Reconstitution of a PolII-like RNA Polymerase and Contribution of Subunit E' and Structural Elements in the Active Center to RNA Polymerase Functions

Dissertation

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I Introduction

Based primarily on 16S rRNA sequence comparisons (Woese et al., 1990), all living organisms have been classified into three domains, Bacteria, Archaea and Eukarya.
Archaea were recently discovered, they are prokaryotes and form a heterogeneous clade characterized by a mosaic of bacterial, eukaryotic and unique features. Archaea and Eukarya share many homologous genes involved in information processing, including DNA replication (Edgell and Doolittle 1997), transcription and translation (Bell and Jackson,1998), whereas Archaea and Bacteria share many morphological structures, metabolic pathways and proteins (Aravind and Koonin, 1999; Kyrpides and Ouzounis, 1999). Archaea possess also unique features such as ether-linked, branched membrane lipids (Albers et al., 2000).
Archaea are found in all ecosystems and often thrive in peculiar environments with extreme temperatures, acidity, pressure and salinity. They are classified into four kingdoms: *Euryarchaeota* and *Crenarchaeota,* which constitute the two major archaeal lineages, *Nanoarchaeota* (so far represented by the single species *Nanoarchaeum equitans*) and *Korarchaeota* (represented by hitherto uncultured organisms).
The archaeon *Pyrococcus furiosus* ("the rushing fireball"), investigated in this work, belongs to the *Euryarchaeota* kingdom. It was isolated two decades ago by Prof. K.O. Stetter from geothermally heated marine sediments at the beach of Porto di Levante in Vulcano, Italy.
Pyrococcus furiosus (*Pfu*) is spherically shaped, 0.8 to 2.5 μm in width and exhibits monopolar polytrichous flagellation (Fiala and Stetter, 1986). It is strictly anaerobic, sulfur-dependent and heterotrophic, growing on starch, maltose, peptone and complex organic substrates. It is capable of growing at pH ranging from 5 to 9 and at temperatures ranging from 70 to 103°C with an optimal growth at 100°C. Its generation time is among the shortest found in Archaea, only 37 min under optimal conditions. The genome of *Pfu* is approximately 1.9 Mb in size and contains 2,192 open reading frames (Poole et al., 2005).
Pfu is also remarkable because it is able to maintain chromosomal integrity at elevated temperatures up to 103°C with very little accumulation of DNA breaks. It is also highly resistant to radiation, suggesting the presence of an efficient DNA repair system. This organism also possesses an array of highly thermostable enzymes that could prove to be important biocatalysts, such as the enzyme studied in the present work: the DNA-dependent RNA polymerase (RNAP).

1. The transcription cycle

A key step in gene expression is transcription, the synthesis of RNA from a DNA template. Transcription is carried out by a complex molecular machine, the DNA-dependent RNA polymerase along with others factors termed general transcription factors. The transcription cycle can be divided into three main stages: initiation, elongation and termination.

During initiation, the transcription factors recognize and bind to specific sequences called promoters within the double-stranded DNA. Transcription factors bound to the promoter recruit RNAP, preluding to the formation of the so called closed complex.
A short portion of the DNA surrounding the transcription start site unwinds, probably through the induction of a torsional strain (Douziech et al., 2000; Forget et al., 2004). During transcription initiation, abortive initiation events occur: RNAP synthesizes and releases small transcripts without disengaging from the DNA template (Vo et al., 2003). When the transcript reaches a length of approximately 10 to 12 nucleotides, RNAP breaks its contacts with the general transcription factors (GTFs) and clears the promoter to start elongation (Craig et al., 1998; Holstege et al., 1997). The transition from initiation to elongation is known as promoter clearance or escape. During elongation RNAP elongates the RNA chain processively while translocating itself and the melted transcription bubble along the DNA template. When a specific termination signal is encountered, RNAP is released and the completed transcript from the DNA. The RNAP is then free and ready to initiate another round of transcription.
All steps in this enzymatic cycle of RNA synthesis can be modulated by regulatory molecules. Transcription and its regulation are very fundamental processes and therefore require biochemical and structural analyses to deepen our knowledge of their mechanism.

2. DNA-dependent RNA polymerase

The overall reaction catalyzed by RNA polymerases is shown in Figure 1. RNAP requires all four ribonucleoside 5'-triphosphates (NTPs) to synthesize a RNA chain complementary to the template strand of duplex DNA. The RNA strand grows is in the 5' to 3' direction (antiparallel to the template strand of DNA). The 3'-hydroxyl end of the growing RNA acts as a nucleophile, attacks the α-phosphate of the incoming NTP and releases pyrophosphate (PPi).

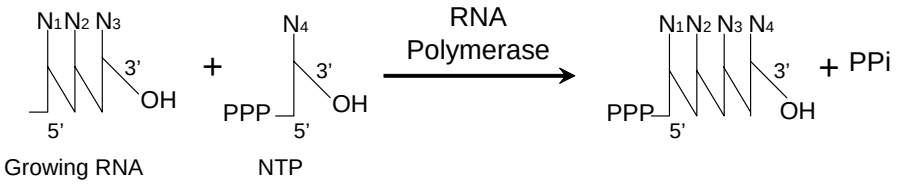


Figure 1. RNA polymerization reaction during transcription. The vertical line represents the pentose and the slanting line denotes the phosphodiester bond. Bases are shown as N_1 to N_4.

Bacteria and Archaea contain only a single RNAP, which synthesizes all classes of RNAs, including mRNAs, rRNAs and tRNAs. Eukaryotes contain three different nuclear RNAPs, termed RNAPI, RNAPII and RNAPIII, each specialized in the transcription of specific classes of RNA.

2.1 Bacterial RNAP

Bacterial RNAP comprises four subunits: α (37 kDa), β (151 kDa), β' (156 kDa) and ω (11 kDa) with stoichiometry $\alpha_2\beta$ β'ω. This complex forms the core of the enzyme with a total molecular mass of around 390 kDa. The core RNAP uses a set of alternative sigma factors for promoter recognition and discrimination. Bacterial promoters consist of two conserved hexamers at positions -35 (5'-TTGACA-3') and -10 (5'-TATAAT-3') relative to the transcription start site (+1). Binding of the sigma subunit to the core enzyme leads to the formation of the holoenzyme, which recognizes the promoter and initiates transcription.
The α subunits play a crucial role during the assembly of RNAPs. Two α subunits form a homodimer that serves as an assembly platform for the incorporation of the two large subunits β and β', which contribute to the formation of the catalytic site (Ishihama, 1981). The ω subunit is not essential for enzymatic activity of the RNAP in *in vitro* transcription experiments but promotes stability and assembly by reducing the configurational entropy of the largest subunit β' (Minakhin et al., 2001).

2.2 Eukaryotic RNAPs

Eukaryotic cells possess three nuclear RNAPs (RNAPI, II and III), which are distinct by their sub-cellular localization, chromatographic behavior, subunit composition, sensitivity to α-amanitin and promoter specifity. Eukaryotic cells are also known to contain separate mitochondrial and chloroplast RNAPs.
RNAPI synthesizes rRNAs, RNAPII transcribes mRNA and some small nuclear RNAs, while RNAPIII is responsible for the synthesis of tRNA, 5S rRNA and most small nuclear RNAs. RNAPs I, II, and III contain 14, 12, and 17 subunits, respectively. These three enzymes are functionally and structurally related: seven subunits are common to all three enzymes (Rbp3, Rbp5, Rbp6, Rbp8, Rbp10, Rbp11 and Rbp12) and three are related (Rbp4, Rbp7 and Rbp9; Carles et al., 1991; Woychik and Young, 1990). Furthermore, the two largest subunits of eukaryotic RNAPs (RNAPI Rpa190 and Rpa135, RNAP II Rpb1 and Rpb2, and RNAP III Rpc160 and Rpc128) share a high degree of sequence similarity, in particular in the region corresponding to the catalytic center of the enzyme.

2.2.1 RNAPI transcription machinery

The promoter elements of RNAPI consist of the core promoter, which extends from -45 to +20, and the upstream control element (UCE), which extends from -180 to -107. Both regions are rich in GC base pairs and they are about 85% identical. Initiation of rRNA transcription in the yeast *Saccharomyces* involves coordinated interactions of at least four transcription factors with promoter elements and RNAPI. The following transcription factors are required: the upstream activating factor (UAF) containing Rrn5, Rrn9, Rrn10, the H3 and H4 histones and Uaf30p; the core factor (CF) containing Rrn6, Rrn7, and Rrn11; TBP, the TATA binding protein; Rrn3p, a factor that binds RNAPI (Nomura,

2001). UAF strongly binds the upstream element and recruits CF with the help of TBP and the Rrn3p-RNAP-I complex to initiate transcription (Keener et al., 1998; Keys et al., 1996, Steffan et al., 1996). Upon transcription initiation, RNAP-I-Rrn3p and CF dissociate from the promoter, while UAF remains bound to DNA to support multiple rounds of transcription.

