Mass Spectrometry Reveals Stable Modules in holo and apo RNA Polymerases I and III

Laura A. Lane, Carlos Fernández-Tornero, Min Zhou, Nina Morgner, Denis Ptchelkine, Ulrich Steuerwald, Argyris Politis, Doris Lindner, Jelena Gvozdenovic, Anne-Claude Gavin, Christoph W. Müller, and Carol V. Robinson

1Department of Chemistry, Chemistry Research Laboratory, Mansfield Road, Oxford OX1 3TA, UK
2Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK
3European Molecular Biology Laboratory, Structural and Computational Biology Unit, Meyerhofstrasse 1, 69117 Heidelberg, Germany
4Present address: Chemical and Physical Biology Department, Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain
5Present address: Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Gottingen, Germany
6Present address: The Research Complex at Harwell, Harwell Science and Innovation Campus, Didcot, Oxfordshire, OX11 0FA, UK
7Correspondence: carol.robinson@chem.ox.ac.uk

DOI 10.1016/j.str.2010.11.009

SUMMARY

RNA polymerases are essential enzymes which transcribe DNA into RNA. Here, we obtain mass spectra of the cellular forms of apo and holo eukaryotic RNA polymerase I and III, defining their composition under different solution conditions. By recombinant expression of subunits within the initiation heterotrimer of Pol III, we derive an interaction network and couple this data with ion mobility data to define topological restraints. Our data agree with available structural information and homology modeling and are generally consistent with yeast two hybrid data. Unexpectedly, elongation complexes of both Pol I and III destabilize the assemblies compared with their apo counterparts. Increasing the pH and ionic strength of apo and holo forms of Pol I and Pol III leads to formation of at least ten stable subcomplexes for both enzymes. Uniquely for Pol III many subcomplexes contain only one of the two largest catalytic subunits. We speculate that these stable subcomplexes represent putative intermediates in assembly pathways.

INTRODUCTION

Three RNA polymerases (Pols) I, II, and III are located in the nuclei of eukaryotes. These enzymes are responsible for catalyzing the DNA-directed synthesis of rRNA; tRNA; mRNA; and other small rRNAs including SS rRNA. Of the three enzymes Pol II from Saccharomyces cerevisiae has the most detailed structural information available, with high-resolution atomic structures of the enzyme (Armache et al., 2005), and its elongation complex (Kettenberger et al., 2004). For Pol I and III, consisting of 14 and 17 subunits, respectively, atomic structures of their stalk subunits (Jasiak et al., 2006; Kuhn et al., 2007) and the Pol I specific A49/A34.5 subcomplex (Geiger et al., 2010) together with cryo-electron microscopy (cryo-EM) reconstructions defining their overall topology (Fernández-Tornero et al., 2007; Kuhn et al., 2007) have been reported. Furthermore, extensive yeast 2 hybrid analysis (Flores et al., 1999) and homology modeling (Jasiak et al., 2006; Kuhn et al., 2007) have defined their stoichiometry and interactions (reviewed in Werner et al., 1992). The assembly mechanism of Pol II has implicated the subunits Rpb3, Rpb10, Rpb11, and Rpb12 (Cramer et al., 2001). The two largest polypeptides are thought to be incorporated early in the assembly process (Ishiguro et al., 1998). Pol assembly has been studied most widely in the bacterial form due to the simplicity of prokaryotic polymerases compared with those in archaea and eukaryotes. A number of subunits are however highly conserved across all three systems (Ebright, 2000; Ishihama, 1981). Furthermore, a handful of studies from both archaea and eukaryotes have identified several subcomplexes which may be highly relevant to the assembly process (Kimura et al., 1997; Rubbi et al., 1999; Werner et al., 2000; Werner and Weinzierl, 2002). Most recently the assembly of RNA Pol II in human cells was studied using quantitative proteomics revealing...
a cytoplasmic complex containing subunits Rpb2-Rbp3-Rbp10-Rbp11-Rbp12 that assemble with the large subunit Rbp1 stabilized by Hsp90 (Boulon et al., 2010).

Here, we apply electrospray mass spectrometry (MS) to define interaction modules generated by partial denaturation in solution. This enables us to assess not only their stoichiometry but also their interaction networks (Zhou and Robinson, 2010). This combination of MS with in-solution disruption techniques, using small organic molecules or the manipulation of ionic strength, has enabled detailed subunit contact maps and subunit architectures to be established (Hernandez et al., 2006; Zhou et al., 2008). Furthermore, a previous study demonstrated reversible disassembly of protein complexes, allowing the proposal that the stable modules generated in solution can represent assembly intermediates during the evolution of protein complexes (Levy et al., 2008). Recently, the assembly of a macromolecular complex has been monitored by the formation of subcomplexes from recombinant subunits (Sharon et al., 2009). Very recently mass spectra of the intact Pol I were recorded and subjected to changes in ionic strength. The resulting mass spectra revealed dissociation of a stable heterodimer A49/A34.5 from the intact Pol I (Geiger et al., 2010).

