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Bqt2p is essential for initiating telomere clustering upon pheromone sensing in fission yeast

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The telomere bouquet, i.e., telomere clustering on the nuclear envelope (NE) during meiotic prophase, is thought to promote homologous chromosome pairing. Using a visual screen, we identified bqt2/im295, a mutant that disrupts telomere clustering in fission yeast. Bqt2p is required for linking telomeres to the meiotic spindle pole body (SPB) but not for attachment of telomeres or the SPB to the NE. Bqt2p is expressed upon pheromone sensing and colocalizes thereafter to Sad1p, an SPB protein. This localization only depends on Bqt1p, not on other identified proteins required for telomere clustering. Upon pheromone sensing, generation of Sad1p foci next to telomeres depends on Bqt2p. However, depletion of Bqt2p from the SPB is dispensable for dissolving the telomere bouquet at the end of meiotic prophase. Therefore, telomere bouquet formation requires Bqt2p as a linking component and is finely regulated during meiotic progression.

Introduction

Homologous pairing during meiotic prophase is essential for homologous recombination and for chromosome segregation during meiosis I; yet, it is largely unknown how homologues approach each other. A chromosomal rearrangement called the telomere bouquet in early meiotic prophase is thought to play a key role in homologous pairing (Scherthan, 2001; Harper et al., 2004). It forms as telomeres cluster to a small region on the nuclear envelope (NE). Though the telomere bouquet is conserved in most organisms, the mechanism of its formation is not well understood.

Fission yeast, Schizosaccharomyces pombe, serves as a good model organism to study telomere bouquet formation because it has a conspicuous method for clustering telomeres. Telomeres are in subclusters scattered on the inner NE in mitotic cells and reorganized upon pheromone sensing (Chikashige et al., 1997), as telomeres start to cluster on the NE adjacent to the SPB. Such reorganization persists during premeiotic S phase and meiotic prophase, as the horsetail-shaped nucleus is driven back and forth by the microtubule arrays attached to the SPB. Therefore, the telomere bouquet could help the homologues approach each other, and the horsetail movement stretches them to facilitate their alignment.

Although many proteins have been identified in the telomere complex and the SPB, the linking components between them for bouquet formation are not well understood, and the forces that cluster telomeres have not been described. Rap1p is a telomere binding protein that is essential for telomere clustering (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). Sad1p, a spindle pole body (SPB) protein (Hagan and Yanagida, 1995) could function as a transmembrane linker for the bouquet formation. It has a transmembrane domain and a Sad1/UNC-like domain, which is an essential part of the UNC-84 protein for nuclear migration in Caenorhabditis elegans (Lee et al., 2002). These two domains are also in the SPB half-bridge protein Mps3p/Nep98p (Nishikawa et al., 2003), the reciprocal best hit with Sad1p in budding yeast. Furthermore, Sad1p interacts with Kms1p (Miki et al., 2004), a protein that is required for telomere clustering (Shimanuki et al., 1997), and with dynin light chain Dlc1p, which is required for the horsetail movement (Miki et al., 2002). Cytoplasmic microtubules are thought to be involved in clustering telomeres in fission yeast. However, this may not be the case in other organisms.

To identify more genes for bouquet formation, we developed a visual screen by monitoring heterochromatin reorganization upon meiosis. One mutant we found, im295, was defective in bouquet formation and was an allele to bqt2 that was identified in genome-wide screens by systematically deleting upregulated genes during meiotic prophase independently in two other groups (Martin-Castellanos et al., 2005; Chikashige et al., 2006). Using multiple GFP- and mCherry-tagged markers for telomeres, the SPB, and the NE, we cytologically proved that Bqt2p specifically functions as a linkage component between telomeres and the SPB. It plays a key role in initiating telomere clustering by generating Sad1p foci proximate to the telomere foci upon pheromone sensing.

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Abbreviations used in this paper: NE, nuclear envelope; SPB, spindle pole body.
Results and discussion

Identification of \textit{bqt2/im295}, a mutant defective in heterochromatin reorganization during meiotic prophase by a visual screen

The dynamic reorganization of heterochromatin during sexual development in the fission yeast can be monitored using GFP-tagged Swi6p, a homologue of heterochromatin protein 1, which binds to all telomeres and centromeres as well as the silent mating type locus (which cannot be detected as a separate identity by light microscopy). During the horsetail stage, telomeres cluster at the leading edge of the nucleus adjacent to the SPB (Fig. 1 A, arrows), whereas centromeres are released into the interior of the nucleus. The attachment of telomeres to the SPB transmits mechanical forces from microtubule arrays to chromosomes, causing stretching and alignment of the homologues (Yamamoto and Hiraoka, 2001). Both telomere clustering and horsetail movement are important for homologous pairing (Ding et al., 2004).