2.2.2 RNAPII transcription machinery

Transcription of protein-encoding genes requires assembly of a preinitiation complex (PIC) composed of template DNA, RNAP II, and five general transcription factors (GTFs). Recognition of the core promoter by the transcription machinery is essential for correct positioning and assembly of RNAPII and GTFs. Eukaryotic DNA is wrapped around a histone octamer, which interferes with many DNA activities. Active promoters are associated with histones, which have been modified in various ways, including acetylation, phosphorylation, and methylation. Sequence elements found in core promoters include the TATA element (TBP-binding site, located ~25 bp upstream of the transcription start site), BRE (TFIIB-recognition element, located just upstream of TATA element), Inr (initiator element, located at or near the transcription start site), DPM (downstream promoter element, located ~30 bp downstream of the transcription start site) and the recently reported MTE (motif-ten-element, located ~22 bp downstream of the start site) (Lim et al., 2004). Most promoters contain one or more of these elements, but none is absolutely essential for promoter function (Hahn, 2004). PIC assembly is nucleated by binding of the TBP subunit of TFIID to the TATA box, the best characterized element, followed by the recruitment of TFIIB and a complex of unphosphorylated RNAP II with TFIIF, TFIIE, and TFIIH. In addition, a 20-subunit Mediator is recruited and transduces regulatory information from activator and repressor proteins to RNA polymerase II (Kelleher et al., 1990; Gustafsson et al., 1998). Mediator is unique to eukaryotes and enables the more intricate gene expression regulation that underlies the development and functioning of complex multicellular organisms.
After RNAPII clears the promoter, TFIIB and TFIIF are released, whereas other factors such as activators, TBP, Mediator, TFIIH and TFIIE remain promoter-associated and form what is termed a reinitiation intermediate or scaffold, to facilitate subsequent rounds of transcription (Yudkovsky et al., 2000).
After promoter melting and transcription initiation, the Rpb1 C-terminal domain (CTD) is phosphorylated by TFIIH and other factors, an event that facilitates promoter clearance and progression into the elongation phase of transcription. Following termination, a phosphatase restores RNAP II to its unphosphorylated form, allowing the GTFs and RNAP II to initiate another round of transcription (Reinberg et al., 1998).

2.2.3 RNAPIII transcription machinery

RNAPIII requires TFIIIB and TFIIIC for the majority of its promoters. In addition, TFIIIA is essential for recognition of the 5S gene promoter (Schramm and Hernandez, 2002). Promoters for 5S and tRNA genes consist of bipartite sequences downstream of the transcription start site with boxA separated from either boxC (type 1 promoters) or boxB (type 2 promoters). 5S rRNA genes have type 1 promoters and tRNA genes have

type 2 promoters. Promoters for snRNA genes consist of separated sequences upstream the transcription start site: Distal Sequence Element (DSE), Proximal Sequence Element (PSE), and the TATA box (Schramm and Hernandez, 2002).
Transcription of Pol III genes also begins with the step-wise assembly of a PIC. When bound to the upstream region of 5S and tRNA genes, yeast TFIIIB correctly positions RNAPIII at the promoter and supports multiple rounds of transcription (Kassavetis et al., 1990). TFIIIB consists of three subunits: TBP, Brf and Bdp1. Brf is related to the TFIIB family. Reinitiation has a higher efficiency than does *de novo* initiation in Pol III dependent transcription (Dieci and Sentenac, 1996). The stable association of TFIIIB with promoter, even after RNAPIII progresses into elongation, bypasses the need for PIC formation, thus accelerating the process of reinitiation (Fan et al., 2005)

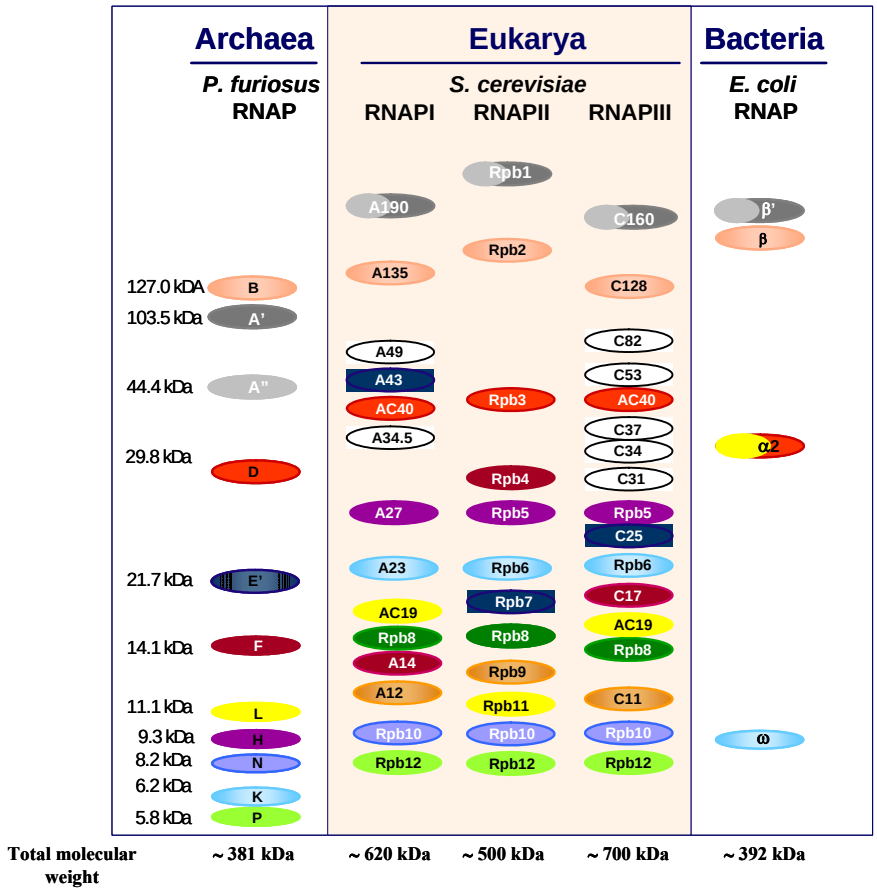


Figure 2. Homologous subunits of RNAP from the three domains of life. Subunit patterns of RNAP from *Pyrococcus furiosus*, RNAPI, II and III of *Saccharomyces cerevisiae* and of the *Escherichia coli* enzyme is shown. Homologous subunits are shown with the same color. The same color coding is used throughout this work. Subunits shown in white are unique to the corresponding RNAP. The molecular masses of *Pyrococcus* RNAP subunits are indicated to the left and the total molecular weight of the RNAPs is shown at the bottom.

2.3 Archaeal RNAP

Archaeal RNAP is more closely related to the eukaryotic nuclear RNAPs than to the bacterial enzyme (Bell et al., 1998). Archaeal RNAP is not affected by the antibiotic rifampicin or α-amanitin, known inhibitors of the bacterial RNAP and RNAPII, respectively (Zillig et al., 1979).
Archaeal RNAP contains from 10 to 12 different subunits (Langer et al., 1995; Darcy et al., 1999), which show high primary sequence similarity to the subunits of eukaryotic RNAPII. Apart from subunits RPB8 and RPB9 (Kaine et al., 1994), homologs of other RNAPII subunits have been identified in all archaeal genomes studied so far (Fig. 1). There are no archaeal homolog of the subunits exclusively found in RNAPI and RNAPIII. This similarity suggests that many of the fundamental structural and functional aspects of contemporary eukaryotic RNAPIIs have remained remarkably unchanged throughout evolution and that RNAPI and RNAPIII evolved from an RNAPII like enzyme after the evolution of eukaryotic cells.
Pyrococcus furiosus RNAP consists of 11 subunits ranging in size from 5.7 to 127.0 kDa. Its largest subunit B is split into two subunits (B' and B") in methanogenic archaea and is homologous to the RNAPII second largest subunit Rpb2 and the bacterial second largest subunit β. The largest RNAPII subunit Rpb1 is represented as two subunits, A' and A", in all archaeal polymerases and is homologous to the bacterial β'. A' and A" are homologous to the C- and N-proximal halves of Rpb1, respectively. B, A' and A", as well as their eukaryotic and bacterial homologs, form the catalytic core of the enzyme. Subunits D and L are homologous to eukaryotic Rpb3 and Rpb11, respectively, and to the amino terminus of the bacterial α subunit, although they lack homology to the α carboxy-terminal domain, which is important in the response of bacterial RNAP to activators (Ebright et al., 1995). DLBA'A'' (or DLB'B''A'A'') is the minimal archaeal configuration with high homology to the bacterial core enzyme α_2β'β (Eloranta et al., 1998; Cramer et al., 2000). Subunit K is homologous to the carboxy-terminal half of Rpb6 and to the bacterial ω subunit.
The remaining archaeal RNAP subunits have homologs only in eukaryotic RNAP. Subunit H is homologous to the carboxy-terminal domain of Rpb5. Subunits N and P are related to Rpb10 and Rpb12, respectively, and form a sub-complex with subunits D and L. The complex D/L/N/P is homologous to the Rpb3/10/11/12 subassembly of eukaryotic RNAP (Werner and Weinzierl, 2003). Subunits E' and F form a stable heterodimeric complex just as their eukaryotic homologs Rpb7 and Rpb4 do. E'/F complex and its eukaryotic homologs Rpb7/Rpb4 (RNAPII), Rpc25/Rpc17 (RNAPIII) and Rpa43/Rpa14 (RNAPI) form heterodimers that are a landmark of all non-bacterial RNA polymerases (Sadhale et al., 1994; Langer et al., 1995; Shpakovski and Shematorova, 1999; Werner et al., 2000; Peyroche et al., 2002; Siaut et al., 2003).
Taken together, the overall high degree of sequence similarity suggests that the archaeal RNAP can be viewed as a simplified version of the eukaryotic RNAPII.

2.3.1 Archaeal transcription machinery

The core archaeal transcription apparatus consists of RNAP and only two initiation transcription factors, TBP and TFB, which are homologous to the core initiation factors

of the eukaryotic RNAPII and RNAPIII and not to the bacterial sigma factors (Thomm, 1996; Bell and Jackson, 1998). Archaeal promoters resemble eukaryotic RNAPII promoters with a highly conserved TATA box sequence (located 25 bp upstream of the transcription start site), an adjacent TFB-recognition element (BRE, located immediately upstream of TATA box) and the initiator element (Inr, located at or near the transcription start site), which contains a pyrimidine-purine dinucleotide, with a purine at position +1 (Bartlett, 2005).