To define subunit contacts in Pol III we investigated complex formation using individual and pairwise recombinant expression of protein subunits. We complemented this data with pull-down assays. We also expressed the Pol III heterotrimer C31/C82/C34 enabling us to define all subunit interactions. These polymerase assemblies are inherently very stable and, as such, rarely yield subcomplexes without perturbation of the solution conditions. We found that by increasing the pH of the buffer, and partially denaturing Pol I and III, we could generate and assign a number of subcomplexes derived from both RNA polymerases and their respective elongation complexes.

RESULTS

Intact Pol I Confirms Unit Stoichiometry for All 14 Subunits

Mass spectra of Pol I isolated directly from yeast cells using an AC40 TAP-tag were recorded under non-denaturing MS conditions (Figures 1A and 1B; see Figure S1 available online). The major charge state series at 11,000 m/z, (gray) dominates the spectrum and corresponds in mass to the intact Pol I. Above 12,000 m/z ''stripped'' complexes are observed formed as a result of the ejection of single subunits from the intact complex under activating MS conditions. Such conditions are required to improve the mass resolution of the intact species. Expansion of the intact region of the spectrum reveals a well-resolved charge state series (green), with a mass of 596,723 ± 78 Da, which is assigned to the 14 component Pol I (Figure 1B).
The less intense series (red, Figure 1B), with a measured mass of 590,690 ± 75 Da is approximately 6 kDa lighter than the intact, and likely corresponds to a form of Pol I in which the 5 kDa TAP-tag is proteolytically cleaved postpurification. Overall, our mass spectra for Pol I are in agreement with the proposed stoichiometry for the 14 component proteins listed in Figure 1A (Buhler et al., 1976; Sentenac, 1985; Russell and Zomerdkj, 2006) and show that all subunits are present simultaneously within the intact assembly, in line with recent studies of this enzyme (Geiger et al., 2010).

A proteomic analysis of the Pol I complex identified a number of posttranslational modifications and the intact masses for each of the Pol I subunits is reported (Table S1). Only in high pH buffer conditions, in which Pol I was disrupted into many subunits and subcomplexes, could the masses of subunit A43 and the two large core subunits be determined as: 36,627 Da for A43; 187,545 Da for A190; and 135,767 Da for A135 (Figure S3). This complete list of subunit masses for Pol I enabled us to assign the subunit composition of all subcomplexes formed (see below).

Dynamic Binding of C53/C37 to Pol III
A mass spectrum of the intact C128-tagged Pol III, isolated directly from yeast cells, has a measured mass of 700,017 ± 87 Da for the predominant charge state series (red) (Figure 1C; Figure S1). This corresponds well with the theoretical mass (698,296 Da) obtained from summing 17 subunits at unit stoichiometry, including the TAP tag, and is consistent with previous studies (Lorenzen et al., 2007). In addition, a minor series (<10% green) is observed in the same spectrum with a measured mass of 620,467 ± 93 Da. The composition of this species does not correspond in mass to the loss of a single subunit. It must therefore result from the loss of multiple subunits from the intact complex. More than one subunit combination is consistent with this mass difference: C37 and C53 (78,809 Da) or C17, 25, and 34 (79,071 Da). To differentiate these two possibilities, we used the C-terminal 5 kDa TAP tag as a mass label, attaching it to one of the subunits within the two groups. After isolation with a C53 tag, the mass spectrum of Pol III revealed two series of almost equal intensity (Figure 1D). The mass difference had increased by approximately 5 kDa from 79 to 84 kDa allowing us to conclude that the C53 subunit, together with C37, dissociate readily from the intact Pol III. This is analogous to the generation of the heterodimer A49/34.5 reported very recently for Pol I by manipulation of ionic strength (Geiger et al., 2010). By perturbing the Pol III structure, through changing the position of the tag, we observed directly the 84 kDa-tagged C53/C57 subcomplex at low m/z (Figure 1E). Together these results confirm direct interaction between C53 and C37, and a labile association of C53/C37 with Pol III.

