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Rtr1 Is the *Saccharomyces cerevisiae* Homolog of a Novel Family of RNA Polymerase II-Binding Proteins

Patrick A. Gibney, Thomas Fries, Susanne M. Baier, and Kevin A. Morano

*Department of Microbiology and Molecular Genetics, University of Texas Medical School, Houston, Texas 77030, and Universitàt des Saarlandes, Medizinische Biochemie und Molekularbiologie, Gebäude 61.4, D-66421 Homburg/Saar, Germany*

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Cells must rapidly sense and respond to a wide variety of potentially cytotoxic external stressors to survive in a constantly changing environment. In a search for novel genes required for stress tolerance in *Saccharomyces cerevisiae*, we identified the uncharacterized open reading frame YER139C as a gene required for growth at 37°C in the presence of the heat shock mimetic formamide. YER139C encodes the closest yeast homolog of the human RPAP2 protein, recently identified as a novel RNA polymerase II (RNAPII)-associated factor. Multiple lines of evidence support a role for this gene family in transcription, prompting us to rename YER139C *RTR1* (regulator of transcription). The core RNAPII subunits RPB5, RPB7, and RPB9 were isolated as potent high-copy-number suppressors of the *rtr1Δ* temperature-sensitive growth phenotype, and deletion of the nonessential subunits RPB4 and RPB9 hypersensitized cells to *RTR1* overexpression. Disruption of *RTR1* resulted in mycophenolic acid sensitivity and synthetic genetic interactions with a number of genes involved in multiple phases of transcription. Consistently, *rtr1Δ* cells are defective in inducible transcription from the *GAL1* promoter. Rtr1 constitutively shuttles between the cytoplasm and nucleus, where it physically associates with an active RNAPII transcriptional complex. Taken together, our data reveal a role for members of the RTR1/RPAP2 family as regulators of core RNAPII function.

Transcription of mRNA and most snRNAs in eukaryotic cells is carried out by the RNA polymerase II (RNAPII) enzyme, consisting of 12 protein subunits (Rpb1 to Rpb12). Five of these (Rpb1, Rpb2, Rpb3, Rpb6, and Rpb11) are homologous to counterparts in bacteria, and six others (Rpb4, Rpb5, Rpb7, Rpb9, Rpb10, and Rpb12) bear resemblance to archaeal RNA polymerase subunits (64). Many of these subunits are also represented by highly conserved homologs in the eukaryotic RNAPI and -III enzymes responsible for rRNA and tRNA synthesis, respectively (52). In addition to the core subunits, numerous additional protein cofactors are required for regulated and accurate gene expression, including the Mediator, Elongator, and SAGA complexes (39). Diverse cellular signals such as environmental stressors (heat, oxidative chemical), nutrient conditions, and proliferation state feed into the transcription machinery at multiple steps (44). One regulatory mechanism involves reversible phosphorylation of the carboxy-terminal domain (CTD) within RNAPII (51). The CTD contains multiple repeats of a conserved motif (YSPTSPS) subject to hyperphosphorylation by RNAPII kinases such as Kin28 and Srb10 (26). Phosphorylation of the Rpb1-CTD at serines in the second and fifth positions within the heptad repeat is associated with transition from preinitiation to a transcriptionally active elongation complex (17). With the scores of known signal inputs, multiple layers of regulation, and hundreds of described components, uncharacterized genes that may play important roles in modulating transcription undoubtedly still exist.

One facet of gene expression that remains largely unexplored is the ability of the transcription machinery to remain active under stress conditions. Both nonessential subunits of RNAPII, Rpb4 and Rpb9, are required for stress tolerance (14, 45, 47, 65, 66). Rpb4 is involved in mRNA export during cellular stress, transcription-coupled DNA repair, sporulation, pseudohyphal growth, recovery from stationary phase, and transcription at elevated temperatures (2, 20, 40, 53, 56). Rpb4 associates with an essential subunit, Rpb7, to form a readily dissociable heterodimer that interacts with the other subunits in a stress-responsive manner (13). This complex engenders tighter interaction between RNAPII and its DNA substrate and is thought to facilitate transcription during both normal and stress conditions. Not unexpectedly, deletion of *Rpb4* results in a temperature-sensitive growth phenotype, likely due to cessation of transcription after shift to the nonpermissive temperature (47). Interestingly, Rpb4 is primarily nuclear in unstressed cells and translocates to the cytoplasm in response to some types of stress (20). Rpb9 has been shown to be involved in accurate start-site selection, transcription through arrest sites, regulation of transcription elongation, and transcription-coupled DNA repair (3, 25, 28, 40). Deletion of *Rpb9* also results in temperature sensitivity, but the specific role of Rpb9 in transcription during periods of environmental stress has not been elucidated (65).

