Rpb3, Stoichiometry and Sequence Determinants of the Assembly into Yeast RNA Polymerase II in Vivo*

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Stoichiometry of the third largest subunit (Rpb3) of the yeast RNA polymerase II is a subject of continuing controversy. In this work we utilized immunoaffinity and nickel-chelate chromatographic techniques to isolate the RNA polymerase II species assembled in vivo in the presence of the His<sub>6</sub>-tagged and untagged Rpb3. The distribution pattern of tagged and untagged subunits among the RNA polymerase II molecules is consistent with a stoichiometry of 1 Rpb3 polypeptide per molecule of RNA polymerase. Deletion of either α-homology region (amino acids 29–55 or 226–267) from the Rpb3 sequence abolished its ability to assemble into RNA polymerase II in vivo.

Yeast Saccharomyces cerevisiae RNA polymerase II is a multisubunit enzyme comprised of 12 core polypeptides (1). With minor variations its subunit composition is characteristic of all eucaryal nuclear RNA polymerases and their archaeal counterparts (1, 2). Despite the trivial quantitative differences, a profound similarity can also be found between these eucaryal/archaeal multisubunit enzymes and eubacterial RNA polymerases. The latter, being heterotetramers of the composition (α)₁β₁β’₁, resemble eucaryal RNA polymerases in their overall appearance (3–6) and in the structural core composition, with the two largest procaryal subunits, β and β’, having fairly conserved eucaryal orthologs, represented by yeast Rpb1 and Rpb2 (reviewed in Ref. 7). Less obvious homology was noted between eubacterial α subunit and yeast Rpb3 (8), consistent with the reported functional equivalence of α<sub>2</sub> and (Rpb3)<sub>2</sub> dimers in assembly of their respective enzymes (9).

Young and co-authors also concluded, based on the [35S]Met labeling of the RNA polymerase II subunits, that two copies of the subunits Rpb3, Rpb5, and Rpb9 were present in each RNA polymerase II molecule, whereas the rest were represented by a single polypeptide each or else recovered in submolar amounts (10). These inferred orthological relations between α<sub>2</sub> and (Rpb3)<sub>2</sub> dimers were later questioned by the reports from several laboratories, that failed to detect homodimerization potential in yeast Rpb3 and its higher eucaryal homologs (11–13). Instead, in an array of in vitro assays, Rpb3 was shown to associate with another apparent α-subunit homolog, Rpb11, with a stoichiometry of 1:1 (11, 13). Human homologs of Rpb3 and 11 were also shown to associate in a complex of unknown stoichiometry in the yeast two-hybrid system (12). It was consequently suggested that the Rpb3-Rpb11 heterodimer serves as a functional analog of the α₂-β homodimer (11); consistent with this hypothesis is the recovery of a Rpb2-Rpb3-Rpb11 core subassembly from a partially denatured Schizosaccharomyces pombe RNA polymerase II (14).

In this work we utilized an independent method to ascertain the stoichiometry of the yeast Rpb3, based on simultaneous expression of the wild-type (genomic) RPB3 gene and its plasmid-borne His<sub>6</sub>-tagged version followed by affinity purification of the in vivo assembled RNA polymerase II species.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The RPB3 expression plasmid p416GAL1::RPB3.2 was created by subcloning of the XbaI-Xhol fragment of the vector pET33::RPB3.2 into XbaI- and Xhol-digested vector p416GAL1 (15). The resulting expression cassette featured the entire RPB3 coding sequence, preceded by pET33-derived sequence MGSSHHHHHHHHHHSSGLVPRGSRRASVH, under control of galactose-inducible/glucose-repressible promoter. RPB3 deletion mutants, lacking the coding sequence for amino acids 29–55 that comprise the NH₄ₐ-terminals α-homology region or the COOH-terminal α-homology region spanning the region from position 226 to 267 were constructed using QuickChange site-directed mutagenesis kit (Stratagene) and pET33::RPB3.2 as a template; sequences of the mutagenic oligonucleotides are available upon request. Mutagenized expression cassettes were excised using XbaI and Xhol enzymes and subcloned into p416GAL1 to yield p416GAL1::RPB3.3M and p416GAL1::RPB3.A2M plasmids, respectively.

