Protein-Protein Interactions in the Archaeal Transcriptional Machinery

**BINDING STUDIES OF ISOLATED RNA POLYMERASE SUBUNITS AND TRANSCRIPTION FACTORS**

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Bernd Goede, Souad Naji, Oliver von Kampen, Karin Ilg, and Michael Thomm

From the Lehrstuhl für Allgemeine Mikrobiologie, Universität Kiel, am Botanischen Garten 1-9, 24107 Kiel and the Lehrstuhl für Mikrobiologie und Archäenzentrum, Universität Regensburg, 93053 Regensburg, Germany

Transcription in Archaea is directed by a pol II-like RNA polymerase and homologues of TBP and TFIIB (TFB) but the crystal structure of the archaeal enzyme and the subunits involved in recruitment of RNA polymerase to the promoter-TBP-TFB-complex are unknown. We described here the cloning expression of subunits E, F, of D and L and a BDLNP-subcomplex were reconstructed. Far-Western analyses identified subunit D as structurally important key polypeptide of RNAP involved in interactions with subunits B, L, N, and P and revealed also a strong interaction of subunits E’ and F. Stable complexes consisting of subunits E’ and F, of D and L and a BDLNP-subcomplex were constituted and purified. Gel shift analyses revealed an association of the BDLNP subcomplex with promoter-bound TBP-TFB. These results suggest a major role of subunit B (Rpb2) in RNAP recruitment to the TBP-TFB promoter complex.

Archaeal RNA polymerases (RNAP) are multisubunit enzymes that resemble in sequence subunit composition and functional aspects eukaryotic RNAP. Fig. 1 shows the subunit structure of eukaryotic RNA polymerase II (pol II), of the *Pyrococcus* RNAP and of the RNAP from *Escherichia coli*. Homologous subunits are indicated by the same colors. Archaeal RNAP display greater similarities with all eukaryotic RNAP than with the four subunits of the bacterial core enzyme. We refer here mainly to pol II as the subunit interactions within this enzyme are known from the crystal structure (1, 2). Archaeal RNAPs have clear homologues to Rpb4 and Rpb7 of pol II, which were first called F and E (3). In the genomes of most Archaea, the gene encoding E overlaps at its 3’-end in a different reading frame with a second gene containing a zinc finger motif. To discriminate between the first gene that is homologous to *rpb7* and the second gene that has no homolog in yeast but is highly conserved in Archaea, the first one is designated in data bases as *rpoE* and the second one as *rpoE’*, and the corresponding proteins as E’ and E”. Only E’ has been detected in purified archaeal RNAP. Two subunits shared by all three eukaryotic RNAP, Rpb12, and Rpb10 have the subunits P and N as archaeal homologues but no bacterial homologues. In addition, subunit H of the archaeal enzyme has a homologue in pol II (and also in pol I and pol III) but not in the bacterial enzyme.

The gene encoding the largest subunit in eukaryotic RNAP, Rpo1 and β’ of the *E. coli* enzyme is split into two genes encoding subunits A’ and A” in all Archaea. The RNAP of *Pyrococcus* and of Crenarchaeota show the subunit composition BA”DE’FLHNKP (4, 5). In methanogens and extreme halophilic Archaea subunit B is split into the subunits B’ and B” (6). This B split defines the second major type of archaeal RNAP with the subunit composition A’B’A”DE’FLHNPK.

The archaeal RNAP is recruited to the preinitiation complex by association to promoter-bound transcription factors TBP and TFB (7, 8), which are interacting with the TATA-box and BRE element of archaeal promoters (reviewed in Ref. 9). Both TBP and TFB consist of two imperfect direct repeats. TFB has in addition an N-terminal domain forming a zinc ribbon and a B-finger (see Figs. 6C, 9, and 10). A third archaeal transcription factor, TFE, is homologous to the N-terminal part of subunit α of eukaryotic TFIIE (11, 12). TFE is not required for promoter-directed transcription but can stimulate the activity of some promoters by a factor of 3–4. TFE can also complement some mutants of TFB indicating that these proteins interact synergistically and contribute to catalytic core functions of RNAP (10). The path of the DNA in the *Pyrococcus* RNAP has been studied by photochemical cross-linking (13, 14, 15). These studies revealed that subunit B of *Pyrococcus* RNAP cross-links the RNAP between the TATA-box and the transcription start site.
Interactions of RNAP Subunits

and that subunits A’, A”, and H contact the DNA downstream of the start site. In vivo and in vitro binding assays were used to investigate the interactions of subunits of the RNAP from Methanocaldococcus jannaschii. The eukaryotic subunits Rpb4 and Rpb7 form a heterodimer that reversibly associated with the pol II core. As predicted from the similarity to the eukaryotic system the archaeal homologues of these polypeptides, E and F, form a complex (3) and archaeal F interacted with human Rpb7 to form an archaeal-human F-Rpb7 hybrid (16). Subunits D, L, N, and P were shown to associate to a tetrameric D-L-N-P complex (16). The eukaryotic homologues of these subunits, Rpb3, Rpb10, Rpb11, and Rpb12 are in close interaction and clustered together in the pol II structure (1). This assembly of the archaeal subunits D-L-N-P was able to recruit the largest subunit B in vitro and used as a frame for the reconstitution of active M. jannaschii RNAP from individual subunits (17).