To identify genes involved in telomere clustering, we developed a visual screen to search for mutants with aberrant GFP-Swi6p pattern during the horsetail stage. A strain expressing GFP-Swi6 (Pidoux et al., 2000) was mutagenized by random insertion of \textit{ura4} fragments (see Materials and methods). Because haploid cells have three chromosomes, 4–7 GFP-Swi6p foci are expected during the horsetail stage, depending on the extent of centromere pairing. From 18,000 \textit{ura4} insertion mutants (i.e., \textit{im} mutants), we found several with defective telomere clustering, including \textit{dos1}, \textit{dos2}, \textit{tel2}, \textit{dhc1}, \textit{taz1}, \textit{trt1}, \textit{eta2}, \textit{matMI}, and \textit{im295}. Among these, \textit{im295} showed the most severe phenotype, as more than seven GFP-Swi6p dots were observed in most severe phenotype, as more than seven GFP-Swi6p dots B and C). Furthermore, in \textit{bqt2-null}, both the telomere bouquet and the microtubule-driven chromosome mass was not distended (Fig. 1, B and D). Therefore, even without vigorous movement of the SPB, the force generated by the microtubule arrays (Fig. 2, I and J), which was labeled with the GFP-tagged \(\alpha\)-tubulin subunit Tub1p (Ding et al., 1998). Therefore, the meiotic SPB and telomeres were no longer connected in \textit{bqt2-null} and, thus, the force generated by the microtubules failed to stretch chromosomes. To see if the disassociation of telomeres to the SPB in \textit{bqt2-null} cells depends on the vigorous movement of the SPB, \textit{dhc1-null} was used because there is only subtle movement in this mutant (Yamamoto et al., 1999). The \textit{bqt2-null}, \textit{dhc1-null} double mutants (Fig. 2 G) showed a similar Taz1p-GFP pattern to that observed in \textit{bqt2-null} (Fig. 2 B) but not in \textit{dhc1-null} (Fig. 2 F). Therefore, even without vigorous movement of the SPB, the connection between telomeres and the SPB is disrupted in Bqt2p-depleted cells.

Sad1p and Taz1p colocalized as a single spot at the leading edge of the elongated nuclei (Fig. 2 A). In \textit{bqt2-null}, however, multiple Taz1p foci were scattered in the middle of the cell, whereas a single Sad1p-mCherry spot moved to the cell tip (Fig. 2 B). Unlike in wild type (Fig. 2 H), the chromosome mass in \textit{bqt2-null} is no longer stretched along with the single Sad1p foci driven by the microtubule arrays (Fig. 2, I and J), which was labeled with the GFP-tagged \(\alpha\)-tubulin subunit Tub1p (Ding et al., 1998). Therefore, the meiotic SPB and telomeres were no longer connected in \textit{bqt2-null} and, thus, the force generated by the microtubules failed to stretch chromosomes. To see if the disassociation of telomeres to the SPB in \textit{bqt2-null} cells depends on the vigorous movement of the SPB, \textit{dhc1-null} was used because there is only subtle movement in this mutant (Yamamoto et al., 1999). The \textit{bqt2-null}, \textit{dhc1-null} double mutants (Fig. 2 G) showed a similar Taz1p-GFP pattern to that observed in \textit{bqt2-null} (Fig. 2 B) but not in \textit{dhc1-null} (Fig. 2 F). Therefore, even without vigorous movement of the SPB, the connection between telomeres and the SPB is disrupted in Bqt2p-depleted cells.

After meiotic prophase, telomeres were detached from the SPB, and the telomere foci and DNA segregates equally in wild
type (Fig. 2 C). However, nearly 40% of the bqt2-null showed abnormal distribution of chromosomes and Taz1p foci (Fig. 2, D and E). This phenotype is typical of mutants deficient in telomere clustering.

