Abstract

Protein interactions among RNA polymerase small subunits from the archaeon *Methanococcus jannaschii* were investigated using affinity pulldown assays in pairwise and higher-order combinations. In the most extensive study of archaeal RNA polymerase subunit interactions to date, including 37 pairs of proteins, 10 ternary combinations, and three quaternary combinations, we found evidence for pairwise interactions of subunit D with subunits L and N, and a ternary complex of subunits D, L and N. No other small subunit interactions occurred. These results are consistent with interactions observed in a crystal structure of eukaryotic RNA polymerase II and support a common archaeal/eukaryal RNA polymerase architecture. We further propose that subunit E is not an integral member of archaeal RNA polymerases. Finally, we discuss the relative accuracy of the various methods that have been used to predict protein–protein interactions in RNA polymerase.

Keywords: Affinity pulldown; Archaeon; Protein interaction; RNA polymerase; Transcription

1. Introduction

Fundamental properties of RNA polymerase (RNAP) are highly conserved among the three domains of life; however, archaeal and eukaryal transcription share a common evolutionary history distinct from bacterial transcription. This common history is illustrated by the requirement of homologous factors for both initiation of transcription [1] and elongation [2] and by the subunit composition of the RNAPs found in the two domains [3,4]. Although a four subunit core of RNAP is conserved in archaeal, bacterial and eukaryal domains, archaeal and eukaryal RNAPs share an additional five subunits (Table 1) [4,5]. The subunits found only in Archaea and Eucarya present us with questions regarding structure and function distinct from bacterial transcription and are the focus of this study. As a step toward understanding functional relationships among these subunits, interactions between small subunits within an archaeal RNAP were investigated. The recent 3-Å resolution crystal structure of *Saccharomyces cerevisiae* RNAP II provides a backdrop for their interpretation [6].

2. Materials and methods

2.1. Cloning and expression of RNAP subunits

PCR products of RNAP subunit genes amplified from *Methanococcus jannaschii* JAL-1T (DSM 2661T) genomic DNA were cloned and overexpressed as recombinant proteins either untagged (in pET-11b, Novagen), or harboring an N-terminal decahistidine (in pET-19b, Novagen) or FLAG (in pF:hTBP-11d [7]) affinity tag. Expression of subunit genes was carried out in *Escherichia coli* BL21(DE3) as described in the pET System Manual (Novagen). Cell pellets from 2.5 ml of culture were harvested 2 h post-induction and stored at –70°C.
Table 1
RNAP subunit composition of *M. jannaschii* and homologous subunits in eukaryal and bacterial domains

<table>
<thead>
<tr>
<th><em>M. jannaschii</em></th>
<th><em>S. cerevisiae</em></th>
<th><em>E. coli</em></th>
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<tbody>
<tr>
<td>A‘-A&quot;</td>
<td>RPB1</td>
<td>β'</td>
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<tr>
<td>B‘-B&quot;</td>
<td>RPB2</td>
<td>β</td>
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<tr>
<td>Dα</td>
<td>RPB3α</td>
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<td>E‘+E&quot;</td>
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<td>K</td>
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<tr>
<td>Lα</td>
<td>RPB11α</td>
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<td>N</td>
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<tr>
<td>P</td>
<td>RPB12</td>
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</table>

*Subunits of RNAP II.

Split subunits represented by one homologous subunit in Eucarya and Bacteria.

Split subunits represented by one homologous subunit in *Pyrococcus* sp., Crenarchaeota, Eucarya and Bacteria.

Contains ‘α-motif’ important for dimerization in Bacteria.

Split subunits represented by one homologous subunit in *S. acidocaldarius*; Eα domain not present in RPB7.

100 mM imidazole. Cleared lysates (200 μl) containing untagged or FLAG-tagged subunits prepared as above were incubated (rotating 1 h at room temperature) with the resin-bound, his-tagged subunit in PBB+0.1% (w/v) bovine serum albumin. After removal of unbound proteins with two, 500-μl rinses of PBB, the his-tagged subunit was specifically eluted in 50 μl PBB with 500 mM imidazole (rotating 10 min at room temperature). SDS-polyacrylamide gel electrophoresis [8] and Western blotting [9] with anti-FLAG-M2 antibody (Sigma) were used to analyze retained subunits.