We are exploring the mechanism and regulation of transcription in Pyrococcus using a cell-free transcription system (7, 18, 20). We report here cloning and expression of all RNAP subunits from Pyrococcus. The interaction of these polypeptides with each other and with the archaeal transcription factors TBP and TFB were studied by far-Western analyses, column chromatography and gel electrophoresis. Our results reveal many interactions predicted from the structural similarities to the pol II system, the existence of various subcomplexes and an interaction of the BDLNP subcomplex with promoter-bound TBP and TFB.

EXPERIMENTAL PROCEDURES

Cloning of RNAP Subunits—The coding region of RNAP subunits B (PF1564), A’ (PF1563), A” (PF1562), D (PF1647), E’ (PF02569), F (PF1036), H (PF1565), K (PF1642), L (PF0050), N (PF16439) and P (PF2009) from Pyrococcus furiosus DSMZ 3638, were PCR-amplified using genomic DNA as template. The oligonucleotides were complementary to the 5′- and 3′-ends of the genes and contained the restriction sites NdeI at the 5′-end and BamHI at the 3′-end. The PCR fragments encoding subunits B, D, E’, F, H, K, L, and P were cloned into the corresponding restriction sites of the expression vector pET-32b (Novagen). The expressed proteins carry a His6 tag and a recognition site for heart muscle kinase (HMK) at the N terminus. The PCR products encoding subunits F and H were also cloned in a modified version of pET-33b resulting in proteins containing the His6 tag and the HMK site at the C terminus. Details of the modified vector and of the oligonucleotide sequences are available from the authors on request. The PCR fragments encoding subunits A’ and A” were cloned in the NdeI site and BamHI site of pET-14b. The expressed subunits A’ and A” contained only a His6 tag at the N terminus and were used as targets in Far-Western experiments. To obtain subunits A’ and A” with a HMK in addition, the PCR products containing the HMK recognition site at the 5′-end were cloned into pET151/D-TOPO (Invitrogen).

Identification of a Consensus Sequence for Subunits of Archaeal RNAP and Bioinformatic Work—Multiple sequence alignments of the genes encoding RNAP subunits of up to 18 Archaea and of the subunits of pol II from S. cerevisiae revealed an amino acid consensus sequence for each subunit with the exception of Rpb8 and Rpb9, which have no homologues in Archaea. Most of the genes encoding archaeal RNAP subunits were extracted from whole genome files available at NCBI or at SRS. BLAST search and other common bioinformatic resources were used to identify unannotated entries. A number of missing genes became available by local BLAST search in Bioedit on the basis of the whole genome file in raw format. Multiple sequence alignments were carried out using Malign, an algorithm especially suitable when genes are compared that show low sequence similarities and different lengths (scoring matrix: PAM250). Because subunit B is split in two parts in several Archaea (rpoB’ and rpoB”) two single alignment steps were carried out and combined in a subsequent step to obtain better results. MAAlign2Msf was used to convert the data into the MSF file type. After import into Bioedit the data were formatted, a consensus sequence was generated and shading was applied. As final step export as RTF file and import into MS Word was performed. The consensus sequence of each alignment was used to generate two-dimensional similarity diagrams (Fig. 3) and to visualize the distribution of identities and similarities in the three-dimensional model of S. cerevisiae polII (PDB: 1NT9; Fig. 4). A small Delphi program was written to draw the two-dimensional diagrams and as a helper tool for sequence analysis and various conversion steps. In the diagrams a vertical line represents identity between the archaeal consensus and the amino acid sequence of pol II. Lines of half-length indicate similarities. To visualize the homologous regions of archaeal RNAPs and of pol II in the three-dimensional structure of pol II, the consensus sequence for each subunit was converted in a ProSite search pattern and applied to the S. cerevisiae pol II model (PDB: 1NT9) using the Cn3D 4.1 annotate function.