Isolation of the in Vivo Assembled RNA II Polymerase Complexes—Yeast S. cerevisiae strain InvSc1 (Invitrogen), transformed with the derivatives of the galactose-inducible/glucose-repressible expression vector p416GAL1 (15) (ATCC 87332), was grown in the yeast nitrogen base CM-URA medium (BIO 101, Inc.) supplemented with 0.5% (w/v) (NH₄)₂SO₄ and 2% (w/v) raffinose with agitation at 26 °C. Cells were harvested at A₅₅₀ = 0.95 by centrifugation at 5000 × g at 4 °C for 10 min, washed with cold TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and resuspended in 0.1 culture volume of cold lysis buffer (50 mM Tris-HCl, pH 8.0, 2% (v/v) glycerol, 0.1 mM 1,4-dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, Complete Mini EDTA-free protease inhibitor mixture (Boehringer Mannheim)). Cells were disrupted by high-speed vortexing with equal volume of acid-cleared glass beads (≈0.4 mm, Thomas Scientific) for 4 min. Lysate was cleared by centrifugation at 15,000 × g at 4 °C for 20 min. Powdered (NH₄)₂SO₄ was added to the cleared lysate to the final concentration of 0.361 g/ml of the lysate, and the precipitate formed after 1-h incubation at 4 °C was pelleted by centrifugation at 15,000 × g at 4 °C for 20 min. The pellet was used immediately for the downstream processing or stored at −80 °C.

RNA polymerase II was purified from the (NH₄)₂SO₄ precipitate according to the modified method of Thompson et al. (16) using immobilized CTD<sup>2</sup>-specific polyclonal-responsive mAb 8WG16. The pellet was resuspended in cold ET buffer (50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA), then diluted with ET to the conductivity of 0.3 m (NH₄)₂SO₄, and incubated with 0.1 volume of 8WG16-Sepharose (16) for 2 h with gentle agitation. Resin was washed with 60 column volumes of 0.5 m (NH₄)₂SO₄ in ET buffer, and the polymerase was eluted in ET buffer containing 40% propylene glycol and 0.5 m (NH₄)₂SO₄. Eluate containing the RNA polymerase was dialyzed against 5000 volumes of the TLG buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride) in the Slide-A-Lyzer 3.5 K (Fisher). To further fractionate immunoaffinity-purified RNA polymerase II, it was diluted four times with TLG buffer and incubated with 0.2

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2. The abbreviations used are: CTD, carboxyl-terminal domain; NiNTA, nickel-nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody.
volumes of Ni-NTA-agarose (Qiagen) for 20 min with gentle agitation. The resin was washed with 20 column volumes of 0.3 M (NH₄)₂SO₄ in TLG, followed by 20 column volumes of 10 mM imidazole, 0.3 M (NH₄)₂SO₄ in TLG buffer. Elution of the nickel-bound proteins was carried out in 160 mM imidazole, 0.3 M (NH₄)₂SO₄ in TLG buffer. Alternatively, the (NH₄)₂SO₄ precipitate was used directly to purify His₆-tagged and associated proteins. To this end, the precipitate was resuspended in TLG buffer (0.1 volumes of the original yeast culture), combined with Ni-NTA-Sepharose and processed as described above.