In an attempt to discover and characterize novel heat shock genes, we identified the open reading frame (ORF) YER139C. Deletion of YER139C results in a temperature-sensitive phenotype, indicating a putative role in heat stress homeostasis. YER139C and putative higher eukaryotic homologs contain a domain of unknown function with significant similarity to zinc-finger motifs, and mutation of conserved cysteine and histidine residues in this region abolished gene function. Initial charac-
terization of this gene strongly links it to regulation of gene expression through genetic interaction with RNAPII and with associated transcriptional regulators. We show that Yer139c shuttles between the cytoplasm and nucleus and physically interacts with the transcriptionally active form of Rpb1. Cells lacking YER139C display a transcriptional defect in inducible expression from the GAL1 promoter, demonstrating the physiological significance of the genetic and biochemical interactions. We have therefore named this gene RTR1 (for “regulator of transcription”). A recent proteomic analysis has identified the human homolog of RTR1 among a group of novel RNAPII-associated proteins, supporting our proposed in vivo role for Rtr1 in transcription (30).

MATERIALS AND METHODS

Sacharomyces cerevisiae methodology. S. cerevisiae strains were grown on media containing 2% dextrose, sucrose, or galactose as indicated. Unless otherwise noted, strains were grown at 30°C. Rich yeast extract-peptone-dextrose (YPD) growth medium was prepared containing 1% yeast extract, 2% peptone, and 2% dextrose (or another sugar, as indicated). Synthetic complete (SC) medium growth medium was prepared containing 1% yeast extract, 2% peptone, and 2% dextrose, sucrose, or galactose as indicated. Unless otherwise noted, transformation procedures were employed (31). Plate growth assays were carried (100

Northern blot analysis of gene expression. Expression of galactose-inducible RTR1 and constitutively expressed genes was determined by Northern analysis as follows. A total of 2.5 μg of total RNA was loaded on a 1% denaturing agarose gel and transferred to a positively charged nylon membrane. The membrane was prehybridized in ExpressHyb (Clontech) at 65°C for 1 h and then incubated with a [32P]dCTP-labeled probe (200 mCi/ml) at 65°C overnight. Membranes were washed with 2xSSC, 0.1% SDS at 65°C and exposed to X-ray film using a phosphor screen, a Nikon DS-5Mc color camera, and NIS-Elements F2.30 software (Nikon). After autoradiography, the membranes were rehybridized with a different probe and reexposed to the film with the same exposure parameters.

Northern blot analysis of the RTR1 transcript. Strain BY4741 grown to an optical density OD600 of 0.5 was frozen in liquid nitrogen and stored at -80°C. Plasmid DNA was isolated using the Qiagen plasmid isolation kit. Northern blot analysis was performed as described above. The blot was probed with a 0.5-kb fragment of RTR1 amplified from BY4741 genomic DNA by use of primer 5′-GACGAAGGCTTACATGCTG-3′ (forward) and 5′-CTTCATGGAGCAATGTCTC-3′ (reverse) using Vent polymerase, and the blot was washed in 0.2xSSC and 0.1% SDS and exposed to X-ray film using a phosphor screen, a Nikon DS-5Mc color camera, and NIS-Elements F2.30 software (Nikon Instruments Europe B.V., Düsseldorf, Germany) and processed in Adobe Photo- shop 4 (Adobe Systems Inc., San Jose, CA).