**Bqt2p is required for generating Sad1p foci next to telomeres upon pheromone sensing but not for attachment of telomeres or the SPB to the NE**

Telomeres start to cluster upon pheromone sensing before conjugation (Davey, 1998). Therefore, we analyzed telomere clustering upon pheromone response in bqt2-null cells using mam2 deletion, which does not affect the response of h+ to the pheromone release by h− cells but blocks pheromone response of h− cells and, consequently, the conjugation between h+ and h− cells. When homothallic h00, mam2-null cells are starved, the cells switched to h− cells can sense the pheromone released by h− cells, but not vice versa. Pheromone sensing induces a tip projection, called schmoo, necessary for cells to approach each other and conjugate. Schmoo and telomere clustering were blocked in starved heterothallic h− cells because of the lack of pheromone (Fig. 3 A). In nearly 30% of the starved homothallic h00, mam2-null population, cells showed either a single fully colocalized Sad1p and Taz1p foci (Fig. 3 B; 50 out of 193 cells) or more than one Sad1p spot that fully (Fig. 3 C; 6 out of 193 cells) or partially colocalized with Taz1p foci (Fig. 3 D; 26 out of 193 cells). This suggests that pheromone sensing generated Sad1p foci next to telomeres, which were pooled together by the microtubule arrays (Goto et al., 2001). The incomplete association of telomere foci and Sad1p foci suggests that the link between them is dynamic or not stable. In contrast, without Bqt2p, no telomere spots colocalized with the Sad1p foci, even in schmooing cells (Fig. 3 E; 137 cells checked). These results demonstrate that Bqt2p is essential for generating Sad1p foci next to the telomeres to initiate telomere clustering.

Although Bqt2p is essential for the association of telomeres and the SPB, it could function in one of two ways, either serving as a component of the linkage or coupling either telomeres or the SPB to the NE. In fission yeast, the SPB were observed to be free floating in some cut11 mutant cells (West et al., 1998). In budding yeast, telomeres are detached from the

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**Figure 2.** Defective telomere clustering and chromosome segregation but normal SPB movement in bqt2-null cells. Meiosis was induced, and cells were imaged after 20–26 h. (A–G) Normal telomere clustering in wild type (wt; A), abnormal telomere clustering in bqt2-null (B), wild type in early meiosis I (C), bqt2-null in early meiosis II (D) and in late meiosis II (E), abnormal telomere clustering in dhc1-null (F) and in bqt2-null, dhc1-null double mutant (G). Taz1-GFP is in green, Sad1-mCherry is in red, and DNA is in blue. (H–J) Normal microtubule behavior and DNA stretching was shown in wild type (H), and normal microtubule and SPB behavior but defective stretching of chromosome mass was shown in bqt2-null (I and J). GFP-Atb2p is in green, Sad1p-mCherry is in red, and DNA is in blue. Bar, 4 μm.
NE specifically upon meiosis entry in the *ndj1* mutant, which results in defective telomere clustering (Trelles-Sticken et al., 2000). To determine whether there was similar defect in *bqt2-null* during meiotic prophase, we used a membrane marker, D817, which is the NH₂-terminal 275 amino acid peptide of NADPH-cytochrome P450 reductase (Ding et al., 2000). In both wild type and *bqt2-null*, a single Sad1p spot remained at the tip of the NE (Fig. 3 F and G), but the NE adjacent to the tip in *bqt2-null* was stretched to form a thin line. From images of single middle z axis slices, all telomere foci were observed to attach to the inner NE in both wild type (Fig. 3 H) and *bqt2-null* cells (I) with plasmid expressing D817-mCherry. Single sections from the middle plane of the nucleus were analyzed. Taz1p-GFP is in green, D817-mCherry is in red, and DNA is in blue. Bar, 4 μm.

Bqt2p localizes to the meiotic SPB, but depletion of Bqt2p is not required for dissolving telomere clustering

The endogenous Bqt2p was COOH-terminally tagged with GFP to check its expression and localization. Its expression was not observed in starved heterothallic h⁻ cells (Fig. 4 A). But upon sensing pheromone, when Bqt2p foci fully colocalized with Sad1p-mCherry in ~40% of the h⁹⁰, mam2-null population (Fig. 4, B and C), most of them were not schmooing yet. Therefore, pheromone induced Bqt2p expression. These results were consistent with the finding that Bqt2p was required to initiate telomere clustering. Compared with data showing partial colocalization of Taz1p and Sad1p in Fig. 3 D, these results also suggest that the colocalization of Bqt2p to Sad1p does not guarantee stable connection of all telomeres to the Sad1p foci.

As expected, Bqt2p-EGFP colocalized with the meiotic SPB during the horsetail stage. However, Bqt2p remained at the meiotic SPB at early meiosis I (8 out of 9 cells with same level of signal as that during meiotic prophase, and 1 with less intense signal) and until the end of meiosis II (23 out of 30 cells; few with same level of intense signal, and most with less intense signal; Fig. 4 D). Therefore, the dissociation of Bqt2p from the meiotic SPB is not required for dissolving telomere clustering at the end of meiotic prophase.