Expression and Purification of Proteins—For the expression of the proteins the plasmids were transformed in the expression strains BL21(DE3)Star-CP (subunits B, A’, D, E’, F, H, K, L, N, and P) and in BL21(DE3)pLysS (subunit A”). The proteins were expressed by inducing exponentially cultures with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. For Far-Western dot blot experiments (Fig. 5A, first three panels), subunit B was purified after SDS-polyacylamide gel electrophoresis. Gel slices containing this subunit were incubated in a solution containing 0.1% (w/v) SDS. Then, SDS was precipitated and the protein refolded by dialysis and dialysis as described by Ref. 21. Subunit B used as probe (Fig. 5A, lower panel) for dot blots and B used for gel blots and for reconstitution of the DLNPB subcomplex and subunits A’ and A” were renatured from inclusion bodies. First, cells were suspended in lysis buffer (20 mM Tris-HCl, pH 8, 1 mM PMSF, 5 mM 2-mercaptoethanol, 0.3 mg/ml lysozyme) and sonicated. After centrifugation the pellets containing the inclusion bodies were extensively washed in purification buffer (20 mM Tris-HCl, pH 8, 0.5 mM NaCl, 0.1% Tween 20, 1 mM PMSF, and 5 mM 2-mercaptoethanol) The inclusion bodies were solubilized in binding buffer (20 mM Tris-HCl, pH 8, 0.5 mM NaCl, 5 mM imidazole, 6 mM guanidine HCl, 1 mM PMSF, 5 mM 2-mercaptoethanol) for 1 h at 20 °C. After centrifugation the supernatant was loaded onto a
Ni\textsuperscript{2+}-NTA column (HisTrapFF, GE healthcare) and washed with binding buffer containing 6 M urea and no guanidine HCl. The refolding of the bound protein was performed on column using a decreasing linear urea gradient (10 column volumes) ranging from 6 M to 0. The refolded proteins were eluted with an imidazole gradient ranging from 5 to 300 mM imidazole. For the purification of subunits D, L, N, E', F, K, H, and P cells were resuspended in a buffer containing 50 mM NaPO\textsubscript{4}, pH 8, 10 mM imidazole, 300 mM NaCl, and 10% v/v glycerol. Cells were disrupted by passage through a French pressure cell. The lysate was clarified by centrifugation at 100,000 \times g for 20 min at 4 \degree C. The supernatant was directly applied to a Ni\textsuperscript{2+}-NTA column (subunits D, E', K) or after heating for 20 min at 80 \degree C (subunits F, H, L, N, and P) and separation of precipitated \textit{E. coli} proteins by centrifugation. Bound proteins were stepwise eluted with 300 mM imidazole. Some subunits were further purified by MonoQ- or Superdex75-chromatography.

**Reconstitution of BDLNP RNAP Subcomplexes**—Equimolar amounts (2.5 nmol) of RNAP subunits were incubated in transcription buffer (40 mM HEPES, pH 7.3, 250 mM NaCl, 2.5 mM MgCl\textsubscript{2}, 10% v/v glycerol, 1 mM EDTA, 1 mM PMSF, 5 mM 2-mercaptoethanol) for 1 h at 20 \degree C. The complex formation was analyzed by Superdex 200 (GE Healthcare) column chromatography. Alternatively, the BDLNP complex was reconstituted by denaturation and renaturation of recombinant subunits. 2.5 nmol of subunits B and D and 5 nmol of subunits N, L, and P were combined in a final volume of 500 \mu l of transcription buffer containing in addition 6 M urea. The mixture was transferred to a dialysis frame (Slide-A-Lyzer 3.5k, Pierce) and incubated for 20 min at 20 \degree C. Then, the mixture was dialyzed against transcription buffer containing 3 \mu M urea for 20 min at 20 \degree C. Renaturation was achieved by dialysis in transcription buffer for 1 h. The renatured subcomplexes were heated for 10 min at 70 \degree C to remove misfolded aggregates. The BDLNP subcomplex was purified by Superdex 200 chromatography (Superdex 200 10/300 GL, GE Healthcare).

**RESULTS AND DISCUSSION**

The Archaeal RNAP Displays High Sequence Similarity with the Catalytic Core of Pol II—To investigate the molecular architecture of an archael RNAP we cloned and expressed 11 subunits of the \textit{Pyrococcus} RNAP (Figs. 1 and 2) ranging in molecular mass from 127 003 (subunit B) to 5757 (subunit P). The sequences of the genes encoding these subunits were aligned with the sequences of subunits of 17 archaeal RNAP and from this an archael RNAP consensus sequence for each individual subunit was derived (“Experimental Procedures” and supplementary data). This consensus sequence was aligned with the pol II sequence and identical amino acids and amino acids highly conserved between archael RNAP and \textit{S. cerevisiae} pol II were indicated by horizontal bars in the schematic representation of
Interactions of RNAP Subunits

| Archaea | Eukarya | Bacteria |
|---------|---------|----------|
| P. furiosus RNAP | S. cerevisiae RNAP | E. coli RNAP |
| Rpb1 | Rpb1 | Rpb1 |
| Rpb2 | Rpb2 | Rpb2 |
| Rpb3 | Rpb3 | Rpb3 |
| Rpb4 | Rpb4 | Rpb4 |
| Rpb5 | Rpb5 | Rpb5 |
| Rpb6 | Rpb6 | Rpb6 |
| Rpb7 | Rpb7 | Rpb7 |
| Rpb8 | Rpb8 | Rpb8 |
| Rpb9 | Rpb9 | Rpb9 |
| Rpb10 | Rpb10 | Rpb10 |
| Rpb11 | Rpb11 | Rpb11 |
| Rpb12 | Rpb12 | Rpb12 |
| A' | A' | A' |
| B | B | B |
| C | C | C |
| D | D | D |
| E' | E' | E' |
| F | F | F |
| G | G | G |
| H | H | H |
| L | L | L |
| N | N | N |
| K | K | K |
| P | P | P |
| 127.0 | 103.5 | 44.4 |
| 29.8 | 21.7 | 14.1 |
| 11.1 | 9.3 | 8.2 |
| 6.2 | 5.8 | 4.4 |