Northern blot analysis of gene expression. Expression of galactose-inducible and constitutively expressed genes was determined by Northern analysis as fol-
TABLE 2. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description (cloning sites)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>p416CUP1</td>
<td>Low copy number; weak constitutive promoter</td>
<td>36</td>
</tr>
<tr>
<td>p416CUP1-RTR1</td>
<td>C-terminal HA-tag fusion (SpeI/XhoI)</td>
<td>This study</td>
</tr>
<tr>
<td>p416CUP1-RTR1C128, H116S-HA</td>
<td>C-terminal HA-tag fusion (SpeI/XhoI)</td>
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<td>pFA0a-3HA-His3MX6</td>
<td>Contains both a triple HA epitope and a hexahistidine coding sequence</td>
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<td>p416CUP1-GFP-RTR1</td>
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<tr>
<td>p423GPD</td>
<td>High copy number; strong constitutive promoter</td>
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<tr>
<td>p423GPD-RTR1-V5</td>
<td>C-terminal V5-tag fusion (SpeI/XhoI)</td>
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<td>N. Woychik</td>
</tr>
<tr>
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<tr>
<td>p16-RTRI</td>
<td>Low copy number; native RTR1 promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pRS315 pNOP-GFP-RTR1</td>
<td>N-terminal GFP fusion (PstI/XhoI)</td>
<td>This study</td>
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<td>pRS315 pNOP-ProA-RTR1</td>
<td>N-terminal protein A fusion (PstI/XhoI)</td>
<td>This study</td>
</tr>
</tbody>
</table>

* MCS, multiple cloning site.

RESULTS

*rtr1Δ* cells are temperature sensitive, whereas overexpression of RTR1 causes a growth defect. In an effort to uncover novel genes required for thermotolerance, we utilized published microarray data to identify unnamed genes induced in response to heat shock (11, 22). In our initial phenotypic characterization of haploid knockouts of these genes, cells carrying a disruption in the YER139C locus were exquisitely sensitive to 41°C (Fig. 1B).

We next examined the consequences of elevated RTR1 expression. Relative growth rates of wild-type cells transformed with an empty vector, a low-copy-number vector expressing *RTR1*, or a high-copy-number *RTR1* expression vector were assessed as shown in Fig. 1C. Increased expression of *RTR1* caused a dose-dependent reduction in growth rate: cells carrying an empty vector (low- or high-copy number) grew with a doubling time of approximately 2.1 h, those carrying an extra copy of *RTR1* on a *CEN* plasmid grew with a doubling time of approximately 2.7 h, and those from a 2-μ-based high-copy-number vector grew with a doubling time of approximately 2.7 h.

*Rtr1* contains a conserved nonconsensus Zn-finger-like motif essential for function. Analysis of the primary amino acid sequence of Rtr1 revealed a cysteine-rich amino-terminal motif reminiscent of a Zn finger (C-x4-C-x27-C-x3-H; Fig. 2A). However, the spacing and arrangement of the putative Zn-coordinating cysteine and histidine residues are novel, with no precise match to known Zn-finger modules. BLAST analysis revealed that this motif, including the intervening amino acids between the cysteine and histidine residues, is highly conserved in a wide range of eukaryotic species, including fission yeast (*Schizosaccharomyces pombe*), amoebae (*Dictyostelium discoideum*), mice, and humans. Homologs are notably absent from both the bacteria and archaea, suggesting that the *RTR1* gene family is unique to eukaryotes. The cysteine residues are invariant in these putative homologs, with a high (approximate) degree of sequence identity in the immediately adjacent regions. *RTR1* sequence similarity drops to negligible levels among the more distantly related species outside of this con-
proteins. Replacement of either cysteine module completely blocked complementation of the rtr1Δ formamide-temperature sensitivity phenotype (Fig. 2B). To determine whether these rtr1 mutant alleles were stably expressed, cells were grown to logarithmic phase, whole-cell extracts were isolated, and Rtr1 proteins were detected by immunoblot analysis. Both the rtr1(C73S) and the rtr1(C112S, H116S) mutants were produced at wild-type levels (Fig. 2C), demonstrating that lack of complementation was not due to destabilization. Moreover, these results suggest that the putative Zn finger is not required for Rtr1 structural integrity and may instead be involved in protein-protein or protein-nucleic acid interactions.