Architecture of the Pol III Initiation Heterotrimer
To probe further subunit interactions and stable modules within Pol III, we perturbed the complex through addition of high pH ammonium hydroxide solution. A large number of subcomplexes were formed including a stable trimer (C31/C82/C34), two heterodimers (C82/C31 and C17/C25), and a number of individual subunits (ABC10z, C11, C82, and C34) (Figure 2A). To probe further the arrangement of these subunits we expressed them recombinantly in Escherichia coli and isolated the trimer (C31/C82/C34). Under low-salt conditions (100 mM ammonium acetate) a single charge series was observed with a measured mass of 138,296 ± 77 Da, consistent with a unit stoichiometry for all three subunits (data not shown). It is not possible, however, to determine the connectivity of the three subunits from MS or tandem MS of the intact heterotrimer. Using a high-salt concentration (1.8 M ammonium acetate) we disrupted the trimer into two dimers composed of C31/C82 and C34/C82 as well as single subunits C34 and C82 (Figure 2B). Crucially, no C31/C34 subcomplex was formed. This is in contrast to interactions deduced from yeast two-hybrid experiments (Flores et al., 1999), implying that any interactions between C31 and C34 are likely to be transient/unstable leading us to establish that the stable arrangement of subunits is C31-C82-C34, with C82 acting as the bridging subunit.

In tandem MS experiments, where a single charge state of the trimer C31-C82-C34 is selected and collisionally activated by inert gas molecules, we found that the first subunit ejected was C34 leaving a complementary subcomplex C82/C31. At higher levels of activation C82 was also detected (data not shown). Similarly, upon collisional activation of the intact 17 subunit Pol III complex, C34 was found to dissociate readily, implying a peripheral location for this subunit (data not shown). Further support for the connectivity between these three subunits is provided by evidence from protein expression. C31 and C82 required coexpression, while C34 could be readily expressed in isolation (data not shown). Together these results indicate not only the strength of the C82/C31 interaction but also the propensity of the C34 subunit to dissociate both from the trimer and the intact Pol III.

The three-dimensional topology of the initiation heterotrimer was probed using ion-mobility (IM) MS. A value of 6549 A˚2 was determined experimentally for the collision cross-section (CCS) of the intact C31/C82/C34. Using a coarse-grained strategy we generated a low resolution model structure of the trimer. This was achieved by utilizing structural information from IM data as size and volume restraints for both individual subunits and subcomplexes C82, C34, C82/C31 and was subsequently correlated to the density difference between the Pol III cryo-EM volume (Fernández-Tornero et al., 2007) and Pol II crystal structure (Cramer et al., 2001) (Figure 2C). Our model for the initiation heterotrimer reveals a linear trimer which has a substantial surface area. This elongated structure most likely reflects its functional role in contacting additional factors and directing DNA to the RNA polymerase core.

Assembling Stable Subcomplexes
To define the smallest possible building blocks, we assembled subcomplexes from subunits and heterodimers generated in vitro. As a control experiment we combined equimolar quantities of subunit C34 and subcomplex C31/C82, to form the initiation heterotrimer characterized above. The series labeled with blue circles (Figure 2D) was assigned to E. coli hsp70, after LC-MS and tryptic digestion (data not shown). As anticipated the initiation heterotrimer, was formed by reconstitution in vitro with a measured mass 139 kDa (Figure 2D). No C31/C82 heterodimer remains confirming its high affinity for C34 and the specificity of these interactions.
Figure 2. Mass Spectra of Subcomplexes Formed under Partially Denaturing Solution Conditions, Topological Model of C34/C82/C31 Trimer, and Interactions Observed from Subcomplex Assembly Experiments

(A) Mass spectrum of AC40-tagged Pol III in 30% v/v ammonium hydroxide (pH 10.9). The 17 subunit Pol III assembly has dissociated into a number of small subcomplexes (C31/C82/C34; C25/C17 and C82/C31) and individual subunits (ABC10a; C11; C34; ABC23 and C82).

(B) Mass spectrum of the Pol III ternary complex from yeast expressed recombinantly in E. coli. This spectrum, obtained in 1.8 M ammonium acetate, shows the C34/C82/C31 trimer and the detection of four additional smaller species as a result of the high-salt conditions. These include two dimers (C34/C82 and C31/C82) and subunits C34 and C82 alone.

(C) A cluster of low resolution models that satisfy the restraints imposed by ion mobility experiments. The solid (opaque) spheres within the shaded area represent the model structures generated from the lower limit of IM restraints for both individual subunits and subcomplexes. The upper limit of the IM data is represented by the shaded area and defined by varying the size of spheres and the distances between the center of masses of subunits. To build these models the relative orientation between the subunits is kept constant and three different views are shown with the EM density of Pol III enzyme. The crystal structure of the Pol II enzyme (PDB reference: 1WCM) was inserted into the EM density of the Pol III species allowing us to view the difference density map which is assigned to the five additional subunits in Pol III. In green are shown the three initiation heterotrimer subunits. C34 is represented by a single sphere, whereas subunits C82 and C31 are represented by two overlapping spheres in order to account for multiple domains. The calculated CCS of the model structure is in good agreement with measured IM/MS value.
Having established this control experiment, we turned our attention to the key C11, C37, and C53 region within Pol III. In addition to the role of C33/C37 in termination recognition, and the C11 responsibility for RNA cleavage, together they are implicated in the process of reinitiation after the first round of transcription (Landrieux et al., 2006). It might be anticipated, therefore, that these subunits would interact. A mass spectrum of an equimolar solution of subunit C11 and the subcomplex C53/C37, incubated prior to analysis reveals two forms of the C53/C37 dimer due to the presence of an N-terminally truncated C53, a population of C37 dissociated from the dimer and the monomer C11 (Figure 2E; Figure S1 and Supplemental Experimental Procedures). However, no stable interaction between C53/C37 and C11 could be identified under these solution conditions. In addition, conventional pull-down experiments between C11 and C37 (Figure S2A), also in the presence of C53, showed no direct interaction between these subunits.

