Activation mutants in yeast RNA polymerase II subunit RPB3 provide evidence for a structurally conserved surface required for activation in eukaryotes and bacteria

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We have identified a mutant in RPB3, the third-largest subunit of yeast RNA polymerase II, that is defective in activator-dependent transcription, but not defective in activator-independent, basal transcription. The mutant contains two amino-acid substitutions, C92R and A159G, that are both required for pronounced defects in activator-dependent transcription. Synthetic enhancement of phenotypes of C92R and A159G, and of several other pairs of substitutions, is consistent with a functional relationship between residues 92–95 and 159–161. Homology modeling of RPB3 on the basis of the crystallographic structure of αNTD indicates that residues 92–95 and 159–162 are likely to be adjacent within the structure of RPB3. In addition, homology modeling indicates that the location of residues 159–162 within RPB3 corresponds to the location of an activation target within αNTD (the target of activating region 2 of catabolite activator protein, an activation target involved in a protein–protein interaction that facilitates isomerization of the RNA polymerase promoter closed complex to the RNA polymerase promoter open complex). The apparent finding of a conserved surface required for activation in eukaryotes and bacteria raises the possibility of conserved mechanisms of activation in eukaryotes and bacteria.

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Eukaryotic RNA polymerase II (RNAP II) acts in concert with a variety of gene-specific activators, repressors, and protein complexes during the regulated synthesis of mRNA (Greenblatt 1997; Myer and Young 1998). Saccharomyces cerevisiae RNAP II is the most extensively characterized eukaryotic RNAP II. It contains 12 subunits, designated RPB1–RPB12 in decreasing order of molecular mass [Young 1991; Sentenac et al. 1992]. RPB1 and RPB2 are homologs of the bacterial RNAP β and β subunits, respectively, whose functions include template, nucleotide binding, and phosphodiester bond formation [Young 1991; Sentenac et al. 1992]. RPB3 and RPB11 are homologs of the bacterial RNAP α subunit amino-terminal domain (αNTD) corresponding to the amino terminal two-thirds of the 329 amino acid α subunit [Young 1991; Sentenac et al. 1992; Ebright and Busby 1995; Zhang and Darst 1998]. αNTD is present in two copies in bacterial RNAP and involved in RNAP assembly and transcriptional activation [Ebright and Busby 1995].

The 318 amino acid RPB3 subunit—and its counterparts in archaeal RNAP (RpoD) and eukaryotic RNAP I and RNAP III (RCP5; also known as AC40, RPA5)—exhibit amino acid sequence similarity to seven discrete conserved regions across the entire length of the αNTD (CR1–CR7; Fig. 1A; see also Zhang and Darst 1998). The 120 amino acid subunit RPB11—and its counterparts in archaeal RNAP (RpoL) and eukaryotic RNAP I and RNAP III (AC19; also known as RPC9)—exhibit amino acid sequence similarity to approximately one-half of αNTD, corresponding to CR1, CR6, and CR7 [Zhang and Darst 1998, and data not shown].

RPB3 and RPB11 heterodimerize both in solution (Ulimasov et al. 1996; Kimura et al. 1997; Larkin and Guilfoyle 1997; Fanciulli et al. 1998; Svetlov et al. 1998) and in the context of RNAP II (Kimura et al. 1997; Ishiguro et al. 1998). The structures of RPB3 and RPB11 can be homology-modeled on the basis of the crystallographic

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Table 1. 

| CR1 | CR2 | CR3 | CR4 |
|-----|-----|-----|-----|
| S2  | S1  | S3  |     |
| S4  |     | S5  | S6  |

Figure 1. (see facing page for legend.)
structure of αNTD (Fig. 1B,C; Zhang and Darst 1998). The RPB3/RPB11 heterodimer in RNAP II corresponds to the αNTDβ/αNTDα homodimer in bacterial RNAP, in which αNTDβ is the αNTD protomer that interacts with the second-largest subunit of RNAP (β) and αNTDα is the αNTD protomer that interacts with the largest subunit of RNAP (β′) (Fig. 1, B and C, and legend to C).