Archaeal TBP belongs to the family of TATA-element-binding proteins that bind to promoter TATA elements. The TFIIB-related transcription factor TFB binds to TBP-DNA complex via its carboxy-terminal domain, recognizing TBP surface and making base-specific interactions with the BRE, which defines the orientation of the ternary complex, and thus, the direction of the transcription (Bell et al., 1999). Once bound, TBP and TFB bend DNA and recruit RNAP, just as eukaryotic TBP and TFIIB recruit RNAPII. The amino-terminal third of TFB contains a zinc ribbon motif and a conserved B-finger motif, and is required for RNAP recruitment (Bell and Jackson, 2000). TBP, TFB and RNAP are necessary and sufficient to direct open complex formation at many archaeal promoters *in vitro*. A third transcription factor, TFE, has been shown to have a stimulatory effect on weak promoters *in vitro* (Bell et al., 2001; Hanzelka et al., 2001).

TFE is homologous of the amino-terminus of the α subunit of the RNAPII transcription factor TFIIE. However, TFE is not essential for *in vitro* transcription (Bell et al., 2001). Unlike eukaryotic systems, no other basal factors (such as TFIIA, TFIIF, and TFIIH) are required for transcription initiation and promoter escape, and none of these steps are dependent on nucleoside triphosphate (NTP) hydrolysis to induce specific conformational changes (Bell et al., 1998). Archaeal RNAP does not have a heptapeptide-repeat-containing carboxy-terminal domain (CTD), the region present in Rpb1 of RNAPII that is phosphorylated in conjunction with the transition from inititation to elongation (Stiller and Hall, 2002). Archaea encode a transcript cleavage factor, designated TFS, that is related to the elongation factor TFIIS in its C-terminal segment and to eukaryotic RNAPIII subunit C11 in its N-proximal portion. The C11 subunit is responsible for the intrinsic cleavage activity of yeast RNAPIII, but archaeal TFS is extrinsic (Hausner et al., 2000). Furthermore, TFS is involved in proofreading by inducing hydrolytic release of dinucleotides from the growing 3'-end of the nascent transcript (Lange and Hausner, 2004).

The events in archaeal transcription beyond initiation have received very little experimental attention, and little is known about the subsequent steps of the transcription.

3. General RNAP architecture

To date, the crystal structure of archaeal RNAP has not been solved yet, however, at least three scientific groups are working hard on determining the first structure of an archaeal RNAP. Archaeal homologs have been reported for all RNAPII subunits, except Rpb8 and Rpb9, and therefore the structure of archaeal RNAP must be very similar to that of the yeast RNAPII. In addition, *Pfu* RNAP subunit-subunit interaction studies show that the overall architecture of the archaeal RNAP resembles very closely the eukaryotic RNAPII (Fig. 3 A; Goede et al., submitted).

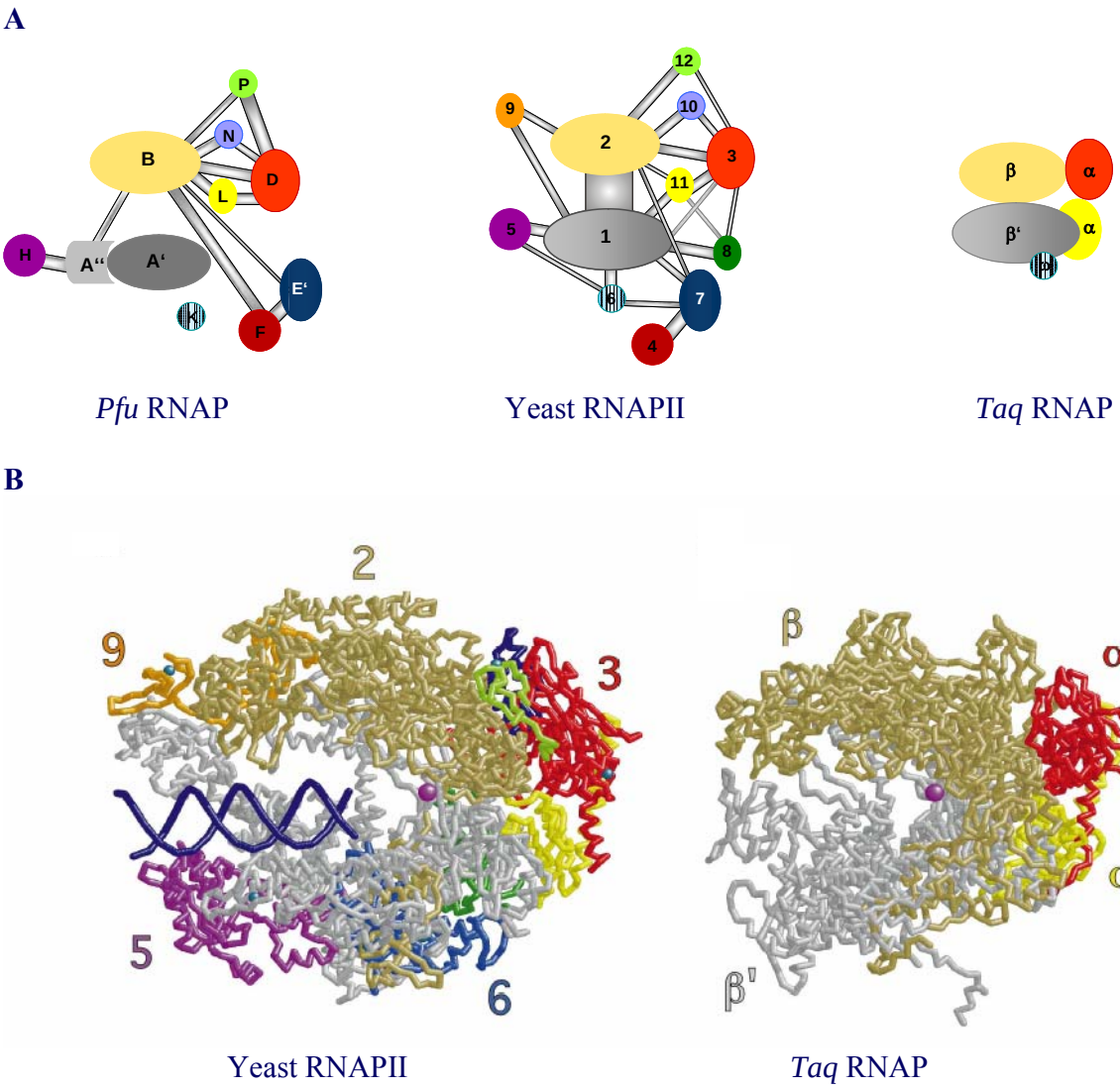


Figure 3. RNAP subunit architecture. (A) Schematic representation of subunit interactions within *Pfu* RNAP (based on biochemical studies; Goede et al., submitted), yeast RNAPII and *Taq* RNAP. Thickness of the white lines approximates the relative amount of surface area buried at the interface between subunits. **(B)** Crystal structures of yeast RNAPII (Cramer et al., 2000) and *Taq* RNAP (Zhang et al., 1999). The structures are shown in similar orientations to reveal the deep cleft. Helical DNA, though not present in the crystal, is docked in the RNAPII cleft, in the location previously indicated by electron crystallographic studies. Corresponding subunits have the same color and are colored according to (A). The active site metal ion A is represented by a magenta sphere. Eight zinc ions are present in RNAPII structure, and several can be seen in this view as blue spheres.

Over the past years, X-ray crystallographic structures have become available for the bacterial RNAP and the eukaryotic RNAPII alone as well as a part of complexes with nucleic acids or regulatory factors. A high resolution structure has been determined for a bacterial RNAP from *Thermus aquaticus* (*Taq*) at 2.6 Å resolution (Vassylyev *et al.,* 2002). The heterogeneity in *Saccharomyces cerevisiae* RNAPII preparations, caused by the variable stoichiometry of the Rpb4/Rpb7 heterodimer, interfered with first crystallization attempts. However, with optimized protocols, it was possible to achieve a 2.8 Å resolution structure of the 10-subunit core (Cramer et al., 2001). The crystal structure of the 12-subunit yeast RNAPII was initially determined at 4.2 Å resolution (Armache et al*.,* 2003; Bushnell and Kornberg, 2003)

Recently, a new structure of free RNAPII subcomplex Rpb4/Rpb7 (Armache et al., 2005) has enabled refinement of an atomic model of complete 12-subunit RNAPII to 3.8 Å resolution (Armache et al., 2005).
The bacterial and yeast RNAP structures reveal that a conserved "core" with a similar architecture (Fig. 3). The two largest subunits, Rpb1 and Rpb2, form the central mass of the enzyme and a positively charged cleft in RNAPII as do their homologs, β' and β, in *Taq* RNAP.
The Rpb1/Rpb2 core is anchored at one end by Rpb3, 10, 11 and 12. In *Taq* RNAP, β' and β are anchored by the N-proximal halves of the two α subunits; indeed, α_2 is the assembly core of bacterial RNAP. The Rpb3-10-11-12 subassembly of RNAPII and the α_2 subunits of *Taq* RNA polymerase occupy comparable positions.
A fifth core subunit (Rpb6 in RNAPII; ω in *Taq* RNAP) further buttresses and stabilizes the large subunits (Minakhin et al., 2001).
Yeast RNAPII shares a core structure with the bacterial enzyme. However, the eukaryotic RNAPII has seven additional small subunits and therefore differs entirely in peripheral and surface structure, where interactions with other proteins, such as general transcription factors, Mediator and other regulatory factors, take place.
Recently, the crystal structure of RNAPIII C17/25 subcomplex was resolved and an 11-subunit model of RNAPIII was established (Jasiak et al., 2006).

4. RNAPII structure

Jean Marx of Science Magazine (Science Apr 20 2001: 411-414) describes RNAPII structure as follows: "If any enzyme does the cell's heavy lifting, it is RNA polymerase II. Its job: getting the synthesis of all the proteins in higher cells under way by copying their genes into RNAs, and doing it at just the right time and in just the right amounts. As such, pol II, as the enzyme is called, is the heart of the machinery that controls everything that cells do, from differentiating into all the tissues of a developing embryo to responding to everyday stresses. Now, cell biologists can get their best look yet at just how the pol II enzyme of yeast and, by implication, of other higher organisms performs its critical role."