Previous yeast two hybrid assays have indicated interactions between C17 and C31, C34, and C11 (Ferri et al., 2000), in addition to the well-established interaction of C17 with C25 (Jasiak et al., 2007). We were unable to confirm a stable interaction between either C17/C25 and C11; C17/C25 and C34; or C17/C25 and C31/C82/C34, under solution conditions of 200 mM ammonium acetate, pH 7.4 (Figures 2–2H). The interaction of C25 and C31/C82/C34, under solution conditions. In addition, conventional pull-down experiments between C11 and C37 (Figure S2A), also in the presence of C53, showed no direct interaction between these subunits.

The Elongation Complexes of Pol I and III

The addition to Pol I and III of an in vitro reconstituted transcription bubble resulted in mass spectra that are remarkably well resolved, despite the difficulties often encountered when obtaining high-resolution spectra of protein–nucleic acid complexes (Figure 3). The mass of the transcription bubble is calculated as 31.6 kDa (Table S3), and the most intense charge state distributions recorded for the Pol I and III elongation complexes are measured as ~32 kDa higher than the masses of Pol I and III alone, 628,188 Da and 730,473 Da, respectively. Comparison of the spectra of Pol I and III in complex with the transcription bubble reveals two interesting features. The first is the loss of 73.8 kDa from the intact Pol I elongation complex (Figure 3A), most likely as a result of the loss of the Pol I subunits A49.5 and A49 (sequence mass: 73,526 Da). This labile binding of A43.5/A49 is reminiscent of the dynamic association of the analogous C53/C37 heterodimer within Pol III (Figure 1E) and was reported previously with increasing ionic strength of solutions containing Pol I (Geiger et al., 2010). Second, a species ~6.0 kDa lighter than the Pol III elongation complex is observed in Figure 3B, most likely as a result of proteolytic cleavage of the TAP tag following purification as observed previously for Pol I (Figure 1B). Additionally a further charge state series at low m/z (data not shown).

The preparation of C82/C31 subunits was contaminated with E. coli hsp70 (blue circles). Individual subunits C37 (32 kDa) and C34/C82/C31, appear insufficient for stable subcomplex formation. A34.5/A49 is reminiscent of the dynamic association of the analogous C53/C37 heterodimer within Pol III (Figure 1E) and was reported previously with increasing ionic strength of solutions containing Pol I (Geiger et al., 2010). Second, a species ~6.0 kDa lighter than the Pol III elongation complex is observed in Figure 3B, most likely as a result of proteolytic cleavage of the TAP tag following purification as observed previously for Pol I (Figure 1B). Additionally a further charge state series at low m/z corresponds to the mass of the initiation heterotrimer, the subcomplex unique to Pol III (Figure 3B). In order to achieve sufficient resolution of the elongation complex this spectrum was recorded under mildly activating MS conditions. This accounts for the further dissociation of the C31/C82/C34 trimer into a C31/C82 dimer and the C34 subunit detected at low m/z (data not shown). It is noteworthy that the loss of the heterotrimer from the Pol III elongation complex is significantly more pronounced than in the apo form, indicating a more labile association of this heterotrimer within the Pol III elongation complex.

(D) Mass spectrum of the subcomplex assembly control experiment in which equimolar quantities of subunit C34 and subcomplex C82/C31 were added, resulting in the formation of the established heterotrimer C34/C82/C31. The preparation of C82/C31 subunits was contaminated with E. coli hsp70 (blue circles).
(E) Subunit C11 and subcomplex C37/C53 were combined with no interaction detected between these two species.
(F) C11 was also combined with subcomplex C25/C17 and no affinity between the two was identified either.
(G) This spectrum shows the combination of the C17/C25 dimer and subunit C34, again no interaction was detected.
(H) Combining subcomplexes C17/C25 and C34/C82/C31 also showed no interaction between these species. See also Table S2.