FIGURE 1. Homologous subunits of RNAP from the three domains of life. The subunit pattern of the RNAP from P. furiosus, pol II of S. cerevisiae and of the E. coli enzyme are shown. Identical colors indicate homology. Note that subunit Rpb1 of eukaryotes is split in Archaea into the two polypeptides, A' and A. Subunit α of the E. coli RNAP is homologous to a part of subunit D of Archaea and of Rpb3 of yeast and to subunits L and Rpb11. The molecular mass of the subunits of Pyrococcus RNAP is indicated to the left.

and a sequence homologous to a part of the wall (from position 852–952) and of the anchor (1127–1151) of pol II (Figs. 3 and 4). Homologous parts of the smaller subunits of archaeal RNAP and of pol II are also depicted in Figs. 3 and 4. It is obvious that the highest degree of sequence conservation was found in the region encoding the major cleft of RNAP harboring the active center which is formed mainly by the two largest subunits of pol II.

Subunits Rbp7 and E', Rpb11 and L and Rpb12 and P are similar in size. The other smaller subunits of pol II are larger in molecular mass (see Fig. 3) mainly because of extensions at the N terminus (Rbp4 and Rpb6) or at the C terminus (Rpb3). These extensions showed no sequence similarity to archaeal RNAP subunits. No homologues of subunits Rbp8 and Rpb9 were detected in the genome of Pyrococcus and of other Archaea. These subunits are indicated in Fig. 4C in light blue. A three-dimensional model showing the amino acids conserved between archaeal RNAP and yeast pol II is available in the supplementary materials.

Far-Western Analyses of Interactions of RNAP Subunits—The 11 cloned archaeal RNAP subunits were used to investigate protein-protein interactions of all components of the basal archaeal transcription machinery. A HMK recognition site was cloned at the N or C terminus of genes encoding RNAP subunits to allow specific labeling of the proteins with 32P. The N-terminally labeled subunits B, D, E', F, L, N, K, and P and C-terminally labeled subunits F and H were used as probes for the detection of interactions of subunits immobilized on nitrocellulose membranes. In one set of experiments the native protein was simply immobilized on nitrocellulose membranes (Far-Western dot blot). In a second approach the RNAP sub-
Interactions of RNAP Subunits

A

Rpb1 \( (\text{rpoA} + \text{rpoA}^\prime) \):

B

Rpb2 \( (\text{rpoB}, \text{rpoB}' + \text{rpoB}') \):

C

Rpb3 \( (\text{rpoD}) \):

D

Rpb6 \( (\text{rpoK}) \):

Rpb4 \( (\text{rpoF}) \):

Rpb7 \( (\text{rpoE}^\prime) \):

FIGURE 3. The consensus of archaeal RNAP subunits shows extensive homology to domains of the active core of pol II. The consensus sequence for the genes encoding archaeal RNAP subunits was aligned with the homologous eukaryotic subunits. The genes encoding the subunits of yeast pol II are shown in the top lane of each panel. DNA regions conserved in the consensus of the archaeal RNAP subunit are indicated in the pol II gene by full-length block bars (identical with archaeal consensus) and half-length bars (similar to archaeal consensus). A, top lanes, comparison of rpoA encoding A' and of rpoA2 encoding A" with the gene encoding the largest subunit Rpb1 of yeast pol II. The extent of the genes encoding subunits A' and A" is indicated in the pol II gene by blue boxes. The C-terminal domain of pol II that is missing in the archaeal enzyme is boxed in red. Second lane, domains or helices identified in the crystal structure of pol II (1); 1, clamp core; 2, clamp head; 3, clamp core; 4, active site; 5, dock; 6, active site (metal A); 7, pore; 8, funnel; 9, cleft; 10, foot; 11, cleft; 12, jaw; 13, cleft; 14, clamp core; 15, linker; 16, CTD. Third lane, amino acids containing hydroxyl groups as potential phosphorylation sites are indicated by bars. B, comparison of Rpb2 and Rpb3 with archaeal subunits B and H. The labeling and symbols are in all the following panels like in the first panel. Domains and helices of subunit Rpb2 in the crystal structure of pol II: 1, external; 2, protrusion; 3, lobe; 4, protrusion; 5, fork; 6, external; 7, external; 8, hybrid binding; 9, wall; 10, hybrid binding; 11, anchor; 12, clamp. Domains and helices in the crystal structure of subunit Rpb5: 1, Jaw; 2, assembly. C, comparison of Rpb3, Rpb11, Rpb10, and Rpb12 with subunits D, L, N, and P of the archaeal enzyme. Domains and helices in the crystal structure of subunit Rpb3: 1, dimerization; 2, domain; 3, zinc loop; 4, domain 2; 5, dimerization; 6, loop; 7, dimerization; 8, tail. Domains and helices in the crystal structure of subunit Rpb11: 1, tail; 2, dimerization; 3, tail; Domains and helices in the crystal structure of subunit Rpb10: 1, zinc bundle; 2, tail. Domains and helices in the crystal structure of Rpb12: 1, zinc ribbon; 2, tail; D, comparison of subunits Rpb6, Rpb4, and Rpb7 with subunits K, F, and E'. Domains and helices in the crystal structure of subunit Rpb6: 1, tail (not contained in the three-dimensional model); 2, assembly.