Since the archaeal *Pfu* RNAP architecture bears striking resemblance to the yeast RNAP II structure, the RNAPII structure will be further described.
RNAPII is composed of four mobile elements termed clamp, jaw-lobe, shelf, and core that can move relative to each other (Cramer et al., 2001). The core module accounts for about half the mass of RNAPII and is composed mainly of subunits common to all cellular RNAPs (Fig. 4). The modules composition is shown in Table 1. The surface charge of RNAPII is almost exclusively negative, while the regions that contact DNA, including the cleft, are positive.
The active site is buried deep into the cleft, near the center of the enzyme (Fig. 5). Beyond the active site, the cleft is blocked at the upstream end by a domain appropriately named the wall, which is part of the second largest subunit Rpb2. The wall contains the flap feature, which might serve as a binding site for transcription factors (Nickels et al., 2005). Just before the active site, a long α helix, called the bridge helix, spans the cleft. This helix and the active site line a perforation referred to as "pore 1", which widens towards the exterior, creating an inverted funnel. The outer rim of the funnel is lined by a pair of α helices in Rpb1, called funnel region. Pore 1 was proposed to allow entry of substrate nucleosite triphosphates (NTPs) and also to allow RNA exit during retrograde movement of the polymerase on the DNA.

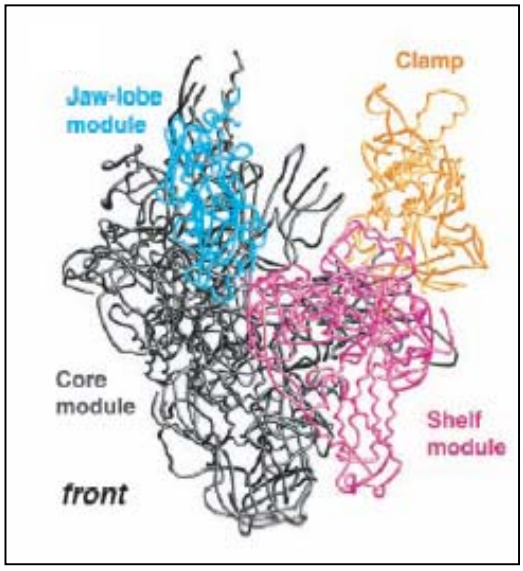


Table 1. Mobile modules

Module	Subunits and regions	Percentage of total mass
Core	All except other three modules	57
Shelf	Rpb1 cleft, Rbp1 foot, Rbp5,Rbp6	21
Clamp	Rbp1 clamp core and clamp head, Rpb2 clamp	12
Jaw-lobe	Rbp1 jaw, Rbp9 jaw, Rbp2 lobe	10

Figure 4. The four mobile elements in RNAPII. Backbone traces of the core, shelf, clamp and jaw-lobe modules of the RNAPII structure, shown in gray, pink, yellow and blue, respectively (Cramer et al., 2001).

4.1 The clamp

The most prominent mobile module, the clamp, forms one side of the cleft and is connected to the remainder of the enzyme through five flexible domains called the switches (switch1-5). The clamp constitutes part of one jaw. The majority of the clamp is formed by Rpb1, a small portion of Rpb2 and the Rpb6 amino-terminal tail (Figures 4 and 5). Crystallographic studies have shown that the clamp can adopt two different conformations, implying a large swinging motion up to 30 Å (Armache et al., 2003). The so called open conformation is thought to allow straight double-stranded promoter DNA to lie along the bottom of the active site cleft and reach the active site (Cramer et al., 2001). In a subsequent step, the clamp would switch to a closed conformation. Rpb4-Rpb7 complex, which is loosely bound to the core RNAP, restricts the clamp to the closed conformation (Fig. 5). It was proposed that the closed state is required for transcription elongation (Armache et al., 2003).

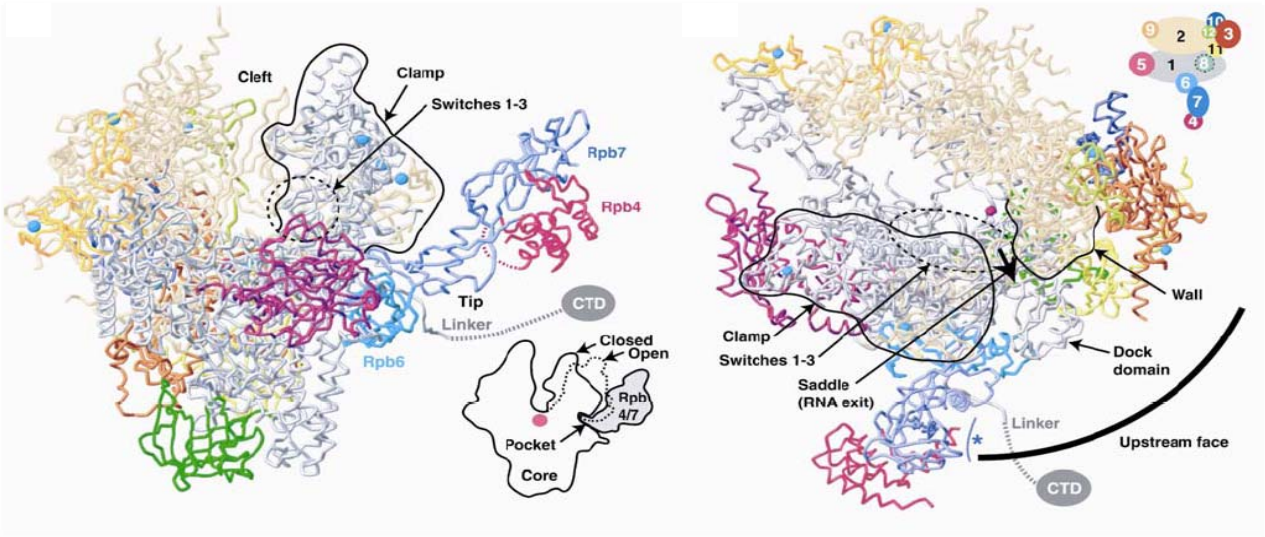


Figure 5. Structure of the 12-subunits initiation-competent RNAPII. The structure was resolved at 3.8 Å resolution and shown as ribbon model (Armache et al., 2003). The 12 subunits Rpb1-Rpb12 are colored according to the key above the views. Cyan spheres and the pink sphere depict eight zinc ions and an active-site magnesium ion, respectively. A black line circles the clamp. The linker to the CTD is indicated as a dashed line. The switches are indicated as a dashed circle. A view of the model from the top is shown on the left. The saddle between the wall and the clamp and the assumed direction of RNA exit are indicated. A schematic cut-away view is shown in the middle. A dashed like indicates the open clamp position observed in RNAPII core structure (Cramer et al., 2001). The presence of Rpb4/Rpb7 complex locks the mobile clamp in a closed conformation. This figure was a very kind gift from Dr. Karim Armache.

4.2 The active site

RNAP catalyzes phosphoryl transfer through a nucleophilic attack of the RNA primer 3'-hydroxyl on the α-phosphate of incoming ribonucleotide triphosphates, resulting in nucleotide incorporation and pyrophosphate release (Fig. 6).
It has been proposed that all types of polymerases catalyze phosphodiester bond formation by a two metal ion mechanism (Steitz, 1998; Fig. 6). A first Mg^{2+} ion, termed metal A, facilitates the nucleophilic attack of the 3′ oxygen on the 5′ α-phosphate. The second Mg^{2+} ion, metal B, is in the vicinity of metal A, at a distance of 5.8 Å, and facilitates pyrophosphate release. In prokaryotic RNAP and eukaryotic RNAP II, metal A is coordinated by three strictly conserved aspartates belonging to the NA*D*F*D*G*D* motif in β′ and Rpb1 subunits, respectively (Zhang et al., 1999; Gnatt et al., 2001; Cramer et al., 2001). This motif is also conserved in archaeal RNAP. The largest subunits, A' and B', contain the metal A and B motifs, respectively (Werner and Weinzierl, 2003).
Metal B seems to have a low affinity for free RNAP II (Cramer et al., 2001) and it has been proposed to enter the active site with the incoming NTP. Metal B is coordinated by three aspartates, two from β′/Rpb1/A' and one from β/Rpb2/B, located in a conserved E*D* motif (Westover et al., 2004). Formation of a phosphodiester bond is followed by translocation of the nucleic acids in order to present the next template register for the next nucleotide addition cycle.

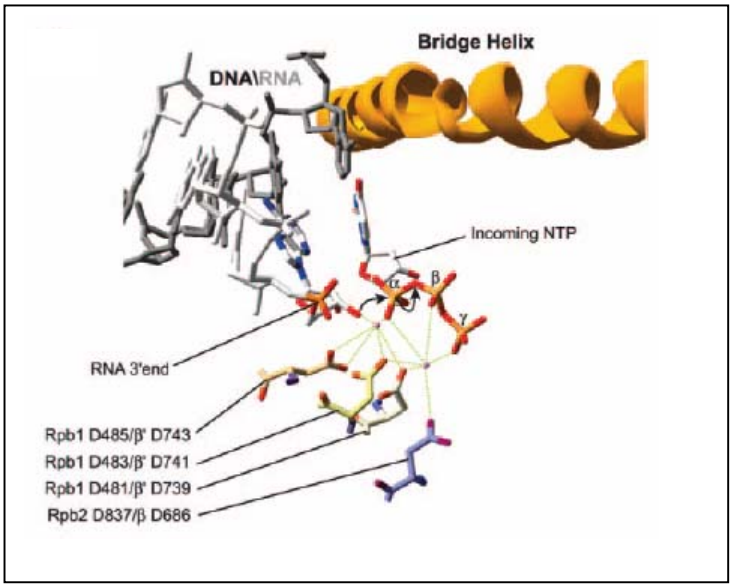


Figure 6. Mechanism of ribonucleotide addition to the RNA chain. Residues known to coordinate the two Mg^{2+} ions (pink spheres) are shown. Coordination bonds are depicted with green dotted lines. DNA and RNA are shown in gray and white, respectively. Black arrows show the nucleophilic attack. This figure was a very kind gift from Dr. Benoit Coulombe.