3. Results and discussion

3.1. Pairwise interactions

Table 2 summarizes the results of affinity pulldown assays of pairwise combinations of small subunits. Interactions were detected for two subunit pairs – D/L and D/N. Resin-bound subunit L retained subunit D, and resin-bound subunit D retained subunit N (Fig. 1A), whereas resin alone did not retain subunits without his-tags (data not shown). The reciprocal experiments also yielded positive results. These interactions were anticipated based on (i) sequence similarity within subunits D and L to bacterial subunit α [4,10], (ii) studies in eukaryal systems [11–15] and (iii) recent studies in an archaeal system [16], verifying that our assay detects known interactions. No other pairwise assay revealed an interaction between subunits (Table 2).

Conflicting data regarding RNAP subunit interactions have been reported [11,12,17–19]. Far Western [12,18] and affinity pulldown assays [11] have reported multiple contacts between eukaryal small subunits, whereas in vivo analyses have detected many fewer interactions [19]. For example, RPB5, the yeast homologue to subunit H, was reported to homodimerize and to interact with as many as four other small subunits [11], but yeast two-hybrid screening suggested interaction with only the largest subunit of the three RNAPs [19]. In our analyses, subunit H did not interact with other small subunits. The yeast RNAP II crystal structure confirms that RPB5 interacts solely with the largest subunit [6], calling into question the specificity of other reported interactions (see Section 3.4).

Although the yeast crystal structure lacks subunits RPB4 and RPB7, the RPB4/7 heterodimer [20–22] has been localized to the largest subunit of RNAP II [6,23]. RPB4 is homologous to subunit F in the Archaea and is not essential for viability in *S. cerevisiae* under normal growth conditions [3,24,25]. RPB7 is related to subunit E’, and our data indicate that subunit E’ does not interact with other small subunits, consistent with association of RPB7 with only RPB4 and the largest yeast subunit. Interestingly, subunits E’ and E” did not interact, even though both subunits are represented as a single polypeptide in * Sulfolobus acidocaldarius* [4]. However, subunit E” was not present in mass spectroscopic analyses of the RNAP subunits of Methanobacterium thermautotrophicum [3] and *M. jannaschii* (Hanjo Lim, John Yates and A.A.B., unpublished). We conclude that E” may not be structurally integral to RNAP, and that in vitro it binds none of the individual small subunits. In addition, analyses of eukaryotic RNAPs and eukaryotic genome sequences have not revealed a peptide with clear similarity to subunit E”.

The RNAP II crystal structure also shows an interaction between RPB12 (subunit P) and the RPB3/RPB10/RPB11 (D/N/L) ternary complex: a β-strand in RPB12 extends a β-sheet in RPB3 [6]. We do not observe an interaction between P and D in the archaeal system, probably because the primary interaction of RPB12 is with RPB2 (in a region corresponding to *M. jannaschii* subunit

Table 2
Results of *M. jannaschii* RNAP subunit pairwise affinity pulldown assays

<table>
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*Not determined due to non-specific interaction with resin or lack of expression from vector constructs.
B'), and it is this latter interaction that is a rate-limiting step during yeast RNAP assembly [19,26]. Although a pairwise interaction between RPB3 and RPB12 has been reported, assay conditions were such that a (presumably non-specific) self-interaction of RPB12 was also observed [11]. Given that both RPB3 and RPB12 make more extensive contacts with RPB2 than they do with each other [6], we expect that a specific interaction of subunits P and D requires the presence of subunit B' (but also see Section 3.4).

In summary, our pairwise analyses (Table 2) detected undisputed subunit interactions, avoided non-specific interactions, and provided data in agreement with small subunit interactions revealed in the yeast RNAP II crystal structure [6]. In saying this, it is important to appreciate that contacts that are primarily organized through large subunit interactions (n.b., subunits P and D) may not be observed.