**Analysis of the Assembled RNA Polymerase II Complexes—**Western blot analysis was performed after protein preparations were separated in 12% or 20% Tris-glycine SDS-PAGE gels (Novex) and electrotransferred onto Protran membrane (Schleicher & Schuell). The membranes were blocked for 2 h in 1% Blotto, rinsed in 1 × TBST, incubated for 90 min with primary murine antibodies (monoclonal antibodies 8WG16 (anti-Rpb1), 1Y27 (anti-Rpb3), 4Y11 (anti-Rpb11), and anti-Rpb2 serum) in 1% primary murine antibodies (monoclonal antibodies 8WG16 (anti-Rpb1), pH 7.4, 150 mM NaCl, 0.1% (v:v) Tween 20), incubated for 90 min with anti-mouse IgG alkaline phosphatase conjugate (Boehringer Mannheim) in 1% Blotto, and washed five times with 1 × TBST. Alternatively, for detection of the His₆-tagged proteins using Ni-NTA alkaline phosphatase conjugate (Boehringer Mannheim) in 1% Blotto, and washed five times with 1 × TBST. Alternately, for detection of the His₆-tagged proteins using Ni-NTA alkaline phosphatase conjugate (Qiagen), membranes were treated according to the manufacturer's recommendations. Blots were developed using 1 × solution prepared from the 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium tablets (Boehringer Mannheim) and scanned at 600 dpi on the Hewlett-Packard Scanjet II.

**RESULTS AND DISCUSSION**

The previously published reports addressing the question of the Rpb3 stoichiometry for the validity of their conclusions implicitly relied either on accuracy and efficiency of RNA polymerase labeling in vivo (10) or upon the actuality and biological relevance of the interactions detected in vitro (11, 13). In this work we extended the elegant combinatorial approach of Guilfoyle and co-authors (11, 13) to the in vivo assembly of RNA polymerase. To that end we utilized a yeast host-vector system allowing simultaneous expression of the wild-type RPB3 gene from its native promoter and the His₆-tagged RPB3 cassette from the vector-borne GAL1 promoter. Initial assumptions made in this work were: (i) stoichiometric incorporation of the Rpb3-tagged and -untagged subunits into RNA polymerase II and (ii) equally efficient incorporation and independent assortment of the tagged and untagged subunits during the polymerase assembly. These assumptions would yield different predictions depending on the stoichiometry of Rpb3 in the assembled polymerase. In the case of 1:1 stoichiometry of the third largest subunit relative to the polymerase core, only two classes of RNA polymerase II molecules should be present in the cell: one with tagged and another with untagged subunit. These two classes can be separated from each other using Ni-NTA affinity chromatography. If the alternative (2:1) proposition were true, independent assortment of the His₆-tagged and -untagged subunits would result in appearance of three distinct classes of RNA polymerase II molecules: two homogeneous classes with both Rpb3 subunits either tagged or untagged and the third class of heterogeneous RNA polymerase II, comprising one tagged and one untagged subunit. Consequently, these heterogeneous molecules would be recovered in the eluate off the Ni-NTA column at nondenaturing conditions, resulting in the appearance of the untagged subunit in the Ni-NTA binding fraction.

By analyzing the relative abundance of the tagged and untagged Rpb3 protein in the crude and fractionated extracts of the yeast cells grown on different carbon sources, we established that RPB3 expression from the plasmid-borne GAL1 promoter is nearly identical to that from its native promoter in the cells grown on 2% raffinose (Fig. 1, lane 1, and data not shown). Western blot analysis of the immunoaffinity-purified RNA polymerase II confirmed our initial assumptions regarding comparable efficiencies of incorporation of the tagged and untagged versions of the third largest subunit into the assembled enzyme, as the relative levels of these subunits in the crude extract were similar to those in the anti-Rpb1 CTD mAb-binding fraction (Fig. 1, lanes 1 and 3). The latter was then subjected to the Ni-NTA column chromatography; unbound RNA polymerase II material (pooled from the flow-through and wash fractions) and nickel-bound retentate from the 160 mM imidazole eluate were probed with anti-Rpb3 antibodies (Fig. 1, lanes 4 and 5). Analysis of these data unambiguously supports the conjecture of the third largest subunit being present in a single copy per molecule of the S. cerevisiae RNA polymerase II, since not only was the untagged subunit not recovered in the nickel-bound fraction, the heterologous (one tagged + one untagged subunit) class of molecules, expected to be the most prominent in the nearly equimolar mixes of the two different, freely assorted subunits, was not found even among the unbound material (Fig. 1, lane 4). Our experiment clearly distinguishes between the two proposed Rpb3 stoichiometries in favor of the presence of only one such subunit per RNA polymerase molecule. This finding is consistent with the Rpb3-Rpb11 heterodimer being an ortholog of the α₁-homodimer in eubacterial RNA polymerases (13).

