 5B). Notably, in all these cases, the TG_{1-3} rearrangement proceeded toward more internal regions than those of their parental clones: for example, the positions 45, 41, 52, 60, 29 in each of pTLU2/C2-2, /C2-4, /C2-5, /C2-6, /C2-8 against the position 60 in pTLU2/C2. The position 60 in pTLU2/C2-6 was identical to that of the parental pTLU2/C2, but the TG_{1-3} organization differed between them. The results imply that the telomere end shortening and repair have continuously advanced toward the inner side during cell growth. Moreover, the stability was extremely reduced in these plasmids: 0.01-0.08% in pTLU2/C2-siblings, 0.01-0.1% in pTLU2/C3-siblings, and 0.01-0.1% in pTLU2/C7-siblings, much lower than data of their parental clones, revealing that the decrease in stability was closely related to the progressive telomeric rearrangements. Throughout this experiment, there was no telomeric rearrangement on the opposite side.

Time course of telomeric rearrangement

The above assay demonstrated that the TG_{1-3} rearrangement occurred in principle on one side of the pTLU plasmid ends during cell growth. To analyze the time-course of this event, the three clones K12-PT2/C2, /C3, and /C7 each were streak-cultured on SC consecutively at intervals of 24 hours (about 10 generations) and examined for the telomeric sequence. The results are given in Fig. 6, showing that the telomeric sequence remained unchanged for the first 10 generations. After 20-50 generations, however, a novel TG_{1-3} organization was detected on the same telomeric sequence, which had a previous rearrangement. It is also notable that, after 50 generations, the second rearrangement occurred from the outside of the previously rearranged telomeric sequence. In contrast, if the above assay was done on SC-Ura, there was no observation of telomeric rearrangement even after 50 or more generations (data not shown). In this case, we presume that telomere rearrangements must actually have occurred in clones growing on SC-Ura as well, but that they could not be detected because the pTLU with rearranged telomeres were highly unstable and thus because the culture was mostly predominated by cells with stably replicating pTLU2 of the original type.

Discussion

Relocation of the yeast cytoplasmic linear plasmids into the nucleus was assayed using a monitor plasmid pCLU1 in terms of the phenotypic change of the integrated markers (the UCS-fused *LEU2* and the native *URA3*) in *S. cerevisiae* (*leu2 ura3*). The nuclearly relocated pCLU1 replicated in either circular or linear form. While the circular form resulted from pCLU1 by ITR-involved intramolecular recombination or transposon-like invasion (18), the linear plasmid pTLU was created by an addition of the host telomeric repeats at both ends of pCLU1 (15, 16).

The telomere addition occurred in *rad52* mutant (16, 17), but not in *tlc1* and *est2* mutants, strongly suggesting the involvement of host telomerase rather than recombination with chromosome telomeric repeats. While the TG_{1-3} organization varied among the individual pTLU plasmids, it was symmetrically identical at the left and the right ends of any one pTLU. This probably arose from a unique process of telomere addition to a racket-frame shaped linear plasmid of an invertron-type, as illustrated in Fig. 3.

Like the chromosome ends, the telomere sequence of pTLU should undergo successive rearrangements due to incomplete DNA replication at the 5'end and subsequent DNA repair by telomerase. In our experiment, the pTLU-derived plasmids with the rearranged TG_{1-3} sequence were unstable, and the instability was much enhanced with increasing in the rearrangement. Thus, when cells were grown on SC-Ura, the telomere of original pTLU type was predominantly selected by surpassing unstable pTLU-siblings. Supposing that the telomeric repeats added to pTLU was about 300-350 bp long, and that the telomere rearrangement resulted from successsive rounds of terminal shortening and end healing by the aid of telomerase, we estimate that the pTLU plasmids underwent the telomere rearrangement over a wide range of around 300 bp inward from the far end during this experiment, reaching the ITR proximal positions between 29 and 77 on the assayed telomeres (Figs. 5 and 6). Such over-shortening or a wide-ranged rearrangement of telomeric sequences might be detrimental to the integrity of pTLU plasmids and may have caused an extreme instability of the linear plasmids.

