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The yeast linear DNA killer plasmids, pGKL1 and pGKL2, possess terminally attached proteins

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ABSTRACT

The terminal structures of linear DNA killer plasmids from yeast, pGKL1 and pGKL2, were analyzed. Results obtained by exonuclease treatments of these plasmids show that both pGKL plasmids have free hydroxyl 3'-ends and blocked 5'-ends. Electrophoretic analysis of the terminal restriction fragments treated with proteases revealed that pGKL1 and pGKL2 have proteins bound at 5'termini and that the terminal protein of pGKL1 is distinct from that of pGKL2. This is the first linear DNA-terminal protein association found in yeast.

INTRODUCTION

A yeast killer system associated with linear DNA plasmids has been recently found in Kluyveromyces lactis (1). The linear DNA killer plasmids, pGKL1 and pGKL2 have sizes of 8.9 kb and 13.4 kb respectively (1, 2). The killer toxin and the resistance or immunity to the killer are coded by pGKL1, while pGKL2 is required for the maintenance of pGKL1 in a cell (3-5). The pGKL plasmids can be transferred into Saccharomyces cerevisiae cells by cell fusion or transformation, where these plasmids can replicate and express the normal K. lactis-type killer character (4, 5).

In the previous papers (2), we have determined the complete nucleotide sequence of pGKL1 (8876 bp) and the sequences of the terminal regions of pGKL2. Nucleotide sequencing has revealed that pGKL plasmid DNAs have inverted terminal repetitions (ITR) of 202 bp (pGKL1) and 184 bp (pGKL2). It has been reported that the linear DNAs of adenovirus (6), phage ø29 of Bacillus subtilis (7), and plasmids from Streptomyces rochei (8) and the linear mitochondrial DNAs from maize (9) have also ITR and terminally attached proteins. This similarity of terminal nucleotide sequence between pGKL plasmids and other linear DNAs having proteins bound at the termini has led us to suggest that pGKL plasmids have also terminal protein. So far,

however, experimental evidences for this postulate have not been presented.

We describe here the experimental results that pGKL plasmids have proteins bound at 5'-termini and that the terminal proteins of pGKL1 and pGKL2 are distinct from one another.

MATERIALS AND METHODS

Yeast linear DNA killer plasmids, pGKL1 and pGKL2 were prepared from Saccharomyces cerevisiae F102-2 which was a fusant between S. cerevisiae AH22 and K. lactis 2105-1D (4), in the absence of pronase or proteinase K as described previously (2). Exonuclease III and λ exonuclease were purchased from Bethesda Research Laboratories. T4 DNA polymerase and restriction endonucleases, AluI and ScaI, were products from Takara Shuzo, Kyoto, Japan. RsaI was from Nippon Gene, Toyama, Japan. Pronase E and proteinase K were obtained from Kaken Kagaku, Tokyo, Japan and Merk, Darmstadt, Germany, respectively. To eliminate possible nuclease activities in pronase E preparation, the stock solution of pronase E (20 mg/ml) was previously incubated for 2 hr at 37°. Trypsin was purchased from Miles-Seravac, Maidenhead Berks, England. [α-^{32}P]dCTP (3000 Ci/mmol) was from Amersham, England.

Digestion of pGKL1 and pGKL2 with exonuclease III and λ exonuclease.

The cell lysate containing 20 μg of DNA obtained from S. cerevisiae F102-2 was incubated with exonuclease III and λ exonuclease. The reaction mixture (50 μl) contained 10 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol and 38 units of exonuclease III. For λ exonuclease digestion, 10 mM NaCl and 13 units of λ exonuclease were used in place of exonuclease III. After incubation at 37°, 5 μl of the reaction mixture was withdrawn into 1 μl of 250 mM EDTA at 2, 5, 10, 15, 20 and 30 min, chilled to 0°, and analyzed by 0.7% agarose gel electrophoresis.

Labelling of pGKL plasmids with [α-^{32}P]dCTP and T4 DNA polymerase and digestion with restriction endonucleases

Both 3'-terminal regions of pGKL plasmids were labelled by replacement synthesis reaction with T4 DNA polymerase. The reaction mixture (11 μl) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 5 mM 2-mercaptoethanol, 3.5 units of T4 DNA polymerase and 0.3-0.6 μg of pGKL plasmid DNA was incubated at 37° for 4 min. One μl of 2 mM each dATP, dTTP and dGTP, and 1 μl [α-^{32}P] dCTP (total 10 μCi, 3000

Ci/mmol) were added to the mixture, and the incubation was continued for 15 min. Then 1 μl of 1 mM cold dCTP was added. After additional 10 min-incubation at 37°, the reaction was terminated by heating at 70° for 5 min. This mixture was treated for 30 min at 37° with 3-4 units of appropriate restriction endonuclease (RsaI or ScaI for pGKL1, and AluI or RsaI for pGKL2) to obtain the short terminal fragment of pGKL DNA. This mixtrue was used directly for the protease assay as described in following seciton.