units were transferred to the membrane after electrophoresis in denaturing polyacrylamide gels (Far-Western gel blot). Detection of interactions was performed after treatment of the transferred proteins by a procedure allowing refolding of protein domains at the membrane (23).

Subunits A' and A" precipitated during the labeling and binding assays and were therefore only used as immobilized targets of binding reactions but not as labeled probes. A typical dot blot experiment is shown in Fig. 5. Subunit D interacted with subunits P and B, subunit L exclusively with D. This result established a strong and specific interaction of the subunits D and L. Subunit E' bound strongly to F and weakly to TBP and TFB. Subunit F bound strongly and exclusively to E'. This result confirmed the specific interaction of these proteins which are known to form a specific subcomplex in the Methanocaldococcus RNAP (25). Subunit N interacted with D and P (Fig. 5). Subunit P bound to D. These result showed that the subunits D, L, N, and P which were used as platform for the reconstitution of Methanocaldococcus RNAP (26, 17) formed also an interactive network in the Pyrococcus RNAP (see Fig. 9). Interactions of labeled D with L and of labeled L with D were confirmed by Far-Western gel blots (Fig. 5, B and C). D as probe interacted with B and L (Fig. 5B). D as probe bound in dot blots to D, F, L, and N, weakly to P and in addition to TFB (Fig. 5A, lower panel). Our results established in addition protein-protein contacts of subunit B with D both when D and B were used as probes (Fig. 5, B and A, lower panel). In addition, contacts of B with N were shown both in dot blots (Fig. 5A) and gel blots (data not shown) with B as probe.

These data establish an interactive network between the subunits BDLNP and between subunits E' and F which were previously shown to form subcomplexes in the Methanocaldococcus system (3, 16, 17). The high sequence similarity of essential parts of the two largest subunits of archaeal RNAP and pol II (see Figs. 3 and 4) suggest that the archaeal subunits B and A', A" form the catalytic center and have many contact sites similar as in pol II. Strong interactions of subunits B with A' and A" could not be shown by Far-Western
and dot blot analyses probably due to the low solubility of the isolated large subunits and because of the tendency of the isolated proteins to precipitate. However, at least weak interactions of subunits B and A\(^{\text{H}}\) could be detected (Fig. 5A) and in the light of the high sequence similarity of the large subunits of pol II and of the archaeal RNAP (Figs. 3 and 4) these were considered as being significant. Additional contacts were found between H and A\(^{\text{H}}\) (Fig. 5A). The homologous...
proteins of pol II, Rbp5, and Rpb1, are in close contact in the crystals of pol II (Fig. 9) and therefore also this H-A interaction observed here was as predicted. Additional contacts, e.g. those of B with N were also as predicted from the crystal structure of pol II.

Fig. 9 summarizes the protein-protein interactions of subunits of Pyrococcus RNAP established in this study in relation to the interactions found in the crystal structure of pol II. The similarities of the interaction patterns of subunits within the archaeal and eukaryotic enzyme are evident. The interfaces between the large subunits B and A’, A” are well conserved between the eukaryotic and archaeal polymerase (Fig. 3). Therefore, the strong interactions and extensive interfaces between the three large archaeal subunits corresponding to Rpb1 and Rpb2 are expected to be very similar in the archaeal and eukaryotic enzymes although we could not show strong protein-protein interactions between these subunits in the present work. The reason for that is probably the low solubility of the isolated large subunits. The subunits B’, A”, and K pre-

3 P. Cramer, personal communication.
Interactions of RNAP Subunits


cipitated during expression in E. coli and had to be renatured from inclusion bodies. We assume that the tendency of these proteins to precipitate and to denature is the major reason for the lack of detected interactions between isolated large subunits. It is interesting to note that subunit H interacted with the A" subunit corresponding to amino acids 100–1430 of Rpb1 as predicted from the crystal structure. The subunit H and Rpb5 are in proximity of the DNA region from +4 to +15 in initiation complexes (27, 13).