We next sought to determine whether overexpression toxicity likewise required the cysteine-rich domain. The conserved region comprises approximately half of the 226-amino-acid sequence of the Rtr1 protein. We therefore generated a series of constructs overexpressing the full-length protein or the amino- or carboxy-terminal halves of the protein. For convenience, the small (14-amino-acid) V5 epitope tag was engineered into the amplifying oligonucleotide primers to facilitate evaluation of protein expression. As a control, full-length Rtr1 was synthesized with either an amino- or a carboxy-terminal V5 tag, each of which was inhibitory to growth when overexpressed, as shown in Fig. 2D (see V5-Rtr1 and Rtr1-V5 data; doubling time, 2.7 and 3.1 h, respectively, versus 1.9 h for strains carrying the empty vector). In addition, the overexpressed nonconserved carboxy-terminal half of the protein was equally toxic (Rtr1141-226-V5; 2.8 h doubling time). In contrast, the amino-terminal half of the protein containing the cysteine-rich domain (V5-Rtr11-140) did not result in a growth defect when overexpressed (doubling time, 2.1 h). This mutant protein was stably produced, as detected by immunoblotting (data not shown). Growth inhibition was further exacerbated at 37°C. Together, these data demonstrate that Rtr1 is the yeast homolog of members of a larger eukaryotic gene family that share a highly conserved cysteine-rich motif in the amino terminus that is required for function in vivo. Moreover, the nonconserved carboxy-terminal half of Rtr1 appears to dramatically hinder cell growth when overexpressed in yeast, suggesting the need for a stoichiometric balance of this protein with other cellular components essential for growth under normal and heat shock conditions.

**Rtr1 constitutively shuttles between the cytoplasm and nucleus.** As part of our characterization of the Rtr1 protein, we determined the subcellular localization of Rtr1 by use of a functional GFP-tagged allele expressed from a low-copy-number vector. Under both normal growth conditions (25°C) and elevated temperatures (35°C), GFP-tagged Rtr1 localized within the cytoplasm, in agreement with a previously published proteome-wide localization study (Fig. 3A) (27). We were unable to detect changes in the fluorescence localization pattern upon exposure to other environmental stresses (data not shown). Yeast two-hybrid analysis suggested that Rtr1 may gain access to the nucleocytoplasmic transport system via interaction with Ran (8). Therefore, to test for nuclear localization of Rtr1, GFP-Rtr1 was expressed in cells lacking Xpo1 (Crm1), the major nuclear export factor that shuttles NES-containing proteins out of the nucleus (60). Inactivation of this protein has been shown to lead to accumulation of substrates with rapid transit kinetics (7, 60). Using the temperature-sen-
sitive allele xpo1-1, we observed prominent nuclear localization within 8 min after shifting to the nonpermissive temperature (Fig. 3B). These data demonstrate that Rtr1 constitutively shuttles between cytoplasm and the nucleus and is actively transported out of the nucleus via the Xpo1 system.

Suppression of rtr1Δ temperature sensitivity by overexpression of RNAPII core subunits. In an effort to understand the cellular role of Rtr1, we undertook a high-copy-number suppressor screening to identify genes whose overexpression could repair the temperature-sensitive growth defect caused by the loss of RTR1. To accomplish this, we transformed a high-copy-number yEP24-based genomic library into the rtr1Δ background and selected for growth at 37°C in the presence of 2% formaldehyde (form) and incubated at the indicated temperatures for 3 days. (C) The strains described for panel B were grown and protein was extracted as described in Materials and Methods. Western blot analysis was used to detect stably produced protein with anti-HA and anti-PGK. WT, wild type; vec, vector. (D) V5-epitope-tagged versions of Rtr1 were constructed as described in Materials and Methods, all by using the high-copy-number plasmid p423GPD as the backbone. These constructs were transformed into wild-type (BY4741) yeast and plated by spot dilution onto selective media (SC-HIS). The plates were incubated for 2 days at 30°C.