Figure 3. Mass Spectra of RNA Pol I and III Elongation Complexes

(A) MS of Pol I elongation complex (orange circles) obtained in an excess of the transcription bubble. The less intense series (blue circles) corresponds to the loss of subunits A49 and A34.5 from the Pol I elongation complex (EC).
(B) The Pol III elongation complex with (orange circles) and without (blue circles) the TAP tag fragment were detected in addition to two other subcomplexes: C31/C82/C34 (138 kDa) (dark green circles) and C82/C31 dimer (102 kDa) (light green circles). Individual subunits C37 (32 kDa) and C34 were also observed at low m/z (data not shown).
Disassembly Reveals Stable Interaction Modules

To disrupt the polymerase assemblies into subcomplexes we added high pH buffer to complex-containing solutions. These basic conditions induced formation of multiple subcomplexes, some identified in our earlier experiments (Figure 2A), but also many larger subcomplexes (Figure 4; Figures S3–S7). Schematics of all the subcomplexes produced under high pH conditions are shown (Figure 5). To assign the subunit compositions of these large subcomplexes, we used tandem MS and spectral deconvolution algorithms reported previously (Hernandez et al., 2009). Many large species, which contain a significant proportion of the overall mass of the polymerase, are especially interesting since they do not maintain the catalytic core formed by the two largest subunits.

The subassemblies of the Pol I elongation complex obtained at high pH were assigned to six subcomplexes which derive from the AC40-tagged Pol I elongation complex (Figure 5A; Figure S3). These include three heterodimers: A190/AC19 (202 kDa); A190/ABC27 (212 kDa); and the core A190/A135 (323 kDa); as well as the heterotrimer A190/A135/ABC27 (348 kDa). In addition, two much larger subassemblies were detected in which the nucleic acid chains were also retained. The largest of these, with a mass of 522 kDa, is a complex in which the only subunits to dissociate are ABC23, A12.2, and the subunits A49 and A34.5 (unique to Pol I). The subcomplex with a mass of 471 kDa forms after the additional loss of the stalk heterodimer (A14/A43). To obtain well-resolved mass spectra under these disruptive solution conditions, it was necessary to apply gas-phase activation. As a consequence some gas phase dissociation of the holo Pol I subcomplexes was observed, giving rise to five gas-phase products which have lost peripheral subunits of Pol I. Interestingly under identical solution conditions the apo Pol I resulted in the formation of a different ensemble of subspecies on activation (Figure 5B; Figure S4). These disruption products originate from the two forms of the intact Pol I which differ in mass by 6 kDa (Figure 1B). The most striking difference between the holo and apo Pol I is the retention of subunits A49 and A34.5 in the apo form, as well as the increased stability of apo Pol I compared with when the transcription bubble is present.

The disassembly products formed from Pol III and the Pol III elongation complex provide further insight into subcomplexes with independent stability (Figure 4B; Figures S5–S7). For both holo and apo forms, we detected eight subcomplexes from solution disruption, all of which contained at least one or other of C160 or C128, without the retention of any nucleic acid chains. The smallest disassembled species, based on a C128 scaffold, is the C128/ABC10β (138 kDa) dimer and the tetramer C128/AC40/AC19/ABC10β. The latter was isolated from both AC40 (197 kDa)- and C82 (192 kDa)-tagged preparations of Pol III (Figure 5C). Similarly, subunit ABC10α was also associated with the tetramer (C128/AC40/AC19/ABC10β) for complexes isolated using both C82 and AC40 tags. The trimer C160/ABC14.5/ABC27 (205 kDa) ejected subunits ABC27 and ABC14.5, during gas-phase activation, giving rise to stripped heterodimers of C160/ABC14.5 and C160/ABC27 respectively (Figure S5C). The three largest subcomplexes (418, 408, and 400 kDa) contain both core subunits as well as variants of the trimeric and tetrameric subcomplexes described above, C160/ABC14.5/ABC27 and C128/AC40/AC19/ABC10β, with and without...
subunit ABC23 (Figures S6 and S7). Interestingly in the apo and holo forms the subunits unique to Pol III (C31, C82, and C34) are not retained. This is analogous to the situation in holo Pol I, in which A49 and A34.5 dissociate readily, implying that interactions with the unique subunits in both polymerases are destabilized in their respective holo complexes.

DISCUSSION

We have shown that well-resolved mass spectra can be obtained for intact Pol I and Pol III, with and without the transcription bubble, confirming their overall stoichiometry and homogeneity. By manipulating the pH and ionic strength of both RNA polymerase-containing solutions, we formed reproducible interaction modules with sufficient intrinsic stability to survive in solution and gas phases. Clear differences were observed in the disassembly of the two polymerases implying that the additional subunits in Pol III (C34, C82, and C31) make a fundamental difference to the overall stability of the complexes. This is an unexpected finding since these subunits are located on the periphery of the complex, and many homologous and analogous subunits are located in the cores of both Pol I and III. Also unexpected was the observation that while the transcription bubble had little impact on the disassembly of apo Pol III, significant differences were observed for the disruption products of the two forms of Pol I. Specifically in the presence of the transcription bubble, interactions with A49 and A34.5 are destabilized relative to the apo form of the complex.