In this work we show that RPB3 contains two determinants specifically required for activator-dependent transcription—that is, required for activator-dependent transcription but not required for activator-independent basal transcription. In the homology-modeled structure of RPB3, the two determinants are located adjacent to each other and, strikingly, in a position that corresponds to the position of a characterized activation target within bacterial RNAP αNTDβ.

Results and Discussion

rp3-2, isolated in a screen for cold- and temperature-sensitive mutants in RPB3, has a severe defect in activator-dependent transcription in vitro

To learn more about the function of the essential RNAP II subunit RPB3, we analyzed the effects on transcription of extracts prepared from conditional mutants of RPB3. We performed random mutagenesis of the entire RPB3 gene using error-prone PCR, screened for mutants displaying cold- (12°C) and/or temperature-sensitive (37°C) phenotypes, and verified that conditional phenotypes resulted from mutations in RPB3. Eight cold-sensitive, 21 temperature-sensitive, and three cold- and temperature-sensitive mutants were isolated. Whole-cell extracts were prepared from RPB3 mutants with the tightest phe-
notypes and tested with an in vitro assay for basal transcription and GAL4–VP16-dependent activated transcription.

One mutant, designated rpb3-2, exhibited a temperature-sensitive, slow-growth phenotype (Fig. 2A,B) and a pronounced defect in GAL4–VP16-dependent transcription (Fig. 3B). The mutant was fully functional in basal transcription—both promoter-independent and promoter-dependent basal transcription (Fig. 3B,C)—but nearly completely defective in activation by GAL4–VP16 (Fig. 3B). Addition of purified RNAP II to mutant whole-cell extracts restored responsiveness to GAL4–VP16, indicating that RNAP II was the source of the activation defect in the mutant (Fig. 3D). We conclude that RPB3 contains determinants specifically required for GAL4–VP16-dependent transcription.

RPB3-2 cells have severe defects in activator-dependent transcription in vivo

We used two approaches to assess the effects of the rpb3-2 mutant on activator-dependent transcription in vivo. First, we tested activator function by measuring expression levels of a lacZ reporter driven by an inducible promoter containing an upstream activation sequence (UAS). Second, we directly measured mRNA levels of inducible genes before and after activation.

Wild-type and rpb3-2 cells containing reporter plasmids for measuring activation by GAL4, PHO4, INO2, and ADR1 were grown at the permissive temperature under nonactivating or activating conditions, and β-galactosidase activity was measured in extracts prepared from each strain. rpb3-2 cells were severely defective in activator-dependent transcription, both at the permissive (Fig. 4A) and nonpermissive temperatures (data not shown).

Northern analysis also confirmed that rpb3-2 is defective in activator-dependent transcription at the INO1, GAL1, and PHO5 promoters (Fig. 4B, left three panels). [We were unable to measure ADH2 mRNA levels by this method, because two closely related transcripts, ADH1 and ADH3, cross hybridize to the ADH2 DNA probe.] Measurement of mRNA levels at the HIS4 promoter indicated that the mutant is not defective in activator-dependent transcription at this promoter (Fig. 4B, right).

To address the possibility that the activation defects seen in the mutant might be a consequence of a defect in some other process, we tested whether rpb3-2 specifically affected activator function. We expressed the test activator LexA–Adr1 fusion protein from a plasmid in isogenic wild-type and mutant strains carrying a second plasmid containing the lexA operators fused to the lacZ reporter gene. The results support a role for RPB3 that is specific to the activation function of LexA–Adr1, as RPB3 mutant cells displayed a sixfold reduction in lacZ expression relative to wild type (Fig. 5A).