Progressive rearrangement of TG_{1-3} sequence on one side of pTLU ends was of special interest, implying that the mode of telomeric replication or end-shortening/repair may be different between both ends. It appears that the event is subject to condition of end-shortening and repair. For example, if the repair at one end is delayed or impaired for some reason, the end-shortening would continue toward more inner region while the opposite end is repaired normally. Thus, in the present study where telomeric sequence assay was conducted for about

100 bp adjacent to the ITR ends, the telomeric rearrangement was detectable on one side, as given in Fig. 7.

A variety of genes or gene products are known to interact with the telomere function and its length control. In *S. cerevisiae*, the *Rap1*, *Est1* and *Est4/Cdc13* products play important roles in regulation of telomere-length via telomerase activity (31-35) while proteins such as Sir3p, Sir4p, Rif1p, and Rif2p interact with Rap1p and are involved in telomere integrity (36-38). The yeast Ku protein is required for telomere-clustering and its functional loss leads to the telomere-shortening (39-42). The *TEL1* and *TEL2* are essential genes required for telomere-length regulation (43, 44). Since pTLU exists in the nucleus as many as 30 copies per cell in a haploid strain of *S. cerevisiae*, the cell should have at least 90 telomeres by including the ends of 16 host chromosomes. Thus, a possibility arises that, under normal condition of cell growth, pTLU-plasmids may be short of such telomere-related proteins mentioned above due to their possible preferential recruitment to host chromosomes, and that it may cause a defect in the telomere function, the length-control or the stability of pTLU-plasmids. Although detailed mechanism for the one-sided telomere rearrangement observed in this study remains to be examined, the pGKL-based linear plasmids are highly unique in that they allow a simultaneous analysis of the telomere sequences at both ends.

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FIGURES AND TABLE

Fig. 1: Plasmid structure and strategy for DNA sequencing of the telomeric repeats at both ends of pTLU. Telomeric DNA sequences at the ITR ends were prepared and analyzed as in MATERIALS and METHODS. Arrow of the primer indicates the direction of the sequence elongation. Closed triangles on the plasmid ends, ITRs (202 bp); striped square, terminally added telomere; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I.

Fig. 2: Telomere-sequences added to the ITR ends. Sequences are presented in the 5' to 3' direction from ITR toward the termini of added telomere $(TG)_{1-6}TG_{2-3}$. L and R denote the left and the right ends of pTLU-type plasmids, respectively. The major telomeric motif (5'TGTGTGGGTGTGG3') is underlined. The TG-dinucleotide tract flanked by TGG and TGGG is shaded. Modified citation from Takata et al. (2000).

Fig. 3: Mechanism of creation of symmetrical telomeric sequences at both ends. A. Racket frame shape model of pTLU. B. Creation of identical telomere sequences at the ITR ends. Telomerase activity requires a single-strand tail as a primer to initiate the telomere elongation, so the ITR ends are deprived of TP and partially digested by a 5' to 3' endonuclease to create a short 3' single-strand overhang with TGTGT nucleotides. See text.

Fig. 4: Telomeric sequences of pTLU2-siblings recovered from slow-growing colonies of K12-PT2. Upper and lower case letters denote the original and rearranged telomeric sequences, respectively. The filled triangles indicate the ITR proximal positions of rearranged telomeric sequence. The nucleotide sequence between positions 6 to 23 was the same in all plasmids and omitted for simplicity. In this study, the telomere sequences were determined for about 100 bp adjacent to the ITR, but the rearrrangement should presumably continue toward the 3'termini of added telomere, which is indicated by the broken lines. Other symbols are as in Fig. 2. Modified citation from Takata and Gunge (2001).

Fig. 5: Clonal analysis of progressive telomere-rearrangements in pTLU2 sibling plasmids. A. Sequences of rearranged telomeres recovered in the first round assay. Data of unstable plasmids, pTLU2/C2, pTLU2/C3, and pTLU2/C7 from the three cloned (K12-PT2/C2,C3, and C7), are shown. Telomeres of stable plasmids from six other clones were identical to those of pTLU2 (data not shown). B. Sequences of rearranged telomere recovered in the second round assay. Note that, in A and B, the number and order of the TG dinucleotide tract in $(TG)_{1\text{-}6}$ $TG_{2\text{-}3}$ varied from each other. Other symbols are as in Figs.2 and 4. Modified citation from Takata and Gunge (2001).