In might be anticipated that changes in stability could be associated with location of the tagged subunit within the complex. This was observed for subunit C53 when introduction of the tag lead to the direct observation of the C53/C37 heterodimer (Figure 1E). This association had been reported using yeast two hybrid methods (Flores et al., 1999). Since the presence of the TAP-tag at the C-terminal end of subunit C53 increased the tendency of the C53/C37 subcomplex to dissociate from the core (Figure 1D), relative to the alternative tagging positions used to isolate Pol III, it is conceivable that the C-terminal anchors the heterodimer to the core of the complex. Additionally, the detection of this heterodimer in the presence of the C53 tag demonstrates that the interaction between C37 and C53 is in all likelihood stronger than that of the heterodimer with the core particle. Interestingly, the ability of C37/C53 to dissociate from
Pol III, and A49/A34.5 to dissociate from the Pol I elongation complex (Figure 3A) may relate to their evolutionary origin as permanently recruited general transcription factor IIIF (Carter and Drouin, 2010).

C11 has a dual function in being both responsible for mediating RNA cleavage activity in Pol III and facilitating transcription reinitiation (Chedin et al., 1998; Landrieux et al., 2006). It was hypothesized that a close connection exists between C11, C37, and C53 in facilitated reinitiation, though it was established that the recognition of terminator elements was achieved by C37/C53 while C11 remained responsible for RNA cleavage (Landrieux et al., 2006). Surprisingly, we found no evidence of a stable interaction between C11 and this heterodimer by both in vitro assembly experiments (Figure 2E) and pull-down assays (Figure S2A). This suggests any communication between C53/C37 and C11 is likely independent of a direct interaction, despite the important cooperation thought to occur in the reinitiation process.

Starting from individual subunits produced by protein expression, we were able to determine the viability of other dimeric and trimeric complexes. Our studies of the Pol III specific initiation trimer (C31/C82/C34) enabled us to elucidate a linear interaction network of C31-C82-C34 with no contacts between subunits C31 and C34 (Figures 2A and 2B). We speculate that the sensitivity to ionic strength of this trimer is due to electrostatic interactions, important in maintaining its independent stability. Particularly stable is the interaction between C82/C31 that provides a plausible explanation for our inability to dissociate C31 under any solution conditions, either from the initiation heterotrimer or from the intact Pol III as isolated subunit. The dissociation of C31/C82/C34 trimers from the Pol III enzyme under high pH conditions is in contrast from previous findings where only C82/C34 heterodimers dissociating from the enzyme were observed (Lorenzen et al., 2007). Genetic evidence implies a close proximity between C31 and C160, through the role of C37 and C11 is likely independent of a direct interaction, despite the important cooperation thought to occur in the reinitiation process.

Using our evidence of subunit contacts and relative positioning of subunits in the initiation heterotrimer, along with IM measurements to define constraints, we propose a topological model (Figure 2C) and incorporated this into the available cryo-EM density map (Fernández-Tornero et al., 2007). This model combines all structural information available and highlights the possible architecture of C31/C82/C34; its large surface area may be important for a flexible interaction and adaptable conformation with DNA at the entry site (Brun et al., 1997; Thuillier et al., 1995). Of particular interest is the observation that for the Pol III elongation complex dissociation of the initiation trimer occurred more easily, while in the case of apo Pol III this dissociation only occurred under high pH conditions. We speculate that this could be a result of a weakening in the interaction between the trimer and the core enzyme, on the binding of the transcription bubble and related to the evolutionary origins of the heterotrimer.

During the reviewing process of this manuscript two additional studies were completed (Fernández-Tornero et al., 2010; Vannini et al., 2010). Based on cryo-EM reconstructions of the Pol III enzyme and the elongation complex (although at different resolutions) homology models of Pol III specific subunits are located in the enzyme at positions that are in accordance with the results reported here.

Assignment of all the subcomplexes generated through solution disruption together with our in vitro assembly experiments allows us to compare these stable modules in the two polymerase enzymes studied here (Figure 6). The major differences arise from the formation of more symmetrical subcomplexes in Pol I. Both subcomplexes, A190/A135 and A190/A135/ABC27, contain the two largest subunits (Figure 4A). Analogous complexes are not observed for Pol III. A plausible explanation for this difference is that A190 and A135 are larger than their Pol III counterparts by ~24 and ~5 kDa respectively. This may account not only for a larger subunit interface between A190
and A135, compared with C160 and C128, but may also explain a second observation from our experiments: that there is greater retention of the transcription bubble by Pol I compared with Pol III. Homologous complexes between Pol I and Pol III include A190/ABC14.5 (202 kDa) and ABC27/135 (212 kDa). Interestingly, one of the most stable subcomplexes formed for Pol I is the 8 mer (471 kDa) which contains the two analogous trimeric and tetrameric interaction modules described for Pol III (ABC27/A190/ABC14.5 and A135/AC40/AC19/ABC10j/ABC10z).