The general architecture of pol II is characterized by a deep cleft between the two large subunits, which are anchored at one end to the subassembly of subunits Rpb3,10, 11, and 12 (the D-L-N-P orthologs; 28; see also Fig. 4D). Rpb5 and regions of Rpb1 form a pair of jaws that appear to guide the DNA to the active center. The cross-linking data of Bartlett et al. (13) and our finding that H interacts with A" as predicted support the conclusion that the archaeal subunit H is located in the three-dimensional structure of the enzyme on a similar position as Rpb5 in the eukaryotic enzyme. Rpb5 forms the end of the lower “jaw” of polII that is in contact with downstream DNA. Interestingly, the highly conserved proline residues 86 and 118 of Rpb5, which are facing the DNA (28) are not conserved in the archaeal subunit H (Fig. 4D and supplemental materials), which lacks the N-terminal domain of Rpb5. Downstream DNA is not involved in sequence-specific contacts with RNAP and therefore subunit H seems to function collectively with A' and A" in guiding DNA toward the active center during elongation of transcription.

In contrast to the larger insoluble subunits, the interaction patterns of the soluble subunits D, L, N, and P described here could be clearly established and the results are as predicted from the crystal structure of pol II. This finding suggests that the same conserved sites in pol II and the archaeal enzyme are involved in interactions of these subunits. It is unclear whether the same amino acids are responsible for the D-L interactions in the archaeal enzyme as in the Rpb3-Rpb11 interactions. The conservation of regions 1 and 3 of Rpb3 and of region 2 of Rpb11, which are involved in dimerization between these subunits (see Fig. 3C with the corresponding regions of subunits D and L is not in particular high. But subunits DL from a stable subcomplex that can be easily identified by gel electrophoresis (Fig. 7). Therefore, these subunits can serve as an excellent model to identify the motifs and principles involved in interactions of a multisubunit enzyme by mutational studies.

The interactions of the 10 subunit core enzyme of RNAP which contains the mobile clamp of RNAP in the open conformation (1) with the subunits Rpb7-Rpb4 which maintains the conformation of a transcribing complex (2, 29) are of particular interest. Rpb7 interacts with a pocket of pol II formed by subunits Rpb1, Rpb2, and Rpb6 (reviewed in Ref. 30). The high structural similarity of Rpb7/4 and of E'/F (3, 25, 31) suggests that the interactions sites are conserved between the archaeal and eukaryotic enzyme, although we could only detect interactions of E' and F with subunit B. Our recent data suggest that E' stimulates transcription of the Pyrococcus core enzyme and that F is not required for this activation but stabilizes the E'-core interaction. The N-terminal region of Rpb4 makes a contact with Rpb1 (29), but a different interaction site must stabilize binding of E' to the archaeal core, because the larger N-terminal domain of Rpb4 is not conserved in the archaeal subunit F, which is much smaller than Rpb4 (see Fig. 3 and supplementary data). In fact, we show an interaction of F with B (Fig. 5) indicating that a different subunit is involved in binding of F to the core enzyme in the archaeal system. Analyses of the interactions of E' and F with core RNAP are of key interest for an understanding of conformational changes in the RNAP during the first steps of transcription. The transcriptional activation of Pyrococcus RNAP by E' and the known crystal structure of the interacting tip domain of E' (3) are excellent tools to unravel the mechanism of E' induced RNAP activation by mutational and further structural studies.

In general, the archaeal subunits are smaller than the corresponding pol II subunits. Rpb2 and B have approximately the same size, the major difference between Rpb1 and A', A" are the splitting of the archaeal protein into two subunits and the lack of the tandem repeats at the C terminus of the archaeal enzyme. Only subunits N and Rpb10 and E' and Rpb7 have similar size. All other archaeal subunits are significantly smaller than their eukaryotic counterparts, in particular H, K, P, and F (Fig. 3).

All these smaller subunits with the exception of subunit K, for which we could not identify a binding partner, seem to interact with each other in a similar manner like their orthologs in pol II. Therefore, the present work suggests that the basic interaction sites between RNAP are conserved between these two domains of life. Hence, the archaeal polymerase seems to represent the ancient version of pol II containing all the structural elements required for formation of a stable structure and catalytic activity. It is most likely that the subunits Rpb8 and Rpb9 and the additional domains found in the eukaryotic polymerase evolved later to cope with the complex regulatory patterns encountered in higher cells. An example for this is the N-terminal domain of Rpb5, which is involved in activation of transcription (33).

