Saccharomyces cerevisiae RNA Polymerase II Is Affected by Kluyveromyces lactis Zymocin

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Daniel Jablonowski and Raffael Schaffrath
From the Institut für Genetik, Biologiestein, Martin-Luther-Universität Halle-Wittenberg, Weinbergweg 10, D-06120 Halle (Saale), Germany

The G₁ arrest imposed by Kluyveromyces lactis zymocin on Saccharomyces cerevisiae cells requires a functional RNA polymerase II (pol II) Elongator complex. In studying a link between zymocin and pol II, progressively truncating the carboxyl-terminal domain (CTD) of pol II was found to result in zymocin hypersensitivity as did mutations in four different CTD kinase genes. Consistent with the notion that Elongator preferentially associates with hyperphosphorylated (II0) rather than hypophosphorylated (IIA) pol II, the II0/IIA ratio was imbalanced toward II0 on zymocin treatment and suggests zymocin affects pol II function, presumably in an Elongator-dependent manner. As judged from chromatin immunoprecipitations, zymocin-arrested cells were affected with regards to pol II binding to the ADH1 promoter and pol II transcription of the ADH1 gene. Thus, zymocin may interfere with pol II recycling, a scenario assumed to lead to down-regulation of pol II transcription and eventually causing the observed G₁ arrest.

Yeast killer toxins are genetically and biochemically diverse. The double-stranded RNA-encoded viral toxins KT28 and K1 from Saccharomyces cerevisiae (1) bind to cell wall mannan and glucan moieties (2, 3), while zymocin, a double-stranded DNA-encoded three-subunit α(βγ) protein complex from Kluyveromyces lactis, requires cell wall chitin for docking (4, 5). KT28 and K1 use retrograde import (6), block DNA synthesis (7), or destroy cytoplasmic membrane function through TOK1 K⁺-channel hyperactivation (8), whereas zymocin arrests S. cerevisiae by a G₁ cell cycle block (9–11). Expression of the γ-subunit of zymocin, the γ-toxin, is sufficient to mimic this arrest (12, 13). As for the target of γ-toxin (TOT), mutations in seven TOT genes lead to zymocin resistance. TOT1–3 and TOT5–7 are identical with ELP3 and ELP1–6 coding for RNA polymerase II (pol II) Elongator and TOT4 (KTI12) specifies an Elongator-associated protein (14–24). In addition, loss of SIT4, KTI11, and KTI13 results in a tot phenotype, suggesting these genes may play a role in TOT function too (25, 26). Elongator is conserved from yeast to man (27), associates with hyperphosphorylated pol II (II0) (16), and is thought to play a role in transcription by virtue of its Elp3p subunit, a histone acetyltransferase (HAT) (17). Consistently, combining deletions of ELP3 and CTK1 coding for the α-subunit of CTDK-I (28), a transcriptionally relevant pol II carboxyl-terminal domain (CTD) kinase, is synthetically lethal (29). Reducing the HAT activity of Elongator phenocopies zymocin resistance (19, 22), implying that the HAT is essential for K. lactis zymocinicity. TOT can be dissociated from Elongator function by ELPS/TOT3 mutagenesis, suggesting that Elongator communicates with γ-toxin in a HAT-dependent manner (23). As Elongator is non-essential, it cannot simply be blocked by γ-toxin. Instead, its function may be modified so that pol II activity becomes poisoned. Evidences demonstrating down-regulation of pol II-dependent genes (22) and expression of partial zymocin resistance on overproducing Fcp1p, the CTD phosphatase, support this notion (23).

To study a link between zymocin action and pol II function, we found progressive CTD truncations and mutations in CTD kinase genes to result in zymocin hypersensitivity. Remarkably, zymocin treatment led to accumulation of pol II form II0. As judged from chromatin immune precipitations (CHIP), zymocin-arrested cells were affected with regards to pol II binding to and transcription of the ADH1 locus. Our data imply that zymocin affects, in a manner presumably dependent on Elongator, pol II function, a scenario assumed to lead to down-regulation of pol II transcription and eventually cause the observed G₁ arrest.