The rpb3-2 mutation does not cause a global effect on transcription in vivo, because total mRNA levels in rpb3-2 cells are 80%–90% of those in wild-type cells—both at permissive and nonpermissive temperatures (Fig. 5B). Furthermore, mRNA levels of a variety of genes—ACT1, U3, TUB2, PDA1, DED1, HIS3, SUA7, SRB5, RPB1, and RPB3—in the RPB3 mutant are not signifi-
cantly lower than those in wild-type cells at permissive and nonpermissive temperatures (data not shown). We conclude that the \textit{rpb3-2} mutant is defective in activator-dependent transcription in vivo, that the defect is severe, and that the defect is pleiotropic (affecting \textit{GAL1}, \textit{PHO5}, \textit{INO1}, and \textit{ADH2}) but not universal (not affecting \textit{HIS4}).

\textit{rpb3-2} has two mutations that are both required for maximal impairment of activation

The \textit{rpb3-2} allele encodes a subunit with two amino acid substitutions: C92R and A159G. To determine whether C92R, A159G, or both substitutions, contribute to the defect in activator-dependent transcription, we analyzed the effects of the C92R and A159G substitutions separately [Fig. 6]. The results indicate that both substitutions are required for the full defect in activator-dependent transcription—both in vitro [Fig. 6A] and in vivo [Fig. 6B]. In vitro transcription with extracts from cells containing either wild-type RPB3, the mutant pair, or either single mutation revealed that only the double mutant is unresponsive to activation by GAL4–VP16 [Fig. 6A]. Consistent with these findings, activated levels of \textit{INO1} and \textit{GAL1} transcripts were typically higher in either single mutant compared with the double mutant [Fig. 6B]. We conclude that both C92 and A159 are residues critical for activator-dependent transcription.

\textit{rpb3-2} cells display activation defects for multiple inducible genes

(A) Impaired activation with four different activator reporter plasmids [activator shown above each graph]. Assays were performed on extracts prepared from cells harvested after growth under activating (+) or nonactivating (−) conditions. Units represented as nanomoles o-nitrophenyl-β-D-galactosidase cleaved per min per mg of protein. [B] Northern blot analysis of \textit{GAL1}, \textit{PHO5}, \textit{HIS4}, and \textit{INO1} transcripts before (−) and after (+) activation. RNA was prepared from cells grown at the nonpermissive temperature (30°C). \textit{TUB2} (tubulin mRNA) and \textit{U3} (RNAP II transcribed snRNA) were included as loading controls. \textit{GAL1}, \textit{PHO5}, \textit{HIS4}, or \textit{INO1} transcripts were normalized to their respective loading controls and graphed relative to the activated wild-type control.

Figure 4.
The gene suggests functional relationship between sites of mutations (Guarente 1993).

We have also constructed and analyzed five additional double mutants altered in the 92 and 159 regions (Fig. 7C). Three of the five double mutants exhibited synthetic lethal phenotypes: Two were lethal at 37°C; one was lethal at 30°C and 37°C (Fig. 7C). In contrast, single mutants in the 92 and 159 regions grew normally, both at 30°C and 37°C (Fig. 7B). These additional examples of synthetic enhancement are consistent with a functional interaction between the 92 and 159 regions.

Finally, we looked for a correlation between growth phenotype and activation phenotype in the single and double mutants. The K160A, K161A, and G162A substitutions resulted in detectable defects in activator-dependent transcription at \( \text{GAL1} \) and \( \text{INO1} \) in vivo, whereas the C95A mutant (like the C92R mutant shown in Fig. 6B) did not significantly affect activation. However, all double mutants tested displayed more pronounced defects in activator-dependent transcription than the component single mutants (Fig. 7D,E), indicating that growth phenotypes and activation phenotypes are, at least in part, correlated. Acquisition of temperature sensitivity was associated with impaired activation; however, the ability to grow at 37°C was not tightly correlated with normal activation of \( \text{GAL1} \) or \( \text{INO1} \).

Overall, we conclude that the residues critical for activator-dependent transcription span (minimally) 92–95 and 159–162 (Fig. 1A, red bars).