Far-Western Analyses of RNAP Transcription Factor Interactions—The archaeal RNAP is recruited to the preinitiation complex by association with promoter-bound TBP and TFB. The nature of this interaction and the subunits involved in RNAP transcription factor contacts are unknown. Therefore, the RNAP transcription factor interactions are of special interest. The structure of the eukaryotic preinitiation complex has not yet been solved and RNAP-transcription factor-interactions elucidated in the archaeal system are also of potential significance for the eukaryotic machinery. Inspection of Fig. 5A reveals that labeled subunits D, E', N and P interacted clearly with immobilized TBP and TFB. In dot blot experiments using labeled TBP and/or TFB as probe, both factors bound to TBP and TFB but only very weak binding signals were detected (TFB bound very weakly to P, D, and K, TBP very weakly to D and P; data not shown). Probably, the dimerization of TBP in solution, the low stability of TFB in solution and its tendency to bind non-specifically to nitrocellulose membranes hampered the

4 S. Naji, S. Gruenberg, and M. Thomm, manuscript in preparation.
detection of interactions of TBP and TFB with RNAP subunits by this approach. When TFB and TBP were used as probes for gel blots the binding signals were still weak, but binding of TFB to A" and E' and of TFB to A" and E' was detected (data not shown).

To investigate the interaction of TBP and TFB with individual subunit of RNAP in a way allowing to estimate the strength of protein-protein interactions, binding of the seven smaller RNAP subunits to serial dilutions of TBP and TFB immobilized on a membrane was analyzed by the far-Western dot blot procedure. When binding of labeled RNAP subunits to immobilized TBP was analyzed, the intensity of binding signals decreased in the order P, N, TFB, E', K, D, H, F, and L. When TFB was bound to nitrocellulose membranes, the binding intensity of labeled probes decreased in the order P, N, D', E', K, TBP, H, F, and L (data not shown).

Considering the crystal structure of pol II and the great similarities of the general architecture of the archaeal and eukaryotic enzyme it is highly unlikely that all these small subunits are in the preinitiation complex in contact with TBP and TFB. It seems more likely that the ability of TBP and TFB to interact with most of the small subunits is restricted to sites exposed only in isolated subunits. Subunit K of Sulfolobus was recently shown to interact with TFB (34) in two hybrid assays. In the light of our findings that many isolated small subunits show a tendency to bind strongly to both TBP and TFB it is advisable to see interactions of isolated small subunits with TBP and TFB with some caution. In pol II, the two larger subunits flanking the deep cleft and forming the active center of the enzyme have been identified as major interaction sites of TBP and TFIIIB by photocross-linking. We have also identified subunit A" as binding target of both TBP and TFB (not shown) and detected also an interaction of labeled subunit B with TFB (Fig. 5A, lower panel). Analysis of a subcomplex containing subunits BDLNP showed that subunits A' and A" are not required for recruitment of RNAP to the TBP-TFB promoter complex (see below). Structural analyses of the archaeal preinitiation complex are required to resolve the transcription factor RNAP interactions sites in more detail.

Interactions of RNAP Subunits

The major conclusions derived from analysis of truncated TFB molecules are that deletions can change the conformation of the protein dramatically and can also enhance the binding capacity of the mutant form of TFB at least in the case of TFB-D interactions by an unknown mechanism. Analysis of the deletion mutant Δ1−39,283−300 lacking parts of the N and of the C terminus suggested that intramolecular interaction of the C terminus and N terminus of TFB molecules are possibly involved in inhibition of the TFB-K interaction, but do not interfere strongly with TFB-D binding.

Analyses of RNAP Subcomplexes—The interactions of some components of the RNAP were analyzed in addition by electrophoresis in native polyacrylamide gels and by Co-immobilization assays on Ni²⁺-NTA columns to confirm and extend the
FIGURE 7. Identification of stable subcomplexes of subunits DL, E’F, co-immobilization of D and P, and identification of a stable BDLNP complex. 

A and B, native gel electrophoresis. The labeled subunit L (70 ng) was incubated for 2 h at 30 °C with increasing amounts of subunit D in TEN buffer containing 10% (v/v) glycerol and 0.5% (v/v) Tween 20. Complex formation was then analyzed on a 8 –15% native PA gel. Lane 1, no D; lane 2, 9 ng of D; the reactions analyzed in lanes 3–10 contained increasing amounts of subunit D (5 ng for each reaction); B, the labeled subunit F (70 ng) was incubated for 90 min at 37 °C in TEN buffer containing 10% glycerol with increasing amounts of subunit E’. Lane 1, no E’; lane 2, 62 ng E’; reactions analyzed in lanes 3–10 contained increasing amounts (62 ng per reaction) of subunit E’. C, co-immobilization of subunits D and P. His6-tagged subunit D (40 ng) was immobilized on a Ni2⁺-NTA resin. After extensive washing of the column, 40 μg of subunit P were added, the column was washed again and immobilized protein were eluted by a linear gradient ranging from 50 mM to 1000 mM imidazole. The proteins in the flow-through fraction and in the fractions eluting from the column were analyzed on an 8–15% PA gel and stained with Coomassie Blue. Lane 1, standard proteins; lane 2, subunit D (fraction applied to the column); lane 3, subunit P (fraction applied to the column); lane 4, flow-through fraction after addition of D; lane 5, flow-through fraction after addition of P; lanes 6–11, fractions eluted by the imidazole gradient. D, identification of a BDLNP subcomplex. Isolated RNAP subunits were reconstituted by denaturation and renaturation and complex formation was identified by Superdex 200 chromatography. The peak elution fractions of standard proteins are indicated by arrows. The fractions eluting from the column were analyzed on an 8–20% PA gel and silver-stained. The fractions containing the BDLNP subcomplex are indicated in the figure.
results that had been produced by Far-Western blotting. When labeled subunit L was incubated with subunit D the formation of a D-L complex was observed after gel electrophoresis (Fig. 7A). A complex formation between labeled subunit F with subunit E' could be shown as well (Fig. 7B). These results confirm that strong interactions between subunits D and L and E' and F exist as observed by Far-Western analyses (see Figs. 5 and 6). An interaction of subunit D with subunit P could be shown when D was immobilized by its His tag on a Ni²⁺-NTA column and subunit P without His tag was added to the column (Fig. 7C). The co-elution of these proteins by a linear imidazole gradient indicated that these polypeptides associate on the column. A complex formation of subunit B with L was also shown by this co-immobilization assay (data not shown). These results are consistent with the Far-Western blotting experiments in respect to binding of subunits P and D (Fig. 5A). Binding of B to L and of B to P was only found when B was used as probe but not in the inverse experiment using P and L as probe. Therefore, the B-L and B-P interactions are less certain and are indicated in Fig. 9 as unidirectional binding.