Protease treatment of the labelled terminal fragments of pGKL DNA

An aliquot (5 μl) was treated with 10 μg of pronase E or proteinase K in 0.5% $NaDodSO_4$ for 30 min at 37° and analyzed by gel electrophoresis. Trypsin treatment was performed in same condition except that 0.5% $NaDodSO_4$ was omitted.

Pronase E treatment of intact pGKL1 and pGKL2 plasmids

0.5 μg of intact pGKL1 or pGKL2 was treated with 10 μg of pronase E in a reaction mixture (10 μl) containing 40 mM Tris-HCl (pH 7.6) and 0.5% $NaDodSO_4$ for 30 min at 37°. Samples were phenolized 3 times, precipitated with ethanol, washed twice with 70% ethanol and lyophilized.

Electrophoresis of DNA

Electrophoresis of short fragments of pGKL DNA was performed in 15% or 20% polyacrylamide slab gel (0.05x12x17 cm) using 89 mM Tris borate/2 mM EDTA buffer (pH 8.3). 0.7% agarose gel electrophoresis for exonuclease digestion assay was performed in the same buffer as above. DNA was detected by autoradiography or ethidium bromide staining.

RESULTS AND DISCUSSION

We examined the terminal structures of pGKL plasmids by using exonucleases. Exonuclease III which cleaves DNA in 3' to 5' direction was able to digest pGKL plasmids progressively (Fig. 1a). λ Exonuclease that hydrolyzes DNA in 5' to 3' direction, on the other hand, did not digest pGKL plasmid DNAs, where the chromosomal DNA was digested (Fig. 1b). Furthermore, the 5'-ends of pGKL plasmids are not accessible for phosphorylation with [γ-^{32}P]ATP and T4 polynucleotide kinase, even after treatment with alkaline phosphatase. Failure in kination of pGKL plasmids was also recently reported by Sor *et al.* (10). These results clearly suggested that both pGKL plasmids have free hydroxyl 3'-ends and blocked 5'-ends. Some linear DNAs have

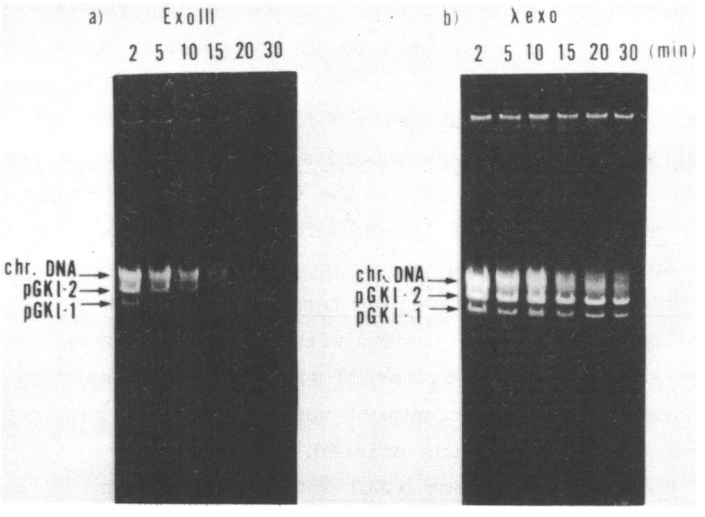

Fig. 1. Digestion of pGKL1 and pGKL2 with exonuclease III and λ exonuclease. The cell lysate containing pGKL1, pGKL2 and chromosomal DNA (chr. DNA) from S. cerevisiae Fl02-2 was incubated with exonuclease III (a) and λ exonuclease (b). Aliquots were withdrawn from the incubation mixture into EDTA solution at indicated time and analyzed by agarose gel electrophoresis.

been reported to have proteins bound at the 5' termini (See the introduction). In the case of these linear DNAs, the presence of protein could be relatively easily proved, since the electrophoretic mobility of protein-bound DNA was much reduced, compared to that of the DNA treated with proteolytic enzyme. This is, however, not the case of pGKL plasmids. We have not detected the clear difference between electrophoretic mobilities of pronase E-treated and untreated pGKL plasmids in agarose gel (data not shown).

One possible explanation of this result is that the protein is attached but not detected in this agarose gel system, probably due to the small size of the protein or the unique composition of amino acids. To examine this possibility we analyzed the very short terminal fragments of pGKL DNAs with and without treatment of proteases by 15% or 20% polyacrylamide gel electrophoresis.