4.3 RNAPII elongation complex structure

During active transcription, RNAPII forms a stable elongation complex (EC). In the EC, incoming DNA is unwound before the polymerase active site and is rewound beyond it to form the exiting duplex. In the transcription bubble, the DNA template strand forms a hybrid duplex with the growing RNA. RNAPII maintains the bubble, selects NTPs in a template-directed manner, synthesizes RNA, transclocates along the DNA and separates RNA from DNA at the upstream end of the DNA-RNA hybrid.
Molecular insights into RNAPII elongation mechanism have been gained from structures of RNAPII in complex with nucleic acids. Kettenberger et al. determined the structure of RNAPII elongation complex in the post-translocation state (Kettenberger et al., 2004). The EC was *in vitro* reconstituted by incubating RNAPII with a synthetic bubble, consisting of template DNA, nontemplate DNA and RNA. The structure of this complex was determined to 4 Å resolution, revealing new important details of the elongation mechanism. This structure clearly shows interactions of the enzyme with both ends of the transcription bubble as well as with the RNA-DNA hybrid (Fig. 7 A).

Four RNAPII loops seem to play a critical role during elongation (Fig. 7 B). Maintenance of the upstream end of the hybrid involves the lid, a prominent loop that protrudes from the edge of the clamp. A phenylalanine side chain of the lid appears to serve as a wedge to maintain strand separation**.** The fork loop 1 and the rudder interact with each other and create two compartments in the cleft to hold the downstream DNA and the hybrid. A primary role of the rudder in stabilizing the unwound DNA beyond the hybrid region is consistent with molecular genetic analysis (Kuznedelov et al., 2002). Fork loop 2 interferes with the non template strand and prevents reassociation of separated strands.
These four loops form a network of protein-protein and protein-nucleic acid interactions to stabilize the elongation complex. In addition, they are well conserved through all archaeal RNAPs.

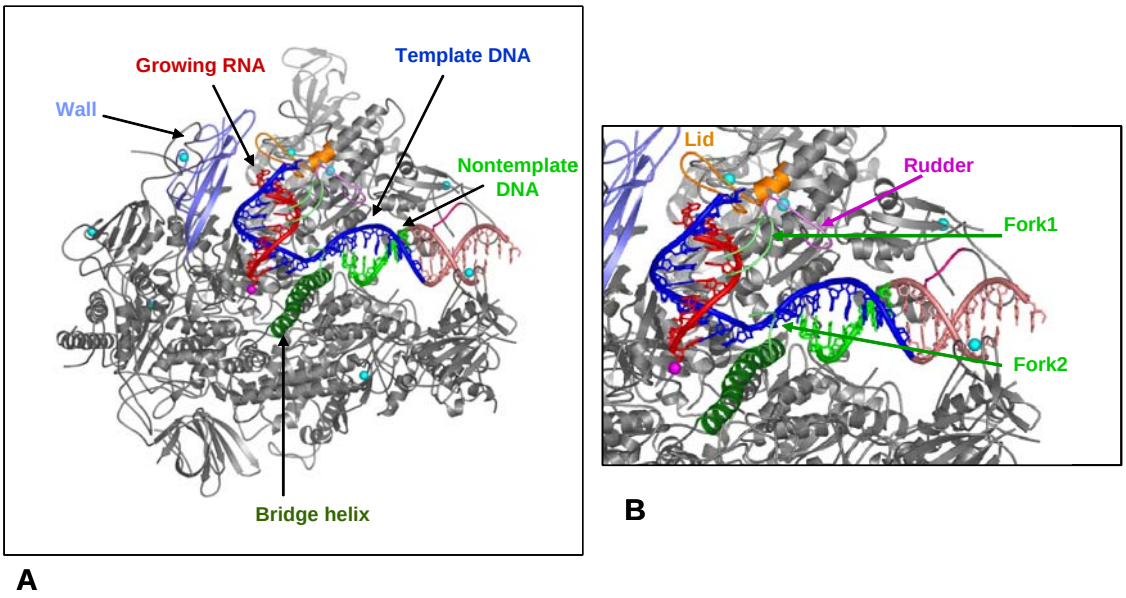


Figure 7. **Structure of the complete RNAPII elongation complex** (Kettenberger et al., 2004). **(A)** Structure of the complete 12 subunit RNAPII bound to a transcription bubble and product RNA. Polymerase subunits are shown as ribbons. 1-12 represent RNAPII subunits Rpb1-Rpb12. Template DNA, nontemplate DNA and RNA strands are shown in blue, cyan and red, respectively. Extrapolated B-form downstream DNA is colored in light pink. The active site metal ion A is indicated by a pink sphere. Eight zinc ions are depicted as cyan spheres. **(B)** Closer view of (A) showing the details of the interactions between RNAPII and the DNA-RNA hybrid in elongating RNAPII. The DNA template and non template strands are in blue and green, respectively, and the RNA is in red. Protein elements that are proposed to be involved in the maintenance of the arrangement of nucleic acids are indicated. Both figures were a very kind gift from Dr. Hubert Kettenberger.

RNAPII is believed to oscillate between forward translocation and backtracking (reverse translocation) at every step of transcription. Forward translocation clears the A site (nucleotide addition site) for entry of the next NTP. Backtracking returns the nucleotide that was just added to the RNA back to the A site. One or more additional steps of backtracking result in a stalled transcription complex, which may persist for many minutes or even, be irreversible. Recovery from pausing generally requires transcript cleavage in the polymerase active center, induced by the general transcription factors IIS (TFIIS); otherwise the polymerase may be ubiquitinated and destroyed, to avoid gene inactivation and cell death.

5. Purpose of this work

In order to gain molecular insights into *P. furiosus* RNAP structure and function, all 11 subunits were over-expressed in *E. coli* and purified to homogeneity. This work presents the successful *in vitro* assembly of a fully active *P. furiosus* RNAP from its recombinant subunits. This reconstitution system allowed us also to study the contributions of the various subunits to RNAP activity.
To date, different high-resolution structures of RNAPII are available and give insights into transcription initiation and elongation (Bushnell and Kornberg, 2003; Kettenberger et al., 2004). However, understanding the functional significance of the structural information requires also biochemical studies. *In vitro* reconstitution of eukaryotic RNAPs has not been successful so far. The high degree of structural and functional similarity between archaeal and eukaryotic RNAPII core transcriptional machineries can be exploited to shed light on transcription mechanisms in Archaea as well as in Eukarya. The availability of *in vitro* reconstituted archaeal RNAP allows subjecting this RNAPII like enzyme to site-directed mutagenesis and functional studies. A broad range of questions can be addressed about interactions and mechanisms within the transcription apparatus, regardless of compatibility with cellular viability. In this work, I investigated in particular the role of specific *Pfu* RNAP structural elements homologous to RNAPII rudder, lid, fork1 and fork2. These loops were altered by site-directed mutagenesis to asses their functions. Recombinant RNAP variants, harbouring the mutated subunits, were produced by *in vitro* assembly and were assayed to evaluate the contribution of these loops to transcriptional activity and to determine at which stage of the transcription cycle they act.
Furthermore, a protocol was developed in order to purify the endogenous *Pfu* RNAP to homogeneity and in large scale.

II Material

1. Suppliers

1.1 Chemicals

All others chemicals used in the work were supplied from Merck (*Darmstadt*) or Sigma (*Deisenhofen*) unless otherwise indicated.

Acrylamide	*Carl Roth GmbH & Co., Karlsruhe*
Agarose	*Invitrogen GmbH, Karlsruhe*
AMP-PNP	*Sigma, St. Louis (USA)*
Ampicillin	*Sigma-Aldrich, Deisenhofen*
APS	*Serva, Heidelberg*
Bacto-agar	*Difco Laboratories, Michigan, USA*
Bacto-yeast extract	*Difco Laboratories, Michigan, USA*
Bacto-tryptone	*Difco Laboratories, Michigan, USA*
Bis-Acrylamide	*Bio-Rad, Munich*
Bromphenol Blue	*Serva, Heidelberg*
CDP-Star	*Roche Diagnostics, GmbH, Mannheim*
DNA ladders	*MBI Fermentas, Vilnius (Litauen)*
dNTPs	*MBI Fermentas, Vilnius (Litauen)*
EDTA	*Serva, Heidelberg*
Ethidium Bromide	*Serva, Heidelberg*
Formamide	*Sigma, St. Louis (USA)*
FUJI NIF RX	*Fuji, Tokyo (Japan)*
GF/F glass filters	*3MM, Maidstone (UK*)
Glycerin (87 %)	*Sigma, St. Louis (USA)*
GMP-PNP	*Sigma, St. Louis (USA)*
Guanidine HCl	*Sigma, St. Louis (USA)*
HEPES	*Sigma, St. Louis (USA)*
Imidazole	*Sigma, St. Louis (USA)*
Isotopes ([α-^{32}P]-UTP; [γ-^{32}P]-ATP)	*Hartmann Analytic, Braunschweig*
KODAK BioMax MR	*Kodak, Rochester (UK)*
Lysozyme	*Boeringer, Mannheim*
ß-Mercaptoethanol	*Roth, Karlsruhe*
NTP-Mix	*MBI Fermentas, Vilnius (Litauen)*
NTPs	*MBI Fermentas, Vilnius (Litauen)*
Phenol/Chloroform/Isoamyl alcohol	*Roth, Karlsruhe*
Poly[d(A-T)]	*Sigma, St. Louis (USA)*
Poly[d(I-C)]	*Roche Diagnostics, GmbH, Mannheim*
Protease Inhibitors Cocktail	*Roche Diagnostics, GmbH, Mannheim*
SDS	*Serva, Heidelberg*