FIG. 2. RTR1 contains an essential, conserved zinc-finger-like motif. (A) Homologs of RTR1 from the indicated species are shown in the diagram. Percent identity with RTR1 cysteine-rich domain is indicated, along with total amino acid length. Clustal alignment of the RTR1 zinc-finger-like motif and its mouse (M.m; NCBI accession BA36627) and human (H.s; NCBI accession NP_079089) homologues (shown at the bottom of the panel) are shown, with Zn-finger-like residues highlighted and their positions in the Δ ceryae sequence indicated. (B) rtr1Δ yeast cells were transformed with vectors containing HA-tagged RTR1 or the mutant alleles constructed as described in Materials and Methods (empty parent vector p416CUP1 was included as a negative control). These strains were spotted onto selective media (SC-URA) with or without 2% formaldehyde (form) and incubated at the indicated temperatures for 3 days. (C) The strains described for panel B were grown and protein was extracted as described in Materials and Methods. Western blot analysis was used to detect stably produced protein with anti-HA and anti-PGK. WT, wild type; vec, vector. (D) V5-epitope-tagged versions of Rtr1 were constructed as described in Materials and Methods, all by using the high-copy-number plasmid p423GPD as the backbone. These constructs were transformed into wild-type (BY4741) yeast and plated by spot dilution onto selective media (SC-HIS). The plates were incubated for 2 days at 30°C.
overexpressed (Fig. 4). These results suggest that RPB5, RPB7, and RPB9 share a common but unknown functional characteristic that permits overexpression of any one of those genes alone to overcome loss of the Rtr1 protein.

Phenotypic and genetic analyses indicate that Rtr1 has a role in transcription. Because overexpression of RPB9 complements the formamide sensitivity phenotype of rtr1Δ, we considered the possibility that these two proteins are functionally linked. rp9Δ cells are defective in start-site selection, utilizing secondary transcription start sites for a number of genes. We performed primer extension analysis using the ADH1 gene and observed the reported defects of an rp9Δ mutant but did not detect obvious transcriptional defects in rtr1Δ cells (data not shown). RTR1 is therefore not required for at least this role of RPB9. To further probe the genetic relationship between RTR1 and RNAPII, we tested whether strains lacking RPB9 or the only other nonessential subunit, RPB4, could be sensitized to moderate overexpression of RTR1 by use of the uninduced CUP1 promoter (36). Surprisingly, at a normal growth temperature of 30°C, both rp9Δ and rp4Δ cells, but not wild-type cells, were exquisitely sensitive to heightened RTR1 levels (Fig. 5). This effect was completely abrogated when either rtr1Δ(C73S) or rtr1Δ(C112S, H116S), the nonfunctional mutant alleles, was likewise overexpressed. These data, along with the results of the overexpression studies represented in Fig. 2, suggest that Rtr1 exists in an optimal stoichiometric balance with functional RNAPII.

A number of transcription mutants are sensitive to the IMP dehydrogenase inhibitors 6AU and MPA (29, 46, 57). These compounds are thought to decrease the nucleotide pool, thereby inhibiting transcription elongation (54). We tested wild type, rp9Δ, and rtr1Δ cells for their relative resistances to these inhibitors and, given our previous results, also included formamide. When cells were plated onto media containing no drug, 6AU, MPA, or formamide, we observed differential sensitivity results, as shown in Fig. 6. At drug concentrations that were permissive for the growth of wild-type cells, rtr1Δ cells were sensitive only to MPA at 37°C and were resistant to 6AU. rp9Δ cells exhibited more severe phenotypes, including sensitivity to 6AU at 37°C and to MPA at both 30°C and 37°C. Interestingly, rp9Δ and rtr1Δ cells exhibited identical sensitivities to formamide only at 37°C. Loss of RTR1 therefore recapitulates only a subset of the phenotypes exhibited by cells lacking RBP9, specifically in combination with elevated temperature.
To further delineate the transcriptional roles of Rtr1, we tested for conditional synthetic interactions between rtr1Δ and deletion of genes involved in various stages of transcription. We observed a number of synthetic growth defects (SGDs), including inviability in the presence of MPA for rtr1Δ rpb4Δ and rtr1Δ elp2Δ double mutants (Fig. 7A and Table 3). ELP2 encodes a subunit of the Elongator complex, which associates with actively transcribing RNAPII and also possesses histone acetyltransferase activity (21). These interactions were not a general result of transcriptional impairment, as RTR1 exhibited no SGD with the initiation factors MED1 and GAL11. RTR1 also exhibited substantial interaction with the uncharacterized ORF YDR066C, which, based on the strong level of homology (89% sequence similarity) and the presence of the RTR1 cysteine-rich domain, may be a recently diverged paralog. Two additional initiation factors, SOH1 and SRB63, and the elongation factors CCR4, CDC73, SPT4, and DST1 exhibited modest SGDs. The strongest interactions were observed with the RNAPII core subunits RPB4 and RPB9. In the course of another line of investigation, we also observed a strong temperature-dependent synthetic interaction between rtr1Δ and an allele of Rpb9 that included the TAP tag RPB9-TAP (Fig. 7B). While RPB9-TAP and rtr1Δ were both viable at temperatures of up to 37°C, deletion of RTR1 in the context of RPB9-TAP resulted in an inability to grow at 37°C. These results suggest that the RPB9-TAP allele may be cryptically hypomorphic and further underscore the close relationship between these two proteins.