EXPERIMENTAL PROCEDURES

All yeast strains used in this study are described in Table I. Standard YPD (1% yeast extract, 2% peptone, 2% dextrose) and SD (0.67% yeast nitrogen base, 2% dextrose) media were prepared as described (30). Zymocin sensitivity or resistance was assessed using the killer eclipse assay (31) and zymocin YPD plate assays. The latter involved partial purification of zymocin from AW137 cell-free culture supernatants and supplementation to YPD plates (5, 23). Zymocin arrest of S. cerevisiae strain LS20 involved partial purification of zymocin from K. lactis killer AW137 grown in YPD flask cultures for 48 h at 30 °C. After centrifugation (6,000 rpm) 50 ml of cell-free supernatants were mixed with 50 ml of 2X YPD medium and inoculated with LS20 cells. These were arrested by zymocin on further incubating for 3–6 h at 30 °C. As controls, untreated LS20 cultures were incubated for the same period of time. Following harvest (6,000 rpm at 4 °C), cells were disrupted by the glass bead method using a multivortex (five 60-s bursts at 4 °C) in 500 µl of breaking buffer B60 (32). After clearing the cell lysates protein content was measured using the Bio-Rad kit. Identical amounts of total protein obtained from three independent experiments of untreated and zymocin-arrested cells were subjected to SDS-PAGE analysis using 6% gels, electroblotted onto polyvinylidene difluoride membranes (Immobilon), and probed with mouse monoclonal anti-pol II IA and anti-pol II antibodies SWG16 and H14 (Covance). Protein loadings were compared using a polyclonal rabbit antibody directed against the α- and β-subunits of yeast Pfk1p (33). Pol II quantitation used the Image Master 2D (Amersham Biosciences) program.

CHIPs were carried out as described (34). In brief, untreated and...
zynocin-arrested yeast cells grown to \( A_{600} \approx 1.0 \) in YPD were cross-linked for 15 min in 1% (v/v) formaldehyde. Following addition of 2.5 ml of 2.5 M glycine and three 1°C TBS (20 mM Tris-HCl, pH 7.6, 157 mM NaCl) washes, cells were lysed with glass beads in FA lysis buffer (34). Sheared chromatin solution was added to the anti-CTD and anti-CTD-SS-P antibodies SWG16 and H14 (Covance) and incubated for 90 min. Following the addition of secondary antibodies coupled to protein A-Sepharose CL-4B beads (Amersham Biosciences) and incubation for a further 60 min, chromatin was immunoprecipitated, washed, and incubated at 65°C for 10 min. Following SDS treatment, chromatin was subjected to protease treatment and reversal of the cross-links (35). For all subsequent PCR reactions a single preparation of immunoprecipitated chromatin served as template. PCR conditions and electrophoresis were as described (34). Primers used were as follows: ADH173, GGT ATA GCT TG; ADH146, ACG CTT GGC ACG GTG ACT G; ADH372, CAA TCA AGG; ADH 1271, ATA AGA GCG ACC TCA TGC TAT ACC; and all subsequent PCR reactions a single preparation of immunoprecipitated chromatin served as template. PCR conditions and electrophoresis were as described (34). Primers used were as follows: ADH173, GGT ATA GCT TG; ADH146, ACG CTT GGC ACG GTG ACT G; ADH372, CAA TCA AGG; ADH 1271, ATA AGA GCG ACC TCA TGC TAT ACC; and IGRVII-2, CCA CTT TCT TCA TTC ACG CAC ACT; ADH SRB10 (34)). Moreover, disruption of either the B to have any effect (Fig. 1).