TEMED	*Serva, Heidelberg*
Tris	*Roth, Karlsruhe*
Tween-20	*Pierce, Rockford (USA)*
Whatman-Paper	*3MM Maidstone (UK)*
Xylene Cyanol	*Serva, Heidelberg*

1.2 Enzymes, antibodies and others proteins

Alkaline phosphatase	*Promega Corp., Wisconsin, (USA)*
BSA	*Roche Diagnostics, GmbH, Mannheim*
DNAse I, RNAse-free (10U/µl)	*Roche Diagnostics, GmbH, Mannheim*
Pfu DNA polymerase	*New England Biolabs, Beverly (USA)*
Prestained Protein markers	*MBI Fermentas, Vilnius (Litauen)*
Protein molecular weight marker	*MBI Fermentas, Vilnius (Litauen)*
Restriction endonucleases	*New England Biolabs, Beverly (USA)*
T4-DNA ligase	*MBI Fermentas, Vilnius (Litauen)*
T4-polynucleotide kinase	*MBI Fermentas, Vilnius (Litauen)*
λ Protein Phosphatase	*New England Biolabs, Beverly (USA)*
Phosphatase, Alkaline−Agarose from calf intestine	*Sigma, St. Louis (USA)*

Primary antibodies

AntiE'	A rabbit polyclonal antibody raised against the *Pfu* subunit E'. *Eurogentec, Seraing (Belgium)*
AntiF	A rabbit polyclonal antibody raised against the *Pfu* subunit F. *Eurogentec, Seraing (Belgium)*
AntiE''	A chicken polyclonal antibody raised against the *Pfu* subunit E". *Davids Biotechnologie GmbH, Regensburg*

Secondary antibodies

Anti-rabbit Alkaline Phosphatase conjugated: *Sigma, St. Louis (USA)*
Anti-chicken Alkaline Phosphatase conjugated: *Jackson ImmunoResearch, West Grove (USA)*

Pyrococcus furiosus transcription factors

TBP and TFB were a kind gift from Dr. Bernd Goede.

1.3 Kits

Champion™ pET Directional TOPO® Expression Kit	*Invitrogen GmbH, Karlsruhe*
Dialysis frame (Slide-A-Lyzer 3.5k)	*Pierce, Rockford (USA)*
PVDF membrane	*Millipore, Bedford (USA)*
QIAquick Nucleotide Removal Kit	*Qiagen, Hilden*
QIAquick® spin Gel Extraction Kit	*Qiagen, Hilden*
QIAquick® spin PCR Purification Kit	*Qiagen, Hilden*

QIAquick® spin Miniprep Kit	*Qiagen, Hilden*
Quick Ligation Kit	*New England Biolabs, Beverly (USA)*
Centrifugal Filter Devices	*Millipore, Bedford (USA)*
Sterile filters 0.22 μm, 0.45 μm pore size	*Millipore, Bedford (USA)*

1.4 Chromatography equipment and columns

AEKTA purifier 12	*GE Healthcare, Uppsala (Sweden)*
Biorex 70 Resin	*Bio-Rad, Hercules (USA)*
HiLoad 16/60 Superdex 75 prep grade	*GE Healthcare, Uppsala (Sweden)*
HiPrep 16/10 Heparin FF	*GE Healthcare, Uppsala (Sweden)*
HisTrap Chelating HP, 5 ml	*GE Healthcare, Uppsala (Sweden)*
HiTrap Protein A HP, 1 ml	*GE Healthcare, Uppsala (Sweden)*
HiTrap SP HP, 1 ml,	*GE Healthcare, Uppsala (Sweden)*
Mono Q 5/50 GL, 1 ml	*GE Healthcare, Uppsala (Sweden)*
MonoQ 15Q Source, 10 ml	*GE Healthcare, Uppsala (Sweden)*
Superdex 200 10/300 GL	*GE Healthcare, Uppsala (Sweden)*
Superose 6 10/300 GL	*GE Healthcare, Uppsala (Sweden)*

2. Materials for cloning

2.1 Archaeal strain

Pyrococcus furiosus DSM3638 (Fiala and Stetter, 1986)

2.2 Bacterial strains

Escherichia coli, JM109 strain (for high copy plasmid preparation; Yanisch-Peron *et al*., 1985)
Escherichia coli, BL21-CodonPlus™ (DE3)-RIL strain (expression strain; *Stratagene, La Jolla, USA)*

2.3 Plasmids

All plasmids used in this work are *E. coli* expression vectors and contain T7 RNAP promoter and Shine-Dalgarno ribosome binding sequence.

Plasmid	Relevant marker	Induction	Company
pET-33b(+)	Kanamycin resistance	IPTG	*Novagen*
pET-30a-c(+)	Kanamycin resistance	IPTG	*Novagen*
pET151/D-TOPO	Ampicillin resistance	IPTG	*Invitrogen*

2.4 Oligonucleotides

All sequences are listed with the standard 5'-3' orientation. All oligonucleotides were provided by MWG, Ebersberg.

Primers for cloning rpoB (Gen PF1564) in pET-33b(+)
RpoB-Fwd-Nhe I**:** 5'-CTAGCTAGCATGAGAGGTCCGACTGTTG-3'
RpoB-Rev-Not I: 5'-AAGGAAAAAAGCGGCCGCTCACACCCTCTCTGAGAGGTTTA-3'
Primers for cloning rpoB (Gen PF1564) in pET151/D-TOPO
151rpoB-Fwd: 5'- CACCATGAGAGGTCCGACTGTTG-3'
151rpoB-Rev: 5'- CTATCACACCCTCTCTGAGAGGTTTA-3'
Primers for cloning rpoA' (Gen PF1563) in pET151/D-TOPO
151rpoA'- Fwd: 5'-CACCATGAAAAAAGTTATTGGAAGTATT-3'
151rpoA'- Rev: 5'- CTATCACACCTTCGCCTTGTTATTTCC-3'
Primers for cloning rpoA'' (Gen PF1562) in pET151/D-TOPO
151rpoA''-Fwd: 5'-CACCATGGTCTCTCTTTCTACTATT-3'
151rpoA''- Rev: 5'-CTATCACACCTCCTCCTTTTCTTTC-3'
Primers for cloning rpoD (Gen PF1647) in pET151/D-TOPO
151rpoD-Fwd: 5'-CACCATGGCCGGAATTGAAGTTCAG-3'
151rpoD-Rev: 5'-CTAAGAGGTCAATTTTTGAAG-3'
Primers for cloning rpoE' (Gen PF0256) in pET151/D-TOPO
151rpoE'-Fwd: 5'-CACCATGTACAAGATAGTCACCGTAAAGGA-3'
151rpoE'-Rev: 5'-TCACTTCTTACCCTCCTCCTTTTCTTTCTT-3'
Primers for cloning rpoF (Gen PF1036) in pET151/D-TOPO
151rpoF-Fwd: 5'-CACCATGATAGGGAGAGGCAAACTCGGGGA-3'
151rpoF-Rev: 5'-TCACTCTCTATACTTGTCAAGGATATCTAA-3'
Primers for cloning rpoH (Gen PF1565) in pET151/D-TOPO
151rpoH- Fwd: 5'-CACCGTGGCGGGGAAAAAGGAATTTAGCATA-3'
151rpoH-Rev: 5'-TTAGTCTTCAACAACAACCCTATAGTAGTAGT-3'
Primers for cloning rpoK (Gen PF1642) in pET151/D-TOPO
151rpoK-Fwd: 5'-CACCATGTTCAAGTATACGAGGTTTGAGAAA-3'
151rpoK-Rev: 5'-TCAGCTCGGTCTGATTACTGTTATTGGGATT-3'
Primers for cloning rpoN (Gen PF1643) in pET-30a-c(+)
RpoN-Fwd-Nde I: 5'-GGAATTCCATATGGGGGCAAGTCCCGTGATTG-3'
RpoN-Rev- Bam HI: 5'-CGGGATCCTCAATACACTCTGTAATGCA-3'
Primers for cloning rpoL (Gen PF0050) in pET-30a-c(+)
RpoL-Fwd-Nde: 5'-GGAATTCCATATGAAGATAGAAGTGATAAAGA-3'
RpoL-Rev-Bam HI: 5'-TTGGATCCTCAGCTCTTCACCGCCTTCTCC-3'
Primers for cloning rpoE" (Gen PF0255) in pET151/D-TOPO
151rpoE''-Fwd: 5'-CACCGTGAGTGAAAAAGCCTGCAGACACTGT-3'
151rpoE''- rev: 5'-TCAGCGCACCCTTATGGCATATTTTCCTGGGACT-3'
Sequencing primers
T7-Promoter: 5'-TAATACGACTCACTATAGGG-3'
T7-Terminator: 5'-GCTAGTTATTGCTCAAGCGG-3'
Sequencing primers for B, A', A" and D subunits were a gift from Dr. Bernd Goede.