Rtr1 physically interacts with active Rpb1. Recent large-scale proteomic studies of both yeast and human cells have indicated that Rtr1 (RPAP2 in humans) physically interacts with multiple components of the core RNAPII enzyme (15, 23, 30). Given the extensive genetic interactions we demonstrated between Rtr1 and RNAPII, we sought to validate and extend these findings in more detail. Protein A-tagged Rtr1 was purified as described in Materials and Methods, and copurifying proteins were visualized using Coomassie brilliant blue staining. Rpb1 and Rpb2 were associated with Rtr1, as evaluated by gel migration investigations (Fig. 8A). Immunoblot analysis of the protein A-Rtr1 affinity purification results by use of the specific monoclonal antibody 8WG16 positively identified Rpb1. Additional proteins were coisolated with Rtr1, including a prominent component of about 180 kDa that currently remains unidentified. We noted that Rpb1 migrated as a doublet, as is consistent with previously documented populations of the protein, one unphosphorylated and the other phosphorylated at serines in the second and fifth positions within the highly conserved heptad repeats of the regulatory CTD. Both protein bands were recognized by the 8WG16 antibody that interacts with nonphosphorylated heptad repeats (Fig. 8B) (9, 51, 62). We also tested reactivity of equivalent amounts of the coimmunoprecipitation mixtures with antibodies specific for phosphorylated CTD. The slower-migrating band reacted strongly with the H5 antibody specific for the CTD phosphorylated at serine 5 in the heptad repeat and weakly with the 8WG16 antibody H14 that recognizes phosphorylation at serine 2. These results are consistent with association of Rtr1 and actively transcribing RNAPII. None of the anti-RNAPII antibodies recognized bands from a parallel control TAP purification (Fig. 8B), and Rpb1 and Rpb2 were not detected by Coomassie staining (data not shown), demonstrating the specificity of the Rtr1-TAP · RNAPII interaction.

Rtr1 regulates transcription from the GAL1 promoter. Considering the genetic and biochemical interactions between RTR1 and components of the transcription machinery, we were interested in determining the transcriptional consequences of RTR1 disruption. We examined both inducible gene expression from the GAL regulon and constitutive expression of a standard “housekeeping” gene, ACT1. Because we anticipated that the requirement for RTR1 might be limited to transcription during heat shock, we also examined gene expression after a 1-h shift to 39°C. As shown in Fig. 8C, expression of GAL1 and GAL7 was strongly induced by a shift from sucrose to galactose in wild-type cells but not in rtr1Δ cells at either normal or heat shock temperatures, indicating a significant role for RTR1 in expression from these loci. Expression of ACT1 and the RNAPIII-dependent RNA component of SCR1, the signal recognition particle, was unaffected in rtr1Δ cells. Complementation of rtr1Δ restored GAL1 induction, verifying that loss of transcriptional activity was due to loss of Rtr1 function (data not shown). Interestingly, RNAPII- but not RNAPIII-dependent gene expression appeared to be inhibited at high temperatures, as is consistent with a previous report (67).
DISCUSSION