**RESULTS**

**Effects of Mutations in pol II CTD Kinase Genes on Zynocin Sensitivity**—Our previous findings that pol II Elongator mutations confer zynocin resistance (22) and that overexpression of Fcp1p, the pol II CTD-phosphatase (36), results in reduction of zynocin hypersensitivity, strongly suggest a functional link between zynocin and pol II, and Elongator. To ask whether pol II activity is necessary for the response of a yeast cell to zymocin, we assayed deletions of **SRB10** and **CTK2** for their sensitivity against zymocin. To test whether pol II activity is necessary for the response of a yeast cell to zymocin, we assayed deletions of **SRB10** and **CTK2** for their sensitivity against zymocin. To test whether pol II activity is necessary for the response of a yeast cell to zymocin, we assayed deletions of **SRB10** and **CTK2** for their sensitivity against zymocin.

![Fig. 1](https://via.placeholder.com/150)

**FIG. 1.** Effect of mutations in the CTD-CDK genes on zynocin sensitivity expression of *S. cerevisiae*. A, killer assay with bar-1 and bar-2 mutants. The indicated strains were tested against a *K. lactis* strain (upper row, killer strain AWJ137; lower row, non-killer strain NK40) and incubated for 1 day at 30°C. Eclipse formation around the killer strain indicates zynocin sensitivity. *total 3A* cells served as zymocin-resistant control. B, YPD zymocin plate assays with *ctk1*, *ctk2*, and *ctk3* cells. The indicated strains were serially diluted and spotted onto YPD-rich medium lacking zymocin (control) or being supplemented with the indicated amounts of zymocin (percent, v/v). C, YPD zymocin plate assays with *srb10* and *srb11* cells. Strains were prepared as in the legend to B. D, killer eclipse assay with the *kin28* mutant. Strains were processed as described in the legend to A. Note that all CTD-CDK mutants express hypersensitivity toward the *K. lactis* zynocin.

**their isogenic wild-type parent, *srb10A* and *srb11A* cells became arrested by less amounts of zymocin. Consistently, a *KIN28* mutant (*kin28*t17D, Ref. 34) with reduced kinase activity was found to be zynocin-hypersensitive, too, when tested in eclipse (Fig. 1D) or zymocin plate assays (not shown). In conclusion, genetic scenarios that impair pol II function by (partial) loss of CTD-CDK activities alter the responsiveness of a yeast cell to zymocin and cause hypersensitive phenotypes. Thus, cells with reduced CTD activities require less amounts of zymocin than wild-type cells to become arrested in G1. This finding strongly suggests a functional link between zymocin mode of action and the CTD of pol II.**

**Effects of pol II-CTD Truncation Mutations on Zynocin Sensitivity**—Inspired by reports that underproduction of pol II (42) phenocopies the effects of CTD truncation mutations, i.e. slow growth, inositol auxotrophy, and thermosensitivity (43, 44), and by our observation that pol II underassembly leads to zynocin hypersensitivity (23), we studied the effects of progressive truncations of the CTD of pol II on the response of a yeast cell to zymocin. As can be deduced from the appropriate YPD zymocin plate assays presented in Fig. 2B, most viable CTD truncations (Ref. 45; Fig. 2, D, pV7 and pV17) behaved comparable with wild-type full-length CTD and unaltered with respect to zymocin sensitivity phenotypes on plates supplemented with 45–50% (v/v) zymocin. However, the penultimate and ultimate viable CTD truncations (Ref. 45; Fig. 2, D, pV20 and pV17), and all progressive ones reported to express...
conditional phenotypes (Ref. 45; Fig. 2, pC2, pC23, pC6, pC1, and pC3) were found to confer hypersensitivity to zymocin (Fig. 2). Thus, hypersensitive phenotypes could be correlated with conditional phenotypes of CTD truncations. This appears to be consistent with the effects of pol II underassembly on expression of hypersensitivity toward zymocin (23). Taken together with the above findings on the effect of CTD-CDK mutants on zymocin sensitivity, other scenarios that lead to an overall reduction of pol II function convert sensitive yeast cells into hypersensitive ones. This reinforces the notion that zymocin action is linked to a functional pol II CTD.

pol II CTD Phosphorylation States in Response to Zymocin Treatment—Since pol II Elongator is essential for zymocicity (22) and stably associates with pol II form II0 (16), we next asked whether zymocin may require Elongator to be associated with II0 and whether zymocin influences the phosphorylation equilibrium of pol II. To do so, identical protein amounts obtained from zymocin-untreated and zymocin-arrested cells after 3 and 6 h were subjected to 6% SDS-PAGE and immunoprobed with monoclonal antibodies specific for the hypophosphorylated CTD of pol II (IIA) and the hyperphosphorylated CTD of pol II (II0). Protein loading was followed using a polyclonal antiserum specific for the α- and β-subunits of phosphofructokinase 1 (Pfk1p) (33).