Primers for generating internal deletions

BΔFork1

Fork1-FwdA: 5'-CACCATGAGAGGTCCGACTGTTGTAGATGTTACTCCCG-3'
Fork1-RevB: 5'-GCAACTGACTAGCTCCAGTTGCAAGAGCATGTTCAATTCT-3'
Fork1-FwdC: 5'-AACTGGAGCTAGTCAGTTGCTAGATAGAACTAACTACATG-3'
Fork1-RevD: 5'-TCACACCCTCTCTGAGAGGTTTAACTTAGGTCTAATAACC-3'

BΔFork2

Fork2-FwdA: 5'-CACCATGAGAGGTCCGACTGTTGTAGATGTTACTCCCG-3'
Fork2-RevB: 5'-GAAGGTCTCTAGACGTGACTCTTCTAAGGTGGGATAATGT-3'
Fork2-FwdC: 5'-AGTCACGTCTAGAGACCTTCACGGAACTCACTGGGGAAGA-3'
Fork2-RevD: 5'-TCACACCCTCTCTGAGAGGTTTAACTTAGGTCTAATAACC-3'

A'ΔRudder

Rudder-FwdA: 5'-CACCATGAAAAAAGTTATTGGAAGTATTGAGTTTGGC-3'
Rudder-RevB: 5'-TCTGAGCTAGAATGTAAGTTGTAACGTGATACTGCAAGAG-3'
Rudder-FwdC: 5'-AACTTACATTCTAGCTCAGAGACTTAAAGGAAAAGAAGGT-3'
Rudder-RevD: 5'-TCACACCTTCGCCTTGTTATTTCCTCTCATCTTTA-3'

A'ΔLid

Lid-FwdA: 5'-CACCATGAAAAAAGTTATTGGAAGTATTGAGTTTGGC-3'
Lid-RevB: 5'-AAGTCTCTAGCAGTTACTGGGGGAACAGGCAAAACT-3'
Lid-FwdC: 5'-CTAGAGACTTAACTCATAAACTTGTTGACATAATAAGGATAA-3'
Lid-RevD: 5'-TCACACCTTCGCCTTGTTATTTCCTCTCATCTTTA-3'

Primers for EMSA template

Gdh-Fwd: 5'-ATAAACAAAAGGATTTCCACTCTTGTTTAC-3'
Gdh-Rev: 5'-CTCAACCATGTTCATCCCTCCAAATTAGG-3'

III Methods

All molecular biology and biochemical methods that are not mentioned here were performed according to Sambrook & Russel (2001) or Coligan et al. (1995).

1. Purification of endogenous *Pfu* RNAP

Pfu cells were grown in a synthetic sea-water-medium under anaerobic conditions at 95°C in a 100 l fermenter as described (Fiala and Stetter, 1986). Cells were harvested during exponential growth phase and collected by centrifugation. All RNAP purification steps were performed at 4°C. Frozen *Pfu* cell paste (100 g) was resuspended in 200 ml ice-cold solubilization buffer (1/2 w/v) by stirring overnight. Cells were homogenized for 3 x 30 s at the maximum speed with an Ultraturrax T-25 grinder (Janke & Kunkel. IKA, Staufen, Germany) and mechanically lysed with a French pressure cell (American Instruments Company) operating twice at 1200 p.s.i. After confirmation of cell lysis by microscopy and centrifugation at 100,000 x g for 1.5 h at 4°C, the supernatant was dialyzed (12-kDa molecular weight cut-off tubing, Serva) against biorex+100 buffer for 2 h. The sample was then loaded onto a 400 ml BioRex70 XK50 column (pre-equilibrated with Biorex+100 buffer) and eluted with a 3 column volumes (CV) linear gradient from 0 to 100% Biorex+1000 buffer. Fractions were analyzed by a promoter-independent *in vitro* transcription assay and fractions containing DNA-dependent RNAP activity were pooled and dialyzed against TMK+100 buffer. The sample was then loaded onto a 20 ml heparin-Sepharose column (HiPrep 16/10 Heparin FF) pre-equilibrated with TMK+100 buffer. Bound proteins were eluted with a 15 CV linear gradient from 0 to 100% TMK+800 buffer. Pooled fractions containing RNAP activity were dialyzed against TMK+100 buffer and applied to a pre-equilibrated 10 ml 15Q Source column (strong anion exchanger). RNAP was eluted with 20 CV linear gradient from 0 to 60% TMK+800 buffer. The eluted fractions were analyzed by 8-20% gradient SDS-PAGE. Fractions containing the RNAP were pooled and diluted with TMK+0 buffer to a final salt concentration of 100 mM KCl. Final purification was achieved on a 1 ml Mono Q 5/50 GL column pre-equilibrated with TMK+100 buffer. RNAP was eluted with 25 CV linear gradient from TMK+100 buffer to 50% TMK+800 buffer. As judged by SDS-PAGE, fractions from the last MonoQ column were containing concentrated and highly pure RNAP. Enzyme concentration was measured by Bradford assay (Bradford, 1976), with BSA as standard. The concentrated RNAP was diluted in RNAP storage buffer to 100 ng/µl concentration, divided in 100 µl aliquots and stored at -80°C.

Solubilization buffer: 50 mM Tris-HCl pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, 20 % v/v glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol, a half Complete protease inhibitor tablet

Biorex+100 buffer: 40 mM K-Hepes pH 7.8, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 20 % v/v glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol, a half Complete protease inhibitor tablet (*Roche Diagnostics, GmbH, Mannheim)*

Biorex+1000 buffer: 40 mM K-Hepes pH 7.8, 1 M KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 20 % v/v glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol, a half Complete protease inhibitor tablet

TMK+0 buffer: 50 mM Tris-HCl pH 7.3, 2.5 mM $MgCl_2$, 10 % v/v glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol

TMK+100 buffer: 50 mM Tris-HCl pH 7.3, 100 mM KCl, 2.5 mM $MgCl_2$, 10 % v/v glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol

TMK+800 buffer: 50 mM Tris-HCl pH 7.3, 50 mM KCl, 2.5 mM $MgCl_2$, 10 % v/v glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol

RNAP storage buffer: 10 mM Tris-HCl, pH 8.0, 350 mM KCl, 0.1 mM EDTA, 0.1 mg/ml bovine serum albumin (BSA), 10 % v/v glycerol

2. Preparation of competent bacterial cells

To increase the efficiency of plasmid DNA uptake (transformation), bacteria were treated with divalent cation solutions. An LB plate was first streaked with cells from a bacterial stock and incubated overnight at 37°C. Multiple colonies were inoculated in 50 ml LB medium and grown at 37°C until the absorbance at 600 nm reached 0.35-0.4. Bacteria were pelleted by centrifugation at 4,500 x g, 5' at 4°C. Supernatant was discarded and cells were gently re-suspended in 1 volume (of the original culture volume) of ice-cold 100 mM $MgCl_2$. Bacteria were centrifuged, as above described, and re-suspended in 0.5 volume of the original culture volume with ice-cold 100 mM $CaCl_2$. After centrifugation and re-suspension of the cells in sterile pre-chilled 2 ml storage buffer by swirling, aliquots of 100 µl were snap-frozen on dry ice and stored at -80°C.

Storage buffer: 10 mM Tris-HCl pH 8.0, 100 mM $CaCl_2$, 15% v/v glycerol

3. Cloning strategies

The open reading frames (ORFs) encoding the *Pfu* RNAP subunits were amplified by PCR from *Pfu* genomic DNA. Oligonucleotides corresponding to the 5' and the 3' end of the ORFs were designed with either restriction sites or the sequence CAAC at the 5' end of the forward primer. *Pfu* genomic DNA was a kindly gift from Dr. Sonja Koning and Dr. Gudrun Vierke.

Table 2. Expression plasmids and restriction enzymes used for cloning

Subunit	**Expression plasmid**	**Restriction enzymes used**	**Fusion-tag**
B	pET-33b(+)	*Nhe* I and *Not* I	PKA and His_6
B, A', A", D, E', F, H, K, E"	pET151/D-TOPO	-	His_6
L	pET-30a-c(+)	*Nde* I and *Bam* HI	-
N	pET-30a-c(+)	*Nde* I and *Bam* HI	-
P*	pET-17b	*Nde* I and *Bam* HI	-

* The construct pET-17b containing the ORF for P subunit was a kind gift from Dr. Bernd Goede.

PCR product corresponding to *Pfu* largest subunit B, was cloned into the expression vector pET-33b(+) containing PKA site in order to radiolabel this protein for Far Western Blot experiments (performed by Karin Ilg; Goede et al., 2006).
For the RNAP reconstitution experiments, PCR products were cloned into pET-30a(+) (subunits L and N) or pET151/D-TOPO (subunits B, A', A", D, E', F, H, K and E").

3.1 DNA digestions and ligations

PCR products corresponding to *Pfu* subunits B, L and N were digested with the enzymes *Nhe*I/*Not*I, *Nde*I/*Bam*HI and *Nde*I/*Bam*HI, respectively. The cloning vectors pET-33b(+) and pET-30a-c(+) (see table 2) were digested with the same enzymes under the reaction conditions suggested by the manufacturer (*NEB*). After digestion, the DNA products were run on agarose gel and purified with QIAquick gel extraction kit. Ligation of the digested PCR product into the expression vector was performed with the Quick Ligation Kit.
PCR products for subunits B, A', A", D, E', F, H, K and E" were directly ligated into pET151/D-TOPO.