This report constitutes the first in vivo characterization of the \textit{RTR1} gene and its protein product. We have elucidated multiple phenotypes associated with loss of \textit{RTR1} and identified a highly conserved amino-terminal motif essential for function. We have further defined multiple genetic and biochemical interactions between \textit{RTR1} and components of the transcription machinery—specifically, subunits of the core RNAPII enzyme. Finally, we observed a defect in transcription from the \textit{GAL1} promoter in \textit{rtr1Δ} cells, demonstrating a functional transcriptional consequence associated with loss of this novel protein. Our analyses indicate that \textit{Rtr1} functions in modulating RNAPII-based transcription, specifically via interactions with RNAPII core subunits. These findings are further bolstered by the identification of C1ORF82, the closest human \textit{RTR1} homolog, as an RNAPII-associated factor. This ORF has been renamed RPAP2 (for “RNAPII-associated polypeptide”) and is located within an expansive network of interacting

\begin{table}
\centering
\caption{Genetic interactions between \textit{rtr1Δ} and other transcription genes}
\begin{tabular}{lccccc}
\hline
Gene & Role (reference) & YPD & Form & MPA & 6AU \\
\hline
\textit{GAL11} & Initiation (49) & — & — & — & ND & — & — & — & — \\
\textit{MED1} & Initiation (49) & — & — & — & ND & — & — & — & — \\
\textit{SOH1} & Initiation (41) & — & SGD & SGD & ND & — & SL & — & SGD \\
\textit{SRB5} & Initiation (61) & — & ND & ND & ND & — & — & — & — \\
\textit{CCR4} & Elongation (18) & — & — & — & ND & — & — & — & — \\
\textit{CDC73} & Elongation (34, 58) & — & — & — & ND & — & SGD & — & — \\
\textit{DST1} & Elongation (50) & — & — & — & ND & — & SGD & — & — \\
\textit{ELP2} & Elongation (21) & SGD & SGD & SGD & ND & SGD & SGD & SGD & SGD \\
\textit{SPT4} & Elongation (42, 55) & — & SGD & SGD & ND & — & — & — & — \\
\textit{RPB4} & Core (16) & — & SGD & SGD & ND & SGD & SL & — & SGD \\
\textit{RPB9} & Core (16) & — & SGD & SGD & ND & ND & ND & — & ND \\
\textit{YDR066C} & Unknown & SGD & SGD & SGD & ND & SGD & SGD & SGD & SGD \\
\hline
\end{tabular}
\end{table}

\textit{YPD}, rich medium, no stress; Form, 1\% formamide; —, no synthetic interaction; ND, not determined (due to single-gene lethality); SL, synthetic lethality.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig8}
\caption{Rtr1 physically interacts with the active form of Rpb1 and regulates transcription from the \textit{GAL1} promoter. (A) Coomassie brilliant blue staining of a sodium dodecyl sulfate-polyacrylamide electrophoresis gel containing protein A-Rtr1 for affinity purification (described in Materials and Methods). The positions of Rpb1 and Rpb2 are indicated. (B) Immunoblot analysis of Rtr1-TAP and control TAP purifications using anti-Rpb1, anti-Rpb1(Ser5P), and anti-Rpb1(Ser2P) antibodies. (C) Northern blot analysis of wild-type (BY4741) and \textit{rtr1Δ} cells grown to logarithmic phase in sucrose (Suc) medium and either maintained at 30\(^\circ\)C or shifted to 39\(^\circ\)C for 1 h followed by a shift to galactose (Gal)-containing medium at the indicated temperatures.}
\end{figure}
protein complexes, as identified by large-scale affinity purification with other transcriptional components (30). However, no functional insights into the roles of RPAP2 have emerged, underscoring the need for further study of the yeast homolog in its in vivo context. For example, comprehensive cataloging of transcriptional defects in \textit{rtr1}Δ cells would aid the assignment of a specific functional role within the RNAPII multi-protein transcriptional complex.

We believe that the Rtr1/RPAP2 proteins may play a unique role in transcription because of the array of genetic interactions and specific physical interactions with core subunits of RNAPII. While many accessory factors and complexes have been identified (Mediator, Elongator, general and specific transcription factors, etc.), few interact robustly with the core subunits of RNAPII. Eight affinity capture interactions with Rtr1 examined in yeast proteome-wide analyses have been described previously—among them, interactions with the nucleolar protein Rp2, with the microtubule-associated protein Bik1, and with the enzyme Ura2, an apparently promiscuous binding protein with 43 unconfirmed affinity interactions (23, 24, 33). The remaining four Rtr1 interactors are RNAPII subunits: Rpb1, Rpb2, Rpb3, and Rpb8 (15, 23). Strikingly, RPAP2 likewise interacts with at least eight core subunits (30).