Having established an efficient system of incorporation of the engineered Rpb3 into the in vivo assembled RNA polymerase II, we next investigated the role of the two segments of this polypeptide (amino acids 29–55 and 226–267), exhibiting some similarity to the α₁-homodimer of RNA polymerases. To that end we utilized a yeast host-vector system allowing simultaneous expression of the wild-type RPB3 gene from its native promoter and the His₆-tagged RPB3 cassette from the vector-borne GAL1 promoter. Initial assumptions made in this work were: (i) stoichiometric incorporation of the Rpb3-tagged and -untagged subunits into RNA polymerase II and (ii) equally efficient incorporation and independent assortment of the tagged and untagged subunits during the polymerase assembly. These assumptions would yield different predictions depending on the stoichiometry of Rpb3 in the assembled polymerase. In the case of 1:1 stoichiometry of the third largest subunit relative to the polymerase core, only two classes of RNA polymerase II molecules should be present in the cell: one with tagged and another with untagged subunit. These two classes can be separated from each other using Ni-NTA affinity chromatography. If the alternative (2:1) proposition were true, independent assortment of the His₆-tagged and -untagged subunits would result in appearance of three distinct classes of RNA polymerase II molecules: two homogeneous classes with both Rpb3 subunits either tagged or untagged and the third class of heterogeneous RNA polymerase II, comprising one tagged and one untagged subunit. Consequently, these heterogeneous molecules would be recovered in the eluate off the Ni-NTA column at nondenaturing conditions, resulting in the appearance of the untagged subunit in the Ni-NTA binding fraction.

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The Rpb3 mutant lacking amino acids 226–267 (Rpb3Δ226–267), when expressed from the GAL1 promoter in presence of raffinose, accumulated in the cell at the wild-type levels (Fig. 2A, lane 1), but in contrast to its full-length counterpart Rpb3* (Fig. 1) was not found in the RNA polymerase II-containing fractions that eluted from the 8WG16-Sepharose (Fig. 2A, lanes 4 and 5). Instead, it appears to be present only in the flow-through fraction (Fig. 2A, lane 2).

The NH₂-terminal α-fragment (8) of Rpb3 (amino acids 29–55) corresponds to the region in the α-subunit where a number of mutations were isolated that affected α-α dimerization (17–20). In order to evaluate the importance of this fragment for the assembly of the RNA polymerase II in vivo, we constructed a deletion mutant of RPB3, lacking the coding sequence for amino acids 29–55 (Rpb3Δ29–55), and cloned it into the p416GAL1 expression vector. Unlike the His₆-tagged derivative of the
Fig. 2. Rpb3 mutants defective in assembly of the RNA polymerase II in vivo. Western blotting with a mixture of mAbs SWG16 (anti-Rpb1), 127 (anti-Rpb3), 4Y11 (anti-Rpb11), and anti-Rpb2 serum following 4–20% Tris-glycine SDS-PAGE. Lanes M, MultiMark molecular mass markers. A: lane 1, (NH₄)₂SO₄ precipitate of the crude extract from the cells expressing untagged wild-type Rpb3 and its His₆-tagged (amino acids 226–267) deletion mutant (Rpb3¹⁰²); lane 2, flow-through of the material in lane 1 loaded on SWG16-Sepharose; lanes 3–5, fractions eluted from the 8WG16-Sepharose in the presence of 40% propylene glycol. Rpb3α, Rpb3 degradation product(s); lane 1, His₆-tagged Rpb3 mutant lacking NH₂-terminal (amino acids 29–55) α-homology region (Rpb3Δ₁₇) isolated at nondenaturing conditions on Ni-NTA-agarose (160 mM imidazole); lane 2, His₆-tagged wild-type Rpb3 (Rpb3*) isolated at nondenaturing conditions on Ni-NTA-agarose (160 mM imidazole).