FIG. 3. Effect of zymocin application on pol II phosphorylation states. A, identical protein amounts obtained from zymocin-untreated and zymocin-arrested cells after 3 and 6 h were subjected to 6% SDS-PAGE and immunoprobed with monoclonal antibodies specific for the hypophosphorylated CTD of pol II (IIA) and the hyperphosphorylated CTD of pol II (II0). Protein loading was followed using a polyclonal antiserum specific for the α- and β-subunits of phosphofructokinase 1 (Pfk1p) (33). B, identical protein amounts obtained from cell fractionation of untreated cells and cells arrested with zymocin for 3 h were subjected to 6% SDS-PAGE and immunoprobed with antibodies specific for IIA and II0 (see A). Protein loading was followed as described in the legend to A.
Zymocin Affects RNA pol II Function

The recent identification of the pol II Elongator complex as TOT, the putative target of the K. lactis zymocin (22), has implied a link between zymocin action and pol II transcriptional elongation (23). Since TOT/Elongator is dispensable for life, its function cannot simply be blocked by zymocin (and its γ-toxin subunit). Instead, it has been proposed that Elongator becomes modified so that pol II activity is poisoned (22, 23). To check this model we asked whether cells, compromised for pol II function, are altered with respect to their response to zymocin too. Genetic scenarios leading to reduced pol II function by mutating individual pol II CTD-CDK genes or by progressively truncating the CTD itself were found to confer zymocin hypersensitivity. Thus, the ability of a cell to express wild-type zymocin sensitivity is at least in part determined by the length of the CTD and the presence of each of the four known yeast CTD-CDK genes. Presumed stability of the CTD of the pol II subunit in these mutants was not affected (and for CTD truncations variants this has been shown; Ref. 44), but rather assembly of pol II holoenzyme was interfered with so that its function was compromised with respect to interaction with mediator (during initiation) or with Elongator (on CTD phosphorylation and promoter clearance); both scenarios, CTD truncation and CTD-CDK mutation, would ultimately lead to a decline of pol II activity. Consistently, CTD truncations and CTD-CDK mutations phenocopy the effects of underproducing the largest CTD-subunit of pol II, Rpb1p, which include slow growth, temperature sensitivity, inositol auxotrophy, and zymocin hypersensitivity (23, 42, 45). Together with the facts that zymocin action requires a functional pol II Elongator complex and that overexpressing Fcp1p, the CTD-phosphatase of pol II, renders cells partially zymocin-resistant (23), these findings suggest that zymocin action requires Elongator to be associated with pol II0 while elongating. Zymocin hypersensitivity due to the above conditions (pol II underassembly, CTD deletions, and CTD-CDK mutations) may imply that in the mutant cells the equilibrium between pol II-free and pol II-bound Elongator is imbalanced, eventually leading to a net increase of Elongator capable of signaling the presence of zymocin. In support of this, the requirement of TOT for γ-toxin and zymocin action was shown to be separable from Elongator wild-type function by TOT3/EFP3 mutagenesis, suggesting that Elongator communicates with γ-toxin (directly or indirectly) in a manner dependent on its HAT subunit (23).