We confirmed the interaction of Rtr1 with Rpb1 in yeast cells and showed that Rtr1-associated Rpb1 is transcriptionally active. Further, \textit{RTR1} genetically interacts with transcriptional components acting at multiple stages in transcription, including initiation and elongation. Core RNAPII subunits RPB4 and RPB9 exhibited some of the strongest genetic interactions with \textit{rtr1}Δ. In addition, the RPB9-TAP allele also rendered cells temperature sensitive in the context of \textit{rtr1}Δ. The heat shock/formamide sensitivity phenotype is shared by a number of other transcription mutants, including those defective in the Pafl complex (Ctr9, Cdc73, Ccr4, Hpr1, Rtf1, Leol1) (12). RPAP2 also appears to associate with a number of other polypeptides in one or more complexes, many of which have yeast counterparts (30). When taken together, these numerous genetic and biochemical interactions place Rtr1/RPAP2 in close proximity to the actively transcribing RNAPII with an as-yet-undescribed molecular role. Interestingly, under all the growth conditions we tested, Rtr1 was largely present in the cytoplasm. The finding that Rtr1 accumulated in the nucleus upon inactivation of the Xpo1-dependent export pathway indicates that nuclear shuttling is constitutive and may be an important aspect of its function. In contrast, nearly all RNAPII subunits are exclusively nuclear. Cytoplasmic localization of a core RNAPII-associated protein has been shown previously for the Rpb4 subunit in both \textit{S. ceresvisiae} and \textit{Schizosaccharomyces pombe}, but the significance of this dual localization is not clear (20, 32).

The presence of the essential Zn-finger-like motif defines a eukaryotic lineage for Rtr1 and its homologous counterparts. This motif is restricted to a single putative homolog in each of the higher eukaryotic species examined. Interestingly, the carboxyl terminus is highly divergent in these putative \textit{RTR1} homologs and is of variable length. This suggests either that this region serves no obvious cellular role or that it mediates interaction with disparate components. The former scenario is unlikely, as we found this portion of the protein to be responsible for growth inhibition upon protein overexpression in wild-type cells. We considered the possibility that Rtr1 interacts with a fungus-specific protein via this region, which could have resulted in titration of a cellular component critical for growth.

We therefore undertook an additional high-copy-number suppressor screening to identify genes that would reverse the growth inhibition caused by \textit{RTR1} overexpression but failed to isolate candidates capable of doing so (data not shown). \textit{S. ceresvisiae} is unique in that it contains two ORFs with the conserved cysteine-rich motif—\textit{RTR1} and YDR066C. This is most likely due to the whole-genome duplication proposed to have occurred approximately 150 million years ago, given the high level of amino acid sequence similarity (89%) that extends throughout the protein (38). Despite the fact that we could not detect any phenotypes associated with deletion of YDR066C, we observed substantial synthetic interactions between \textit{rtr1}Δ and \textit{ydr066cΔ}, indicating possible functional redundancy between the two proteins (Table 3). We therefore propose renaming the uncharacterized ORF YDR066C \textit{RTR2}, although at this time we do not have evidence for direct interaction with RNAPII.

Zn-finger motifs are predominantly involved in nucleic acid binding; as a result, the spacing between cysteine and histidine residues contributes to binding specificity (37). However, unlike many Zn fingers, the residues between the putative metal chelating cysteines and histidine in Rtr1 are also highly conserved. It may also be noteworthy that \textit{RPB5} and \textit{RPB9}, two of the three high-copy-number suppressors, are themselves Zn-containing proteins (19, 63). In fact, \textit{RPB9} is a small 122-amino-acid subunit that harbors two distinct Zn-binding domains organized into what has been termed a “zinc ribbon.”

Because \textit{RPB5} and \textit{RPB9} are both located at the “jaws” of RNAPII and function as part of the DNA clamping mechanism, it is tempting to speculate that Rtr1 may function as an accessory DNA binding factor for RNAPII in a mechanism requiring its cysteine-rich motif. Further analysis of the precise protein-protein interactions of Rtr1/RPAP2 with RNAPII, of its presence or absence on the actively transcribing enzyme, and of gene-specific transcriptional requirements are needed to provide a full understanding of this protein family.

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