Ectopic expression of meiosis-specific proteins sometimes mimics meiotic cellular activity in vegetative cells. This is not the case for Bqt2p, as it was preferentially located in the nucleus but did not concentrate at the SPB when expressed in vegetative cells (Fig. 4 E). Therefore, other meiosis-specific factors may be required for docking Bqt2p to the SPB.
Localization of Bqt2p to the meiotic SPB depends on Bqt1p but not on Rap1p and Kms1p

We analyzed the localization of Bqt2-GFP in several different mutants, including kms1 (Shimanuki et al., 1997), rap1 (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001), and bqt1 (Martin-Castellanos et al., 2005; Chikashige et al., 2006), all of which are defective in telomere clustering. Without Kms1p, multiple Sad1p foci are present, and Bqt2p colocalized to all the Sad1p foci (Fig. 4 F). Also, Bqt2p localized to the single Sad1p foci in the rap1-null (Fig. 4 G). These data demonstrate that Bqt2p localization to the Sad1p foci is independent of Kms1p, Rap1p, or telomeres. However, Bqt2p was no longer localized to the single Sad1p-mCherry spot in the bqt1-null (Fig. 4 H). Therefore, colocalization of Bqt2p to the meiotic SPB requires Bqt1p, and Bqt1p may link and/or stabilize the Bqt2p association with the Sad1p foci.

We have shown that Bqt2p is specifically essential for linking telomeres to the SPB and for transmitting force from the microtubule arrays to the chromosomes. Bqt2p is essential to initiating telomere clustering upon pheromone sensing, and it remains so after telomeres leave the SPB. Our observations agreed with the model for telomere clustering recently proposed by Chikashige et al. (2006). First, upon pheromone sensing, Bqt1p-Bqt2p binds to Sad1p. Once telomere binding proteins, such as Rap1p, interact with Bqt1p-Bqt2p, more Bqt2p-Bqt1p-Sad1p and other factors aggregate and attract Kms1p as well as other components of the microtubule cytoskeleton. However, other factors may be required to stabilize the connection between telomeres and the Sad1p foci. Kms1p may also function as one of these stabilizers because, in kms1 mutants, there are multiple Sad1p foci not localized with the telomere foci (Shimanuki et al., 1997), which is totally different from the pattern in dhc1 mutants (Fig. 2 F). Finally, the telomere foci are pooled together. Other forces, together with the forces generated by the cytoplasmic microtubules, are required to cluster telomeres. If this were not the case, we would expect that the Sad1p spot at the mitotic SPB would not colocalize with any telomere spots in dhc1-null, but this colocalization was observed (Fig. 2 F). Our hypothesis is consistent with the telomere bouquet formation in other organisms, which can occur without nuclear movement in meiotic prophase. At the end of meiotic prophase, the telomere bouquet is dissolved possibly by regulating the stabilizers for the linkage between telomeres and the SPB.

Materials and methods

General techniques, plasmid, and yeast strain collection

Methods and media for fission yeast were as described previously (Moreno et al., 1991). Molecular cloning was done as described previously (Sambrook and Russell, 2001).

The following plasmids were used in this study: pUC18, p3BV, p4BV, p5BV, p6BV, pFA6a-kanMX6, p17kanMX6, p13his5, pKSura, p16ura4, p18hph, p31tadh, p32Pnmt1, pEFGP, p42egfp, pFakanMX6, p46mCherry, p157b2egfpOE.
The following fission yeast strains were used in this study: YS127 [his6, nmt41::gfp-swi6::LEU2 (at ars1 locus), leu1-32, ura4D18], IM295 [ura4D18 insertion in b2t2 from the screen in Y127], Ty104 [his6, sad1-mCherry::ura4, tat1-gfp::his5, his5, ura4D18], YS783C3 [his6, tat1-gfp::his5, his5, ura4D18], YS788 [his6, tat1-gfp::his5, his5, ura4D18], YS128 [his6, sad1-mCherry::ura4, tat1-gfp::his5, his5, ura4D18], Ty103 [his6, tat1-gfp::his5, his5, ura4D18] (p157D817gfp, p157D817mCherry, p23bq2 and p157B2gfpOE).

Plasmid construction
Fragment was amplified by PCR using pUC18 as the template and two primers (5'-gaagagctctgcgaatcgcggatcagttcagagcagttcagagcagttcagagcagccgcaagca-3' and 5'-gaagagctctgcgaatcgcggatcagttcagagcagttcagagcagttcagagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagccgcaagccaggcaacagcagccgcaagccaggcaacaaggcaagccggcaagccagggcaagccaggcaacagcagc
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