How to integrate these findings in a model that explains both requirement of Elongator for and involvement of pol II in zymocin action? Our observations that zymocin-arrested cells accumulate pol II form I10 and that binding of pol II to the ADH1 promoter is dramatically reduced following zymocin treatment are indicative for pol II function being affected. Consistently, we have demonstrated down-regulation of pol II activity (22) in the presence of zymocin, while pol I activity

Fig. 4. Effect of zymocin treatment on pol II binding to and transcription through the ADH1 locus. A, a schematic representation of the primer pairs used to PCR-amplify regions upstream of and within the ADH1 locus following CHIP experiments on untreated and zymocin-arrested cells. The open bar represents the open reading frame (ADH1); the gray bars indicate PCR products with coordinates relative to the initiation codon of the open reading frame. B, CHIP/PCR. Cross-linked and sheared chromatin obtained from zymocin-untreated and zymocin-arrested cells was immunoprecipitated with the indicated antibodies. After reversal of cross-linking and purification of the DNA, PCR was used to test for the presence of promoter or coding sequences (CDS). Each CHIP/PCR panel contained a second primer pair that amplifies an intergenic region (IGR) of chromosome VII, thus providing an internal background control. Input (bottom) shows the signal from the chromatin before immunoprecipitation.

untreated and zymocin-arrested cells while being bound to and transcribing through the ADH1 locus. To do so, CHIPS were carried out with anti-IIA (8WG16) or anti-II0 (H14) antibodies and subjected to PCR using primers to amplify DNA fragments specific for the ADH1 promoter or its coding sequences (Fig. 4A). Consistent with a previous CHIP study (34), our analysis demonstrated that in untreated cells the predominant fractions of pol II form IIA are found at the ADH1 promoter and the early coding sequence (Fig. 4B, left upper panel), while hyperphosphorylated pol II form I10 was preferentially found to associate with the promoter. As soon as it clears the promoter there is a significant decrease of H14-mediated recognition, indicating that the CTD of pol II is subject to dephosphorylation (Fig. 4B, left middle panel). Thus, initially in early elongation pol II is heavily phosphorylated on the Ser5 of CTD, whereas later in elongation Ser5 becomes dephosphorylated (34). In striking contrast to this pattern were CHIPS performed with zymocin-arrested cells; while pol IIA bound to the promoter was drastically reduced compared with untreated cells, it was found to be predominantly associated within the early coding sequence of the ADH1 gene after zymocin application (Fig. 4B, right upper panel). As for pol II form I10, zymocin treatment significantly interfered with its capability to bind to the ADH1 promoter, resulting in a steep decrease compared with the parallel experiment without zymocin (Fig. 4B, right middle panel). While the latter finding suggests that zymocin-arrested cells are transcriptionally impaired with regards to recruiting pol II form IIA to the ADH1 promoter, the former observations may be interpreted as pol II recognized by the 8WG16 antibody being stalled during early ADH1 transcription elongation. Taken together, these CHIP studies suggest that zymocin action is linked to pol II function and imply that zymocin negatively interferes with the pol II transcription cycle by affecting the recycling of initiation-competent IIA from elongation-competent I10.

DISCUSSION

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largely remains unaffected (22). Thus, zymocin appears to preferentially affect pol II rather than pol I (or pol III) function. An attractive, though speculative, model is how zymocin might work at the molecular level involves γ-toxin to transform Elongator so that pol II becomes limiting. If this conversion conferred a dominant negative effect onto pol II function for example by blocking pol II in its II0 form, zymocin-treated cells would be expected to be seriously affected with regards to pol II recycling during transcription. In favor of this are our findings showing that the IIA/IIO ratio is imbalanced toward form II0 following zymocin application. Also, the CHIP studies demonstrate that form IIA predominantly accumulates during early transcription elongation within the ADH1 gene, while pol III is significantly impaired with regards to ADH1 promoter binding and clearance. Thus, pol II recycling necessary for preinitiation complex formation is likely affected by zymocin. Such a scenario can be ultimately envisaged to result in a decline of overall pol II activity and might eventually, due to a transcriptional shutdown of the START-specific gene program, culminate in the observed G1 cell cycle arrest. Consistent with this notion are temperature-sensitive mutations in the largest CTD subunits of yeast and hamster pol II, which cause a conditional G1 arrest at START (46, 47). Thus, cell cycle progression and START execution appear to be particularly sensitive to an impaired pol II function.

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