The 92 and 159 regions are adjacent to each other

The 92 region is located in a nonconserved region between CR2 and CR3 (Fig. 1A). Residues 92 and 95 are part of the sequence \( ^{G6C}X-CX_7-CX_9-C^{G95} \), a presumed zinc binding site (Treich et al. 1991). The 159 region is located at the end of CR5 (Fig. 1A). Residues 159–162 map to the end of \( \beta \)-strand 9 in bacterial RNAP αNTD (Fig. 1A).

In the homology-modeled structure of the RPB3/RPB11 heterodimer, the 92 region and the 159 region are adjacent and likely to interact with each other (Fig. 8B)—consistent with the observed synthetic enhancement of phenotypes in the 92 region and 159 region mutants (see preceding section). We infer that the 92 and 159 regions constitute a single determinant specifically required for activator-dependent transcription, that is, a single activation target.

In the homology-modeled structure of the RPB3/RPB11 heterodimer, the 92 and 159 regions are prominently exposed on the surface of RPB3/RPB11 and on the
face of RPB3/RPB11 opposite that predicted to interact with the second-largest and largest subunits of RNAP II [Fig. 8B]. We infer that the 92 and 159 regions are available, in principle, to participate in macromolecule–macromolecule interactions directly involved in transcription activation.

The 92 and 159 regions are well separated from all determinants of RPB3 known (Kolodziej and Young 1991; Ulmasov et al. 1996; Svetlov et al. 1998; Mitobe et al. 1999) or predicted (Fig. 8B) to be involved in RPB3/RPB11 heterodimerization and RNAP II assembly—consistent with immunoprecipitation results demonstrating assembly of the \textit{rpb3-2} (C92R;A159G) subunit with RNAP II and correct stoichiometry of the RNAP II in mutant cells [data not shown]. We infer that the defect in activator-dependent transcription of \textit{rpb3-2} (C92R;A159G) is not a secondary consequence of defects in RPB3/RPB11 heterodimerization or RNAP II assembly.

Figure 7. Synthetic enhancement between residues from the RPB3 92 and 159 regions. (A–C) Equal amounts of yeast cells from strains with the indicated mutant forms of RPB3 were spotted onto YPD plates containing 5-FOA and tested for growth at the temperatures indicated. The arrow highlights the mutant strain that is synthetically lethal at both temperatures. (D,E) RNA was prepared from the wild-type cells or selected double mutants (grown under activating conditions at 30°C) in the 92 and 159 regions as indicated. Histograms represent activated levels of expression of either the \textit{INO1} [D] or \textit{GAL1} [E] mRNA relative to the activated wild-type control normalized to the \textit{U3} (RNAP II-transcribed snRNA) loading control.

Figure 8. Location of activation target in αNTD\textsuperscript{I} and RPB3. (A) Crystallographic structure of the \textit{E. coli} RNAP αNTD\textsuperscript{I}/αNTD\textsuperscript{II} homodimer [two orthogonal views; Zhang and Darst 1998]. αNTD\textsuperscript{I} is in light blue; αNTD\textsuperscript{II} is in gray; regions that interact with the second-largest and largest subunits of RNAP II are highlighted in green and blue, respectively [Heyduk et al. 1996]. The characterized activation target within αNTD\textsuperscript{I}—required for response to CAP at class II CAP-dependent promoters [Niu et al. 1996; Busby and Ebright 1997,1999]—is represented as an orange sphere. (B) Homology-modeled structure of \textit{S. cerevisiae} RPB3/RPB11 heterodimer [two orthogonal views; see Fig. 1C]. RPB3 is in light blue; RPB11 is in gray. Regions predicted to interact with the second-largest and largest subunits of RNAP II are highlighted in green and blue. The two regions that define the activation target in this work—required for response to GAL4–VP16, GAL4, PHO4, INO2, and ADR1 at tested promoters—are represented as red spheres (with the 92 region slightly above and to the left of the 159 region in each view).
The 92 and 159 regions correspond to a characterized activation target in bacterial RNAP αNTD