Comparing our results with those established for bacterial polymerase assembly which is well documented, both in vitro (Igarashi and Ishihama, 1991) and in vivo (Hayward et al., 1991), α2 dimerization (Zhang et al., 1999), is followed by the addition of β, then β′. The pivotal role of the subunits equivalent to α2 and conserved as subunits D/L in archaea (Goede et al., 2006) (homologous to Rpb3/Rpb11 in Pol II [Cramer et al., 2001] and AC40/AC19 in Pols I and III) were also identified as the platform for the assembly of the archaeal enzyme core (Hirata et al., 2008). Furthermore, studies of Pol II from Schizosaccharomyces pombe established that under high denaturant concentrations core subassemblies of Rpb2/Rpb3/Rpb11 (remi-

er II assembly mechanism (Cramer et al., 2001). The D/L/N/P and Rpb12 from S. cerevisiae, which are homologous to Pol II subunits Rpb3, Rpb10, Rpb11 have been shown to form a tetramer (Werner et al., 2000), and are also homologous to Pol II subunits Rpb3, Rpb10, Rpb11 and Rpb12 from S. cerevisiae, which were implicated in the Pol II assembly mechanism (Cramer et al., 2001). The D/L/N/P tetramer was also shown to recruit subunit B (Werner and Weinzierl, 2002) and the resulting pentamer is analogous to the C128/AC40/AC19/ABC10j tetramer observed in our experiments (Figure 4B; Figures S5–S7). In addition, the subunits D, L, N, and P from Methanococcus jannaschii have been shown to form a tetramer (Werner et al., 2000), and are also homologous to Pol II subunits Rpb3, Rpb10, Rpb11 and Rpb12 from S. cerevisiae, which were implicated in the Pol II assembly mechanism (Cramer et al., 2001). The D/L/N/P tetramer was also shown to recruit subunit B (Werner and Weinzierl, 2002) and the resulting pentamer is analogous to the C128/AC40/AC19/ABC10j tetramer observed in both C82- and AC40-tagged preparations of Pol III (200 and 204 kDa, respectively) (Figure 4B; Figures S5–S7). Together with previous experiments in analogous systems, and those reported here, our results highlight the intrinsic stability of the asymmetric pentameric and tetrameric modules and their possible role in the assembly pathway of the euakaryotic Pol III.

Our ability to study highly heterogeneous systems has enabled us to examine the disassembly of both Pol I and III directly in complex with their transcription bubbles. The interaction modules that we observe are both in keeping with intermediates proposed for the simpler bacterial system, where available, and are consistent with subcomplexes which have been isolated from archaeal or eukaryotic Pol II systems.

The remote homology of C37/C53 and A49/A34.5 with the N termini of Pol II specific general transcription factors TFIIID subunits x and β, and an even more remote homology of Pol III subunits C82 and C34 with TFIIIE subunits x and β (Carter and Drouin, 2010; Geiger et al., 2010; Kuhn et al., 2007), have led to the suggestion that these specific subunits correspond to general transcription factors permanently recruited during evolution (Carter and Drouin, 2010). The facilitated dissociation of the heterotrimer C82/C34/C31, and heterodimers C37/C53 (Pol III) and A34.5/A49 (Pol I) (Lorenzen et al., 2007; Geiger et al., 2010), therefore, implies that similarly to Pol II, assembly and disassembly cycles could occur for each productive transcription event (van Werven et al., 2009). As a consequence our results contribute to a dynamic view of the enzymes in which yeast derived Pol I and Pol III specific subunits are not in a stable complex, but rather form stable interaction modules that are free to cycle during multiple transcription events.

**EXPERIMENTAL PROCEDURES**

**Isolation of RNA Pols and Elongation Complexes**

S. cerevisiae RNA Pol samples were isolated directly from cells according to previously described methods (Fernández-Tomero et al., 2007). Yeast strains were modified to express endogenous proteins fused with a C-terminal TAP-tag, on subunits C128, C82, or C53 for Pol III and AC40 for both Pol I and Pol III. An additional chromatography step using an anionic exchange mono Q column (GE), according to a previously described protocol (Huet et al., 1996), was added in the AC40 TAP-tag preparation to enable separation of Pol I and Pol III. The artificial transcription bubble (Table S2) was prepared as previously described (Kettenberger et al., 2004), and added to purified RNA Pol I or III in a 5:1 molar excess. The transcription bubble excess was removed using size exclusion chromatography on a Superose 6 column (GE) in 10 mM Tris (pH 7.5), 100 mM ammonium sulfate, 10 mM DTT.