wild-type Rpb3, whose calculated molecular mass was significantly larger than that of its untagged counterpart (38,034 versus 35,300 Da), His₆-tagged mutant subunit comigrated with the untagged wild type polypeptide during SDS-PAGE (their calculated molecular masses being 35,077 and 35,300 Da, respectively). In order to distinguish between the two, we utilized a combination of Rpb3-specific antibodies and His₆-specific Ni-NTA-alkaline phosphatase conjugate. When subjected to the immunoaffinity chromatography on the 8WG16 column, all the Rpb3-associated Ni-NTA-binding potential eluted in the flow-through (data not shown), whereas a majority of the RNA polymerase II (the fraction containing nonproteolyzed Rpb1 CT.D) eluted only after addition of propylene glycol (data not shown). Conversely, when the crude lysate from the cells, expressing both the untagged wild-type and the His₆-tagged Δ29–55 mutant subunits, was passed through the Ni-NTA-agarose column, the bound material included the mutant His₆-tagged protein, but not other Rpbs, such as the two largest, Rpb1 and Rpb2, and another α-ortholog, Rpb11 (Fig. 2, lane 1, and data not shown), that copurified in similar conditions with the wild-type His₆-tagged Rpb3 (Fig. 2B, lane 2, and data not shown).

Both mutant Rpb3 proteins accumulated in the cell at approximately the same level as the wild type and did not significantly affect growth even when overexpressed in the medium containing both raffinose and the inducing carbon source, galactose (data not shown).

Thus, deletion of either α-homology region in the yeast Rpb3 effectively abolished its incorporation into the RNA polymerase II (in presence of the comparable amount of the wild-type protein). This finding is consistent with the relatively innocuous phenotype of these mutants in the merodiploid (relative to the Rpbs gene) yeast. The exact nature of the interactions disrupted by these mutations will be addressed in a series of the pairwise far-Western blot and pull-down experiments involving RNA polymerase II subassemblies and individual subunits. Our experimental approach serves as a complement to the techniques already in use in the research of RNA polymerase II (11, 13, 14). Among the substantial advantages of this approach is its operational simplicity, compared with the gene transplacement method used by Ishihama and co-workers (14) to produce His₆-tagged yeast RNA polymerase II. The fact that expression of the engineered subunit in yeast can be regulated over several orders of magnitude by changing the carbon source in the growth media or by switching to another of the many “sister” plasmids (15) allows not only the adjustment of the expression to the wild-type level, but also staging the in vivo “competition” experiments and overproduction of the His₆-tagged subunits in yeast and E. coli using the same expression cassette.³

Our results together with data from other laboratories (11–13) strengthen the hypothesis that the homology between the α-subunit of the eubacterial RNA polymerase and eucaryal subunits 3 and 11 reflects not only the past common origin, but also persisting similar functional roles in determining the architecture of their respective RNA polymerases. The question remains unanswered of the evolutionary bifurcation that separated eucaryal and archaeabacterial enzymes with their α-like heterodimers (subunits 3/11 and D/L, respectively (Fig. 3)) from eubacterial RNA polymerases, built around α₉-homodimer. Based on the phylogenetic clustering of the polypeptide sequences for the cloned α-homologs (Fig. 3), we find it plausible that a heterodimeric enzyme, similar in architecture to the eucaryal/archaeabacterial RNA polymerases, existed prior to the separation of the three evolutionary lineages, with subsequent loss of the smaller (Rpb11- or λ-like) subunit in the early history of eubacteria. This thesis is in agreement with previously proposed hypothesis of the reductionist evolution leading to the modern eubacterial RNA polymerases (2).

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