Gel filtration experiments revealed that only a few soluble subcomplexes are stable during chromatography. Complex formation between subunits D and L and between subunits E' and F was clearly demonstrated by Superdex 75 chromatography (data not shown). When the subunits B, D, L, N, and P were incubated and applied to a Superdex 200 column a stable BDLNP complex could be identified (data not shown). The same complex was formed after denaturation of recombinant subunits in 6 M urea and stepwise renaturation by dilution in buffers containing 3 M and no urea (“Experimental Procedures,” Fig. 7D). The same procedure was successfully used to reconstitute a highly active RNAP from 11 isolated subunits⁵ but the BDLNP complex observed during reconstitution of the Methanothermobaculum enzyme (17) could not be identified in reconstitution experiments with recombinant Pyrococcus RNAP subunits (Fig. 7D; data not shown). Most combinations of two subunits were unstable and precipitated during size exclusion chromatography, e.g. when P was incubated with D, B, with A' and B with A^+ (data not shown).

The BDLNP Subcomplex Associates with Promoter-bound TBP-TFB—Analyses in the pol II system indicated that the two largest subunits Rpb3, homologous to A' and A^+, and Rpb2, homologous to subunits B of the archaeal enzyme (Figs. 1, 3, and 4), are mainly involved in binding of transcription factors (19, 36). The archaeal RNAP forms a large complex of low electrophoretic mobility in mobility shift assays with promoter bound TBP-TFB (13; Fig. 8, lane 6). To investigate whether this isolated BDLNP complex can associate with the promoter-bound TBP-TFB platform, the subcomplex was added to DNA binding reactions containing TBP and TFB and DNA alone. The BDLNP subcomplex produced a smear with a probe harboring the Pyrococcus gdh promoter (Fig. 8, lane 3) suggesting that it interacted weakly and in a nonspecific manner with DNA (compare lanes 1 and 3). TBP-TFB form a distinct complex with the gdh promoter (Ref. 32; Fig. 8, lanes 4 and 5). When the purified subcomplex was added to reactions containing TBP and TFB a third complex was observed that showed lower electrophoretic mobility than the DNA-TBP-TFB complex (compare lanes 4 and 5 with lane 6). The purified RNAP forms a slower migrating complex indicating the difference in molecular mass between the subcomplex and the complete enzyme (Fig. 8, lane 2). This finding demonstrated that the BDLNP subcomplex was able to form a stable complex with promoter-bound transcription factors TBP and TFB.

⁵ S. Naji, manuscript in preparation.
Interactions of RNAP Subunits

Our results show that subunits A', A'', H, K, E', and F are not required for RNAP recruitment. Rpb1 represent the major mass of pol II in the region below the cleft. It forms an essential part of the clamp, of the active center and of the upper and lower jaw. More importantly, the “dock” domain formed by Rpb1 has been suggested as binding site of TFIIB in the preinitiation complex (1, 2). Rpb1 and Rpb2 of pol II were found in the eukaryotic preinitiation complex in close proximity of the three TFIID domains (19, 36). Hence, our finding that stable recruitment to promoter-bound factors requires only subunit B (the Rpb2 ortholog) bound to the D-L-N-P anchor is highly unexpected. Our novel findings suggests that the dock domain is not essential for RNAP binding and that the important interactions sites with promoter-bound transcription factors reside in the outer surface of the B-D-L-N-P (Rpb 2-3-10-11-12) subcomplex (Fig. 7). The gel-shift assay established for binding of the B-D-L-N-P subcomplex to promoter-bound TBP-TFB (Fig. 8) might be a useful tool to identify the sites involved in recruitment of a pol II-like RNAP by mutational analyses.

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