Fig. 2A shows the physical maps of the very terminal regions of pGKL1 and pGKL2. Restriction endonucleases, RsaI and ScaI treatments should produce 5'-end blocked terminal fragments of 21 bp from pGKL1.

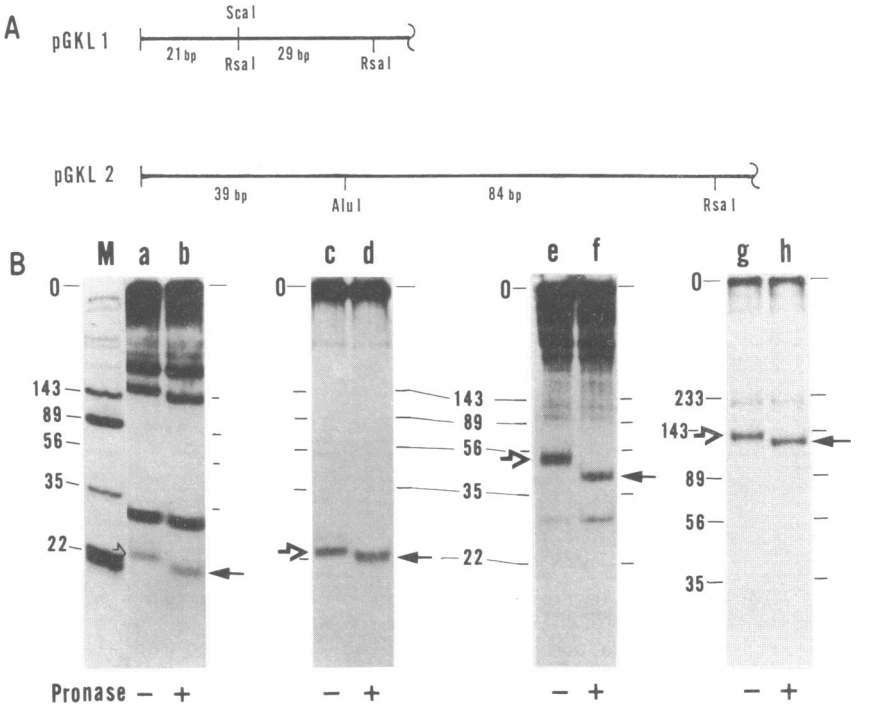

Fig.2. A. Physical maps of the terminal regions of pGKL plasmids. The restriction sites were first determined by computer analysis of the nucleotide sequence (2). B. Electrophoretic analysis of the terminal fragments of pGKL plasmids. Each pair of pronase E treated (+) and untreated (-) fragments was electrophoresed in 20% (lanes a-f) or 15% (lanes g and h) polyacrylamide gel. O indicates the origin of electrophoresis. Lanes a and b, RsaI fragments of pGKL1; c and d, ScaI fragments of pGKL1; e and f, AluI fragments of pGKL2; g and h, RsaI fragments of pGKL2; M, size markers. Numbers indicate the sizes (bp) of øX174RF DNA Taq I fragments labelled with [α-^{32}P]dNTP and T4 DNA polymerase. The white and black arrows indicate untreated and pronase E treated terminal fragments, respectively. In lanes a and b, the terminal fragments of pGKL2 (5'-end blocked 123 bp) are also visible, because that the preparation of pGKL1 used here was contaminated by pGKL2 plasmid.

AluI and RsaI produce 5'-end blocked 39 bp and 123 bp terminal fragments, respectively, from pGKL2. These terminal fragments should be produced from both ends of plasmid DNAs, since pGKL1 and pGKL2 have long inverted terminal repetitions(2, 10).

3'-Terminal regions of pGKL DNAs were labelled with T4 DNA polymerase and [α-^{32}P]dCTP. Each sample was treated with appropriate

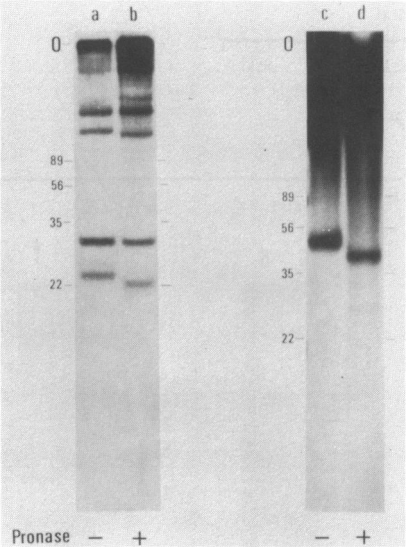

Fig. 3. Electrophoretic analysis of the terminal fragments of pGKL plasmids treated with pronase E. Intact pGKL1 and pGKL2 were first treated with pronase E, and then lalbelled, fragmented and electrophoresed (lanes b and d) with untreated control samples (lanes a and c) in 20% polyacrylamide gel. Lanes a and b, RsaI fragments of pGKL1; c and d, AluI fragments of pGKL2. Numbers are same as in Fig. 2.

restriction endonucleases and divided into two equivalent samples. One sample was incubated with pronase E and each pair of samples was then electrophoresed in adjacent lanes (Fig. 2B). The electrophoretic mobility of only one band corresponding to terminal fragment increased after pronase E treatment.