In the homology-modeled structure of the RPB3/RPB11 heterodimer, the location of the 92 and 159 regions of RPB3 corresponds to a characterized activation target within bacterial RNAP αNTD [Fig. 8, cf. A and B; Niu et al. 1996; Busby and Ebright 1997, 1999]. The 92 and 159 regions are located in the subdomain of RPB3 that corresponds to the subdomain of αNTD that contains the activation target, and on the face of the subdomain that most nearly corresponds to the face that contains the activation target [Fig. 8]. The 159 region of RPB3 is located in CR5, which, in αNTD, immediately precedes the first residue of the loop containing the activation target [Fig. 1a, cf. second red bar with orange bar].

In bacterial RNAP, this activation target mediates response to transcriptional activation by catabolite activator protein (CAP; also known as cyclic AMP receptor protein CRP) at one class of CAP-dependent promoters [Niu et al. 1996; Busby and Ebright 1997, 1999]. The activation target functions to direct protein–protein interactions with a four amino acid determinant within CAP (activation region 2, AR2; Niu et al. 1996; Busby and Ebright 1997, 1999). Experiments with oriented αRNAP derivatives carrying one wild-type and one mutant α subunit indicate that the activation target is functionally presented in only one of the two copies of αNTD in RNAP, that is, αNTD [Niu 1998; Busby and Ebright 1999].

Implications for activation

Our results indicate that the 92 and 159 regions of RPB3, together, constitute an activation target specifically required for activator-dependent transcription both in vitro and in vivo. We propose that this activation target participates in a direct protein–protein interaction required for transcriptional activation. Possible candidates include an RPB3-activator interaction, an activator-dependent RPB3-coactivator interaction, or an activator-dependent RPB3 general transcription factor interaction. The observation that the activation target in RPB3 is required for response to at least five different activators—GAL4–VP16, GAL4, PHO4, INO2, and ADR1—leads us to disfavor models involving direct RPB3-activator interaction and to favor models involving an activator-dependent RPB3 coactivator or RPB3 general transcription factor interaction. The location of the activation target in the homology-modeled structure of RPB3 corresponds to the location of a characterized activation target within the structure of bacterial RNAP αNTD [Fig. 8; Niu et al. 1996; Busby and Ebright 1997, 1999]. The activation target within αNTD functions through a protein–protein interaction with a factor that binds to a DNA site that overlaps the core promoter and has a precisely defined spatial relationship relative to the core promoter [such that repositioning the DNA site by a single base pair reduces or eliminates function [Gaston et al. 1990; Valentin-Hansen et al. 1991; Busby and Ebright 1999]]. To the extent that the activation target within RPB3 is analogous to the activation target in αNTD, this would further tend to disfavor models involving direct RPB3 activator interaction [because the activators shown to be affected—GAL4–VP16, GAL4, PHO4, INO2, and ADR1—function from DNA sites at varying distances from the core promoter].

We note that the activation target within αNTD functions at a step subsequent to recruitment of RNAP to promoter DNA, facilitating isomerization of the RNAP promoter closed complex to the RNAP promoter open complex [Niu et al. 1996; Busby and Ebright 1997, 1999; Rhodia et al. 1997]. It is attractive to speculate that the activation target within RPB3 likewise functions at a step subsequent to recruitment, and that at this region within RNAP—bacterial or eukaryotic—there is a conserved, specific structural feature (a button or switch) that permits response to postrecruitment activation. This speculation would provide an explanation for the conservation of an activation target in bacteria and eukaryotes.