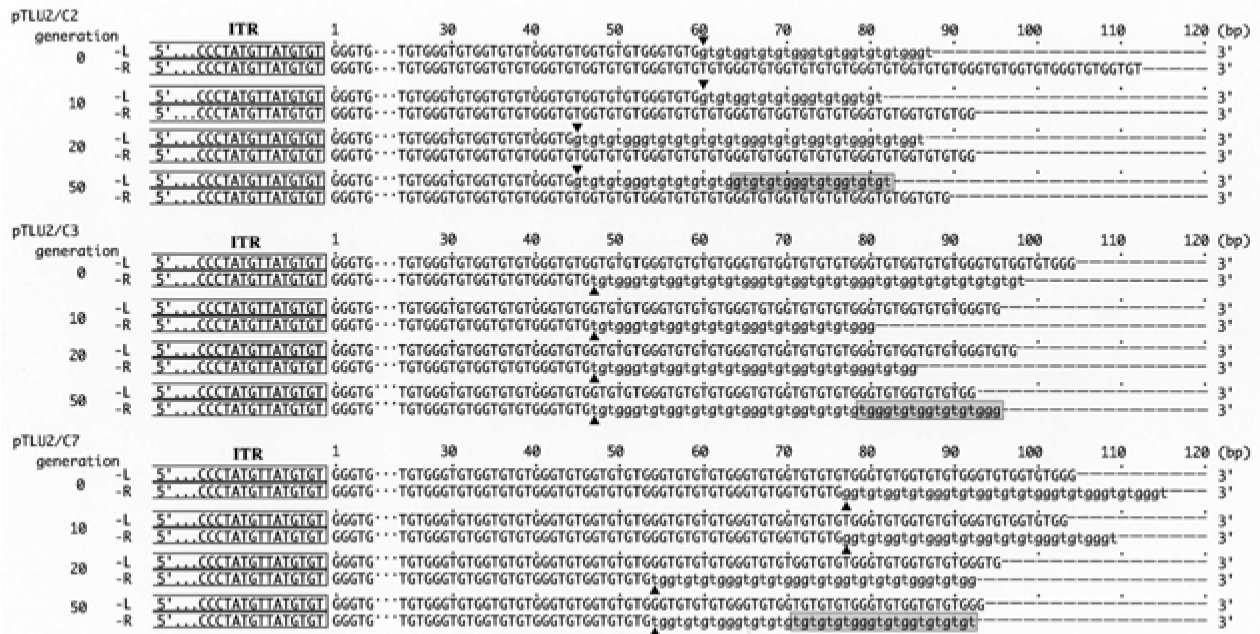

Fig. 6: Time course of progressive telomeric rearrangement. Additional telomeric rearrangements were detected after 20 and/or 50 generations. The shaded box (50 generations) indicates novel telomeric rearrangements occuring on the previous rearrangements. Other symbols are as in Fig. 4. Modified citation from Takata and Gunge (2001).