In this experiment, the sample treated with pronase E was directly electrophoresed in the presence of pronase E, whereas control sample contained no pronase E. To rule out a possibility that the electrophoretic pattern was changed by the presence of pronase E, although this is not likely, another experiment was performed. Intact pGKL DNAs were first treated with pronase E and phenolized. This sample was labelled, digested with restriction endonucleases, and electrophoresed with control samples. Hence, these two samples for electrophoresis, pronase E treated and untreated control, had the same composition. As shown in Fig. 3, the electrophoretic patterns practically same as in Fig. 2B were obtained.

These results clearly show that pGKL1 and pGKL2 have proteins

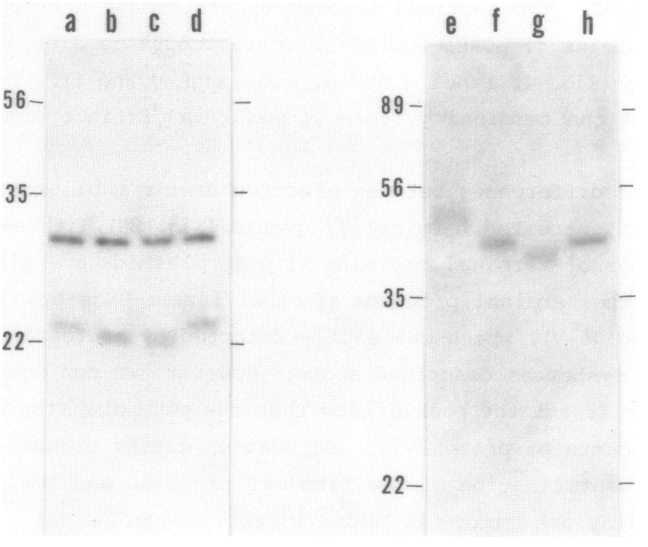

Fig. 4. Cleavage of the terminal fragments of pGKL plasmids by proteases. RsaI fragments of pGKL1 (lanes a-d) and AluI fragments of pGKL2 (Lanes e-h) were treated with proteinase K (lanes b and f), pronase E (c and g) and trypsin (d and h) and electrophoresed in 20% polyacrylamide gel. Lanes a and e were untreated control samples. Numbers are same as in Fig. 2.

bound at the 5'-termini. It is also important to note that the right and left ends of each pGKL plasmid have probably the same protein, because that the both terminal fragments of each pronase E-untreated pGKL plasmid migrated as single band (Fig. 2B, lanes a, c, e and g).

The differences between electrophoretic mobilities of pronase E treated and untreated terminal fragments of pGKL plasmids indicate that the proteins bound at 5'-termini correspond to 2 bp of DNA for pGKL1 and 12 bp of DNA for pGKL2 in the polyacrylamide gel system used (Fig. 2B). Although one can not estimate the size of the terminal proteins from these results, these values of differences suggest interestingly that the terminal protein of pGKL1 is distinct from that of pGKL2.

This was further confirmed using different proteolytic enzymes, proteinase K and trypsin. Fig. 4 shows that the terminal protein of pGKL2 was cleaved by trypsin (lanes e and h), while no difference between the mobilities of trypsin treated and untreated terminal fragments of pGKL1 was observed (Fig. 4, lanes a and d). Proteinase K treatment also revealed the distinction between terminal proteins of

pGKL1 and pGKL2. The portions removed by proteinase K from the terminal proteins of pGKL1 and pGKL2 corresponded to 2 bp and 8 bp, respectively (Fig. 4, lanes a and b, and lanes e and f). These results indicate that the terminal protein of pGKL1 is distinct from that of pGKL2.

From the differences between electrophoretic mobilities of pronase E treated and untreated terminal fragments (Fig. 2B), it seems likely that the sizes of terminal proteins of pGKL plasmids are quite small, compared to the terminal proteins of other linear DNAs previouly characterized (6-9), which was easily detected by protease assay in agarose gel system as described above. However, we can not yet completely rule out the possibility that the pGKL plasmids used here were the products of proteolytic degradation during preparation of DNAs. The identification of the terminal proteins and the precursors of the proteins are currently under investigation.

The terminal proteins of pGKL plasmids may have functions to protect against degradation of DNA and to be involved in the replication as primers, like the terminal proteins of other linear DNAs(6-9).

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