Materials and methods

Media, yeast strains, and plasmids

Rich medium (YPD), synthetic complete (SC), and minimal medium were prepared from standard recipes [Treco and Lundblad 1993]. 5-Fluoro-orotic acid (5-FOA) used in plasmid shuffle experiments was added to 1 mg/ml [Boeke et al. 1987]. The wild-type and isogenic mutant rpb3-2 strains were named WY-95 (MATa ura3-52 leu2-3,112 HIS4-912 lys2-1286 rpb3D1::LYS2 [pRP37]) and WY-96 (MATa ura3-52 leu2-3,112 HIS4-912 lys2-1286 rpb3D1::LYS2 [pRP38]), respectively. pRP37 is a LEU2 CEN plasmid with a 1.9-kb RPB3-containing SacI–PstI fragment ligated to the corresponding sites of pRP415 [Sikorski and Hieter 1989]. pRP38 was isolated after PCR mutagenesis [Muhlrad et al. 1992] and is the same as pRP37 but contains two mutations resulting in two amino acid changes, C92R and A159G. WY-95 and WY-96 were created by transformation of Z242 [Kolodie and Young 1989] with pRP37 and pRP38, respectively, followed by exposure to 5-FOA. All other mutants used in this study were prepared by site-directed PCR mutagenesis [Ho et al. 1989] of pRP37. Once the changes were verified by sequence analysis, the plasmids were transformed into Z242, followed by exposure to 5-FOA.

Sequence alignment

Sequences of bacterial αNTD from Escherichia coli [RPOA_ECOLI], Haemophilus influenzae [RPOA_HAEIN], Bacillus subtilis [RPOA_BACSU], Mycobacterium tuberculosis [RPOA_MCYCU], Borelia burgdorferi [RPOA_BORBU], Mycoplasma pneumoniae [RPOA_MYCPN], Thermotoga maritima [RPOA_THEMA], Archaeal RpoD from Methanococcus jannaschii [RPOD_METJA], Halococcus marismortui [RPOD_HALMA], Pyrococcus horikoshii [RPOD_PYRHO], Sulfolobus acidocaldarius [RPOD_SULAC], Sulfolobus solfataricus [RPOD_SULSO], Methanobacterium thermoautotrophicum [RPOD_METTH], Archaeoglobus fulgidus [RPOD_ARCFU], Aeropyrum pernix [BAA80745], RBP3 from S. cerevisiae [RBP3 YEAST], Schizosaccharomyces pombe [RBP3_SCHPO], Mus musculus [RBP3_MOUSE], Homo sapiens...
mRNA for 3 hr at 30°C. The mutant and isogenic wild-type strain have a higher (HIP4-912) that results in a His- phenotype due to the preferential synthesis of an aberrant mRNA (Winston et al. 1984). However, a small percentage of normal mRNA is also synthesized, allowing us to compare induction of HIP4 in the mutant relative with the isogenic wild-type strain.

For PHO5, cells were grown at 30°C in either low-phosphate YPD or high-phosphate YPD, cultures were started at OD₆₀₀ of 0.01, and harvested at an OD₆₀₀ of ~0.5. In low- and high-phosphate YPD, potassium phosphate was added to a final concentration of 0.1 and 7.5 mM, respectively. For ADH2, cells were grown at 30°C in medium containing 2% ethanol plus 2% glycerol to an OD₆₀₀ of 0.6.

Total RNA was prepared by standard methods [Kaiser et al. 1994]. Total RNA (15 µg) was loaded into each lane in Northern gels, and 20 µg was loaded to each compartment for dot blots. mRNA levels for dot blots were assessed by hybridization with an excess of [poly]dT oligonucleotide. Both Northern and dot blots were hybridized with radioactively labeled DNA and bands intensities quantified with a PhosphorImager. The probe names and fragment sizes varied as gene-specific probes were as follows: INO1 p[N333] 0.9-ko HindIII-Clai, GAL1 p[GAL1- GAL10] 2.1-kb EcoRI, TUB2 p[VST138] 0.25-kb HindIII–KpnI, U3 [pID161] 0.5-kb BamHI–HpaI, PHO5 p[N973] 625-bp BamHI-SalI, HIS4 p[FW45] 1.4-kb EcoRI–SalI.

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