Fig. 1. Affinity pulldown assays with subunits D, L and N. Western blots against FLAG-tagged subunits were used to determine if proteins remained free in solution (lanes marked S) after incubation with resin-bound his-tagged subunits or were retained by and specifically eluted with the his-tagged subunit (lanes marked E). SDS-polyacrylamide gels (18%) were stained with Coomassie blue to identify his-tagged subunits in the eluate fractions. Each subunit harbors an N-terminal affinity tag as indicated above each panel, and bands corresponding to specific subunits are marked. (A) Assays identifying interactions between subunits L and D and subunits D and N. (B) Assays indicating formation of a ternary complex between subunits D, L and N. The molecular masses (kDa) of standards (lanes marked M) (Benchmark Protein Ladder, Life Technologies) are as indicated.

Fig. 2. Affinity pulldown assay with subunits P, D, L and N. This assay demonstrates that his-tagged subunit P does not retain FLAG-tagged subunits D or N when presented with the D/L/N ternary complex. SDS-polyacrylamide gels (18%) were silver-stained to detect the his-tagged subunit P in the eluate fraction. Lane designations are as described in the legend to Fig. 1.
3.2. Higher-order interactions

Weak interactions requiring stabilization from other subunits or creation of binding sites upon association of multiple subunits could cause some interactions to be missed in pairwise analyses. Therefore, selected higher-order combinations of subunits were tested. A ternary complex between subunits D, L and N is predicted from the pairwise data. Our results (Fig. 1B) clearly show retention of subunit N by resin-bound subunit L only when subunit D is also present, in agreement with previous studies [14,16] and the RNAP II crystal structure [6]. However, ternary or quaternary complexes of subunit H and combinations of subunits D, L and N, which were suggested by previous studies [11], were neither detected in our studies of the archaeal enzyme nor are they consistent with the RNAP II structure [6]. Similarly, subunit K did not bind any combination of subunits D, L and N (data not shown), in agreement with the location of corresponding proteins in the RNAP II crystal structure [6].

Although interactions between subunit P and subunits D, L or N were not detected in pairwise assays (Table 2), both the contact between RPB3 and RPB12 [6] and the knowledge that subunit D is part of a D/L/N complex led us to test for higher-order interactions among these small subunits. However, no ternary complexes were observed between subunit P and any pairwise combination of subunits D, L and N (data not shown). Likewise, his-tagged subunit P did not retain the D/L/N ternary complex (Fig. 2).

3.3. Evaluation of protein interaction prediction methods

At the inception of this survey, we derived hypotheses regarding interactions among subunits from genomic organization of subunit genes (see [27]), gene fusion events (see [28,29]), biochemical assays [11–13,15–18,21], yeast two-hybrid analyses [14–16,19,22,26], and second site suppression data [14,26]. With the inclusion of the large subunits, at least 36 pairwise interactions were suggested (Fig. 3A). We can now evaluate these hypotheses and their underlying methods using the yeast RNAP II and Thermus aquaticus RNAP crystal structures as guides [6,30].

Six interactions are suggested by adjacency of subunit genes in three operons: H–B"–B′–A"–A′, N–K, and E′–E" [5]. Protein–protein interactions of four of these pairs are further supported by gene fusions (β/β′ in Helicobacter [31] and E′/E" in S. acidocaldarius [4]) and split subunit pairs (A′/A" and B′/B") (Fig. 3A). Overall, of these six proposed interactions, only three (A′/A", B′/B" and B′/A′) are supported by the structures. The fusion of domains corresponding to archaeal A′ with A" and B′ with B" is clear in both structures. Similarly, the bacterial crystal structure confirms intimate contacts between regions corresponding to archaeal subunits B′ and A′. In contrast, RPB5 (subunit H) interacts with RPB1 (subunit A"), not RPB2 (B") as suggested by gene organization. Likewise, subunits N and K do not interact in our results above, and RPB10 and RPB6 are far apart in the RNAP II structure. Most surprisingly, although gene organization and the reported fusion of subunits E′ and E" in S. acid-
oacidarius suggest a direct interaction, none is observed in pairwise assays (above) or the peptide composition of archaeal RNAPs. In summary, in the RNAP, adjacency of genes as a sole criterion is a remarkably poor indicator of protein contacts, though it might help to define functional relationships. In contrast, adjacency of subunit genes coupled with observed gene fusions is a strong indicator of protein–protein interaction. The limits of genome-organization-based conclusions can be further underscored by noting that in most bacterial genomes, the genes for the β and β′ subunits of RNAP are found in the midst of ribosomal proteins.