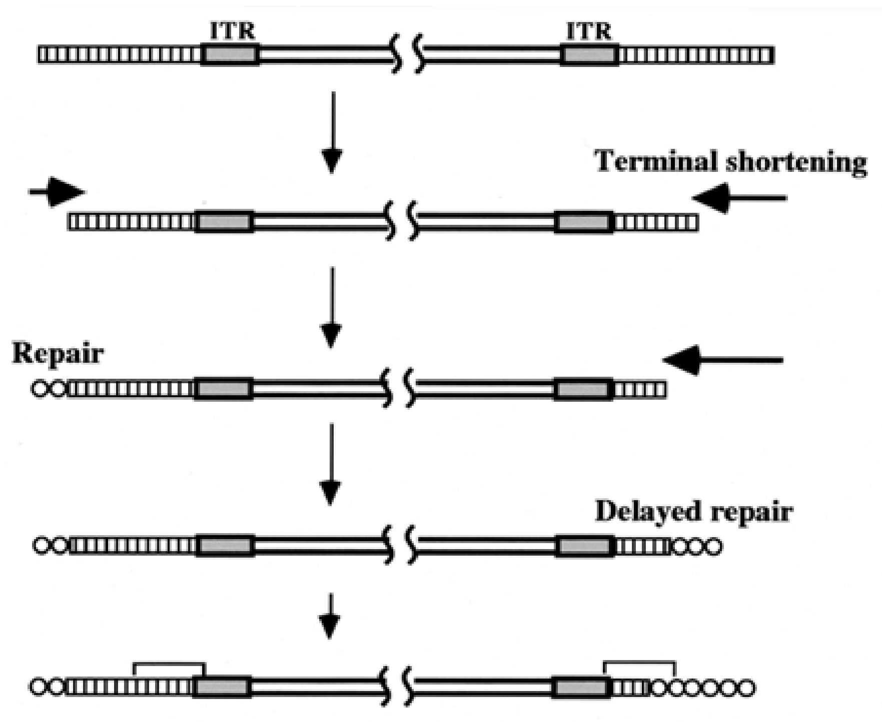

Fig. 7: A model of telomeric sequence rearrangement on one side. Open circles on telomere indicate rearranged telomeric repeats due to delayed repair. The square bracket, sequenced telomeric region.

Table 1: Frequencies of nuclear-relocation of pCLU1 and telomeric attachment at the ends

Strain	Nuclear relocation	% of pTLU-type *
K12-2A-PC (wild-type)	1.1×10^{-3}	14 (48)
A364a-PC (wild-type)	1.1×10^{-3}	11 (48)
S288C-PC (wild-type)	1.0×10^{-3}	12 (16)
W303-PC (wild-type)	1.1×10^{-3}	14 (16)
XS95-6C-PC (*rad52*)	1.1×10^{-3}	13 (32)
YPH499-PC (*tlc1*)	1.0×10^{-3}	0 (48)
BY4741-PC (*est2*)	1.4×10^{-3}	0 (48)

*** Number of plasmids examined is given in parenthesis.**

PROTOCOLS

Sequencing of telomeric DNA attached to the left and the right ITR ends of the yeast linear plasmid pTLU

Strain used for telomere-sequencing

Saccharomyces cerevisiae K12-PT2 (*Matα leu2 ura3* ρ^{+} pTLU).

This strain should be kept on medium SC-Ura to maintain the telomere-associated plasmid pTLU (pTLU carries a selective marker Sc*URA3*).

A. Isolation of DNA (A modification of Miniprep method in Methods in Yeast Genetics, CSH Laboratory, 1994)

1. Grow cells overnight in 5 ml of medium SC-Ura.
2. Collect the cells in a microfuge at 2000 rpm for 5 minutes. Discard the supernatant.
3. Resuspend the cells in 0.5 ml of buffer (10 ml EDTA, 2.5% 2-mercaptoethanol, 0.6 M KCl, pH 8.0). Hold at room temperature for 5 minutes.
4. Centrifugation at 2000 rpm for 5 minutes and discard the supernatant.
5. Add 0.3 ml of a lysis buffer (0.1 M EDTA, 33 mM Tris-HCl (pH 8.0), 0.6 M KCl, 0.03 % 2-mercaptoethanol, 0.4 mg/ml Zymolyase 20T) and incubate at 37°C for 30 minutes.
6. Centrifuge in a microfuge at 3000 rpm for 1 minute. Discard the supernatant.
7. Resuspend the cells in 0.3 ml of 0.1 M EDTA, 50 mM Tris-HCl, pH 8.0, and mix well.
8. Add 0.03 ml of 10% SDS, mix well and incubate the mixture at 65°C for 20 minutes.
9. Add 0.11 ml of 5 M potassium acetate and place the microfuge tube on ice for 60 minutes.
10. Centrifuge in a microfuge at 15000 rpm for 10 minutes.
11. Transfer the supernatant to a fresh microfuge tube and add one volume of 100% cold ethanol at room temperature. Mix and allow it to sit at room temperature for 5 minutes. Centrifuge in a microfuge at 15000 rpm for 10 minutes. Discard the supernatant.
12. Add 100µl 70% cold ethanol.
13. Centrifuge at 15000 rpm for 5 minutes. Discard the supernatant and air-dry the pellet.
14. Resuspend the pellet in 0.3 ml of distilled water.
15. Add 1µl of a 10mg/ml solution of RNase A and incubate at 37°C for 30 minutes.