**Subcomplex Assembly**

Subunits C11, C34, and C37 and subcomplexes C37/C53, C17/C25, and C82/C31 were expressed recombinantly; for details of purifications, see Supplemental Experimental Procedures. To identify binding between different species equimolar quantities (excepted for experiments between C34 and C17/C25; and C34/C82/C31 and C17/C25 where a 5:1 molar ratio was used) were combined in their original buffer and incubated at 37 °C for 30–40 min. The mixtures were then buffer exchanged into 200 mM ammonium acetate (pH 7.4) using centrifugal ultra-filtration columns with molecular weight cut-offs of 3 or 5 kDa (Sartorius).

**MS of RNA Pols, Elongation Complexes, and Subcomplexes**

For MS of the intact complexes, 30 μl of Pol or Pol elongation complex were buffer exchanged into 200–250 mM ammonium acetate (pH 7.4–7.5) using 3 or 5 kDa vivaspin columns (Sartorius). Nano-ES mass spectra were acquired on a Synapt HDMS (Waters), a modified QSTAR XL (Applied Biosystems) or a modified Q-ToF II mass spectrometer (Waters) using a previously described protocol (Hernandez and Robinson, 2007).

Typical instrument parameters used, in positive ion mode, on the Synapt HDMS (Waters, Manchester, UK) were: capillary voltage 1.7 kV; sample cone up to 170 V; extraction cone 5 V; accelerating voltage into the trap HDMS (Waters, Manchester, UK) were: capillary voltage 1.7 kV; sample cone up to 170 V; extraction cone 5 V; accelerating voltage into the transfer cell (termed “trap collision energy” as per the manufacturer’s terminology) 20–150 V; accelerating voltage into the transfer cell (transfer collision energy) 10–100 V; bias voltage 50 V; backing pressure 6.1 mbar; T-wave drift cell pressure 5.1 mbar, trap and transfer pressure 5.2 × 10⁻⁶ mbar (argon), T-wave drift cell pressure 5.1 × 10⁻⁶ mbar (nitrogen), and TOF pressure 2.2 × 10⁻⁶ mbar.

Typical instrument parameters used, in positive ion mode, on the QSTAR XL (MDSci, Applied Biosystems, Toronto, Canada) were: capillary voltage 1.4 kV; declustering potential 100–150 V, focusing potential 150–200 V; declustering potential 2 15 V. quadrupole voltage (Q0) 100–200 V, collision gas (CAD) 6–12. In tandem MS experiments, ions were isolated in the quadrupole and subjected to collision-induced dissociation (acceleration energy up to 200 V).

Typical instrument parameters used, in positive ion mode, on the modified Q-ToF II mass spectrometer (Waters, Manchester, UK) were: capillary voltage 1.8 kV; sample cone up to 160 V; extraction cone 0–5 V; collision energy up to 180 V; collision gas pressure 15–21 mbar; analyzer pressure ~1.9 × 10⁻⁶ mbar; backing pressure ~1.1 × 10⁻⁶ mbar; TOF pressure ~2.0 × 10⁻⁶ mbar; hexapole region pressure 3.6 × 10⁻⁶ mbar.

To generate subcomplexes, high pH ammonium acetate solutions were used. Pol I and Pol III elongation complex solutions were diluted with ammonium hydroxide (Sigma) at a ratio of 10%, 20%, and 30% (v/v) (pH 10.2–11.0). For the initiation heterotrimer expressed recombinantly, intact spectra were acquired in 100 mM ammonium acetate (pH 8.0). High-salt
conditions of 1.8 M ammonium acetate were used to partially denature the subcomplex. For details of LC-MS, IM-MS, and instrumental parameters, please refer to Supplemental Experimental Procedures.

Data Analysis

Data was acquired using Analyst QS software (Applied Biosystems) or MassLynx v4.1 (Waters), all other data processing was performed using MassLynx. In-house developed software (MASSIGN) was used to assign subcomplex composition, taking into consideration: masses, mass shifts, charge states, as well as tandem MS data, where available.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at doi:10.1016/j.str.2010.11.009.

ACKNOWLEDGMENTS

We thank Michel Werner for the kind gift of cDNA; Christophe Romier and Gunther Stier for kind gifts of subunit expression vectors; Gabriela Ridiola for LC-MS analysis; and Gill Hilton for critical reading of the manuscript. We acknowledge with thanks funding from the EPSRC (L.A.L.), the BBSRC (A.P.), the Royal Society (C.V.R.) and the EU grants “3D repertoire” LSHG-CT-2005-512028 (C.F.-T., M.Z., D.P., D.L.) and PROSPECTS (PROteomics SPECification in Time and Space), which stems from the European Commission’s FP7 (GA-HEALTH-F4-2008-201648) (N.M.). The authors declare no financial conflict of interest.

Received: June 4, 2010
Revised: October 15, 2010
Accepted: November 9, 2010
Published: January 11, 2011

REFERENCES