Yeast two-hybrid and genetic experiments fared well with respect to the yeast crystal structure, with eight of 10 predicted interactions borne out by structural studies (Fig. 3). Previous biochemical assays (e.g. far Western blotting or co-expression of proteins within a single cell followed by affinity purification) provided at least 30 predictions of archaeal subunit interactions (Fig. 3A), most of which are misleading (Fig. 3B). When a published biochemical assay was the sole basis for predicting an interaction, only four of 24 interactions were supported by the structures. It is clear that in vitro surveys of interactions among proteins must be executed under conditions minimizing potential non-specific interactions and, where possible, verified with a complementary in vivo approach.

3.4. Conclusions

Previous studies have reported multiple RNAP subunit contacts [10–22] (Fig. 3A), including those seen here. However, several of the reported interactions among eukaryal small subunits are contradictory, possibly owing to differences in methodology or low stringency of some assays allowing detection of non-specific interactions. In light of the yeast RNAP II crystal structure [6], it is clear that most previously reported interactions are not present in the polymerase (Fig. 3B). Our analysis of an archaeal RNAP is the most comprehensive survey of interactions between small subunits and yields results completely consistent with both the crystal structure [6] and a protein interaction map of yeast RNAP III [19]. These data suggest that archaeal RNAP subunit architecture closely resembles that seen in Eucarya – a subcomplex of three small subunits (D/L/N) associated with the large subunits (A′, A″, B′ and B′′), with the remaining small subunits individually dispersed around the large subunit core.

While this manuscript was in review, additional data on archaeal subunit interactions were reported [32]. In contrast to the present broad screen for possible interactions, Werner et al. tested only a limited number of specific combinations. Notably, an interaction between subunits F and E′ was demonstrated, a pairwise combination we were unable to test (Table 2). The authors also examined the predicted interaction between subunits D and P. Yeast two-hybrid analysis produced a good signal in one bait/target pairing, but yielded a much reduced signal in the reciprocal pairing, consistent with a weak interaction. It was further shown that purified subunit P co-elutes with the D/L/N ternary complex when subjected to gel filtration chromatography. Because Werner et al. only report results for subunit combinations that are expected to interact, it is possible that their assay is less stringent than the assay reported here. This would be consistent with our discussion above that the primary interaction of subunit P during assembly is with subunit B′, and that it makes a secondary, more limited, contact with subunit D.

Our results are reproducible and the differences between positive results (e.g. Fig. 1A, D/N panel) and negative results (e.g. Fig. 1B, L/N panel) are dramatic. However, prior to the publication of the yeast RNAP II crystal structure [6], the small number of interactions troubled us. We now see that all archaeal subunits with homologues in the crystal structure behave exactly as would be expected if eukaryal and archaeal polymerases share a common architecture. This leaves us with a model of RNAP evolution in which all the small subunits are independent accretions on a core polymerase corresponding to the bacterial subunits β′, β and α2 (i.e. archaeal A, B, D and L; or yeast RPB1, RPB2, RPB3 and RPB11). Further, the data to date [3] (Hanjo Lim, John Yates and A.A.B., unpublished; this study) suggest that when archaeal RNAP ‘subunit’ E is not fused with subunit E′ (into subunit E, as in S. acidocaldarius [4,10]), then it is not an integral part of at least some archaeal polymerases.

Acknowledgements

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References