16. Add one volume of 100% cold ethanol. Mix and allow it to sit at temperature for 5 minutes. Centrifuge in a microfuge at 15000 rpm for 10 minutes to harvest the pellet of DNA.
17. Discard the supernatant and add 100µl 70% cold ethanol.
18. Centrifuge at 15000 rpm for 5 minutes. Discard the supernatant and air-dry. Resuspend the pellet of DNA in 100µl of distilled water.

B. Telomeric sequences of pTLUs are amplified by PCR

PCR Amplification

1. Prepare a solution (49µl): 5µl of 10xKOD buffer (1.2mM $MgCl_2$ at the final concentration) Toyobo), 5µl of 2mM dNTPs, 1µl KOD Dash (2.5U/µl, Toyobo), 2.5µl each of forward and reverse primers (50pmol), 34µl of distilled water, and mix well.

Note: The left plasmid end containing telomeric repeats is amplified using the primers, #1 (5'CACACACCACACCCACACAC3') and #2 (5'GAATCAAA GTCTTCATATAC3'). The primer #1 is designed to contain the nucleotides complementary to a major telomeric motif 5'TGTGTGGGTGTGG3', which was revealed previously (Takata *et al.* 2000). The primer #2 is complementary to a part of pTLU (nucleotide positions 276 to 295 inward from the left ITR end, see Fig. 1).

The right plasmid end containing telomeric repeats is amplified using the primers, #1 and #3 (5'GGACTTAATGATATACCTAGAG3'). The #3 primer is designed to be complementary to a part of pTLU (nucleotide positions 471 to 492 inward from the right ITR end, see Fig. 1).

2. Add 1µl (10ng) of the template DNA (DNA in TE/10) to the PCR solution above. After careful mixing by hand and thermocycling is performed.
3. The PCR amplification (50µl in total) is carried out as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 2 seconds, extension at 74°C for 30 seconds, repeating of 30 cycles, and hold at 4°C.
4. After amplification, the PCR products are placed into a 1.5 ml microfuge tube.
5. Add 5µl 3M sodium acetate (pH 4.8) and 130µl 100% cold ethanol, and mix well.
6. Incubate at -90°C for one hour.
7. Centrifuge at 15000 rpm for 10 minutes. Discard the supernatant.
8. Add 100µl 70% cold ethanol.
9. Centrifuge at 15000 rpm for 5 minutes. Discard the supernatant.
10. The DNA pellet is dissolved in 10µl of distilled water (final concentration of ca.30ng/µl of DNA).

C. Telomere-sequencing is analyzed by the chain termination method

Sequencing is done by the chain termination method using primer #4 (5'GTATAATAAAATGACTTATAGG3'), which is designed to be complementary to the nucleotides 171-192 inward from each ITR end (see Fig. 1).

The sequencing solution contains the following in a final volume 20µl: 8µl Terminator premix,[a)] **,** 3.2pmol primer #4, 3µl (ca.90ng) purified PCR products), distilled water (20µl in final volume).

a) Terminator premix: 4µl 5xSequencing buffer, 1µl dNTP mix, 0.5µl Dye deoxy terminators, 1µl AmpliTaqFS in 8µl)

1. Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems), 3.2pmol primer #4 and 3µl purified PCR products.
2. Sequence reaction (Dye Terminator Cycle Sequencing): The amplification profile is as follows. Initial denaturation at 96°C for 1min. Denaturation at 96°C for 10sec., annealing at 50°C for 5sec., and extension at 60°C for 4min. The reaction was repeated 25 cycles. In our experiment, the TAKARA PCR Personal Thermal Cycler (TAKARA Shuzo) was used.
3. PCR products were purified by ethanol precipitation. Transfer the PCR products to a 1.5ml microfuge tube. Add 2µl 3M NaOAc(pH4.8) and 50µl 100% cold ethanol. Incubate on ice for 1hr.
4. Centrifuge for 20min. at 15,000rpm. Transfer the supernatant. Add 250µl 70% cold ethanol.
5. Centrifuge for 5min. at 15,000rpm. Transfer the supernatant.
6. Pellet was used for automated sequencer (ABI373S, PE-Applied Biosystems).