3.2 Construction of B and A' mutants with site directed mutagenesis

To study the function of specific amino acids in the largest subunits B (fork 1 and fork 2 domains) and A' (rudder and lid domains), internal deletions were introduced by PCR-fusion (Fig. 8)

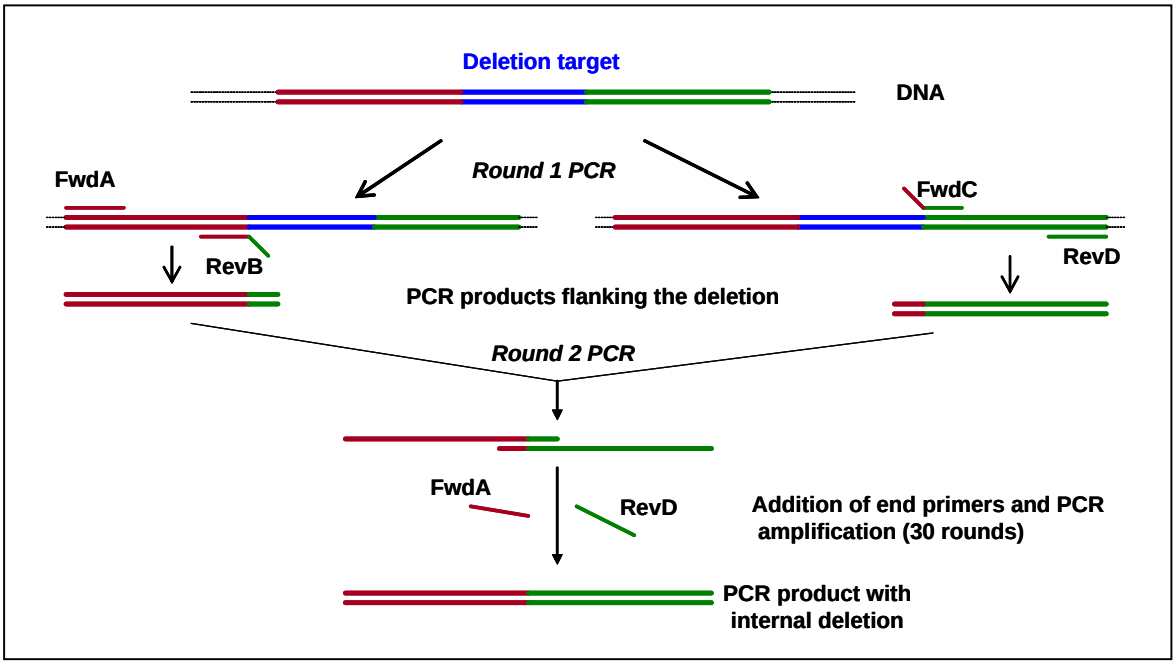


Figure 8. Schematic representation of the strategy for deletion mutagenesis by PCR-fusion. The sequence to be deleted is shown in blue. FwdA and RevD are end primers; RevB and FwdC represent primers flanking the deletion.

For construction of BΔFork1, BΔFork2, A'ΔRudder and A'ΔLid, deletion mutagenesis was performed by overlap extension PCR using a two-round, four-primer technique. In *round 1*, two PCR products were generated in separate reactions. Each PCR reaction was carried out using genomic DNA as template, an end primer (FwdA or RevD) and a primer flanking the internal deletion sequence (RevB or FwdC). The resulting products were analyzed by agarose gel electrophoresis and purified using the QIAquick® spin Gel Extraction Kit.
Purified segments were added jointly for *round 2* of PCR. Fusion of the two intermediate segments was achieved as a result of overlapping complementary regions in the products produced in *round 1*. These products paired during the annealing phase of PCR *round 2* and were amplified by addition of end primers (primers FwdA and RevD). After analyzing PCR products by agarose gel electrophoresis, the resulting mutant insert was purified with the QIAquick® spin Gel Extraction Kit, and ligated into pET151/D-TOPO.

3.3 Bacterial Transformation

The *E. coli* strain JM109 was used as a host for standard sub-cloning and production of large amounts of cloned DNA. 2 µl of ligation mixture was added to 40 µl of competent cell suspension and incubated on ice for 20 min. Cells were then subjected to heat shock at 42°C for 50" and incubated again on ice for 2 min. 400 µl of LB recovery medium was added to cells and incubated at 37°C for 1 h to allow the expression resistance genes conferred by the plasmid. 100 µl of suspension was plated onto LB agar plates containing the appropriate antibiotics and grown at 37°C overnight.

3.4 Screening of transformants

Positive transformants were analyzed by PCR. For this purpose, 20 colonies were picked and grown overnight at 37°C in 5 ml LB medium containing the appropriate antibiotics. 25 µl of each overnight culture were centrifuged. Cell pellets were resuspended in no salt lysis buffer and heated at 95°C for 10 min. After centrifugation, 1 µl of the supernatant, which contained plasmid DNA, was used as a template in PCR reactions. T7 promoter and T7 terminator were used as primers and PCR products were visualized by agarose gel electrophoresis. Plasmids containing positively identified inserts were purified with QIAquick® spin Miniprep Kit and verified by sequencing (*Geneart*). No nucleotide mutations in the coding region of full-length subunits or any deletional constructs were detected.

No salt lysis buffer: 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1 % triton X-100

4. Overexpression of recombinant subunits

For protein overexpression, 1-50 ng of the resultant plasmid was transformed into *Escherichia coli* strain BL21-CodonPlus™ (DE3)-RIL (following the protocol described in 2.2), which contains a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control (Studier et al., 1990). Expression of the subunits under control of strong bacteriophage T7 promoter was induced during cellular logarithmic growth by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.5 mM followed by an overnight incubation at 20°C. The cells were collected by centrifugation for 10 min at 5,000 *g* and washed with washing buffer. After centrifugation, cells pellets were stored at -80°C until use.

Washing buffer: 50 mM Tris-HCl pH 8.0, 0.1mM EDTA, 5 % v/v glycerol, 0.5 mM PMSF, 5 mM β-mercaptoethanol

5. Recombinant subunit purification

5.1 Purification of insoluble subunits

The his_6-tagged subunits B, A', A'' and K were highly insoluble and expressed in inclusion bodies (IBs) in *E. coli* cells. It was necessary to purify them under denaturing conditions (Fig. 9).

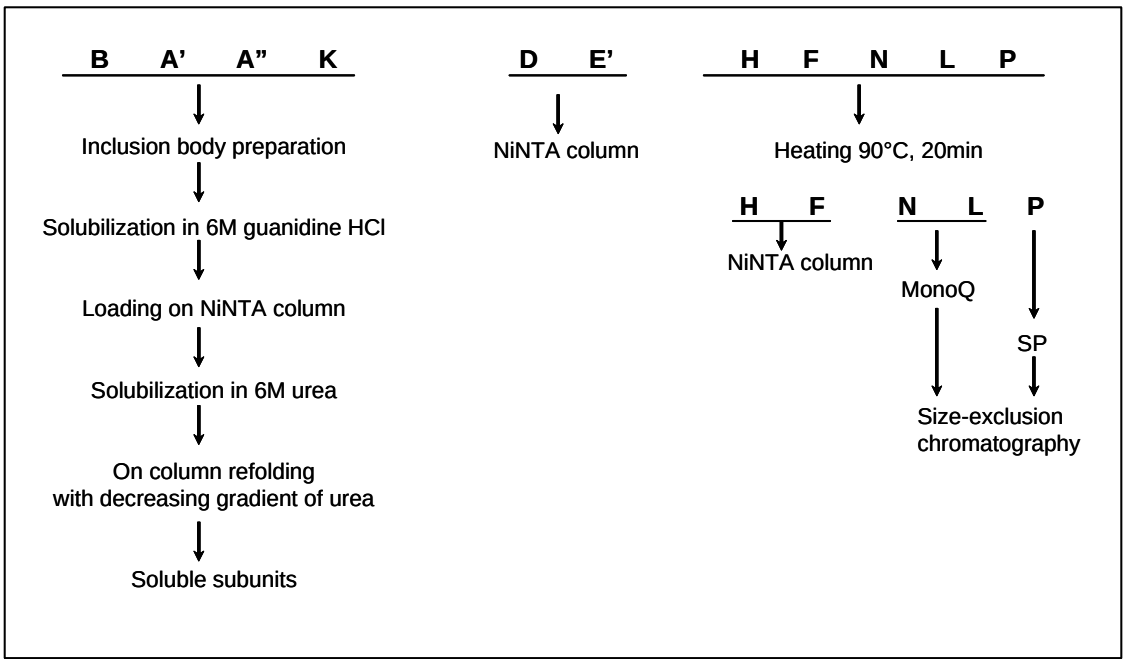


Figure 9. Purification strategies for recombinant *Pfu* RNAP subunits.

The cell pastes were resuspended in IB-lysis buffer, sonicated on ice and centrifuged at high speed for 10 min at + 4°C. The pellets, which contained IBs, were extensively washed with IB-washing buffer, sonicated and centrifuged as above.
Solubilization of IBs was performed first in denaturing-buffer-1 for 1h at room temperature (RT). After centrifugation, the supernatants were passed through a 0.22 μm filter to remove remaining particles, then loaded on nickel-nitrilotri-acetate-agarose (Ni-NTA) column (HisTrap Chelating HP**)** and further washed with denaturing-buffer-2. Refolding of the bound proteins was performed on column with a decreasing linear urea gradient, from the denaturing-buffer-2 to the refolding buffer. The refolded proteins were eluted with an increasing imidazole gradient, from 10 to 300 mM imidazole in the same refolding buffer. No further purification of these subunits was required, as revealed by SDS-PAGE analysis.

IB-lysis buffer:	20 mM Tris-HCl pH 8.0, 1 mM PMSF, 5 mM β-mercaptoethanol, 0.3 mg/ml lysozyme
IB-washing buffer:	20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 0.1% Tween 20, 1 mM PMSF, 5 mM β-mercaptoethanol
Denaturing-buffer-1:	20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM PMSF, 5 mM β-mercaptoethanol
Denaturing-buffer-2:	20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5 mM imidazole, 6 M urea, 1 mM PMSF, 5 mM β-mercaptoethanol
Refolding buffer:	20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 10 mM imidazole, 1 mM PMSF, 5 mM β-mercaptoethanol

5.2 Purification of soluble subunits

Recombinant RNAP subunits D, E', H, F, N, L or P were overexpressed in a soluble form. Cell pellets were resuspended in lysis buffer, sonicated on ice and centrifuged at high speed for 10 min at +4°C. The supernatants containing soluble his_6-tagged D and E' subunits were loaded on Ni-NTA column (HisTrap Chelating HP) pre-equilibrated with Ni-NTA binding buffer and the proteins were eluted with an imidazole gradient from 10 to 300 mM in Ni-NTA elution buffer.
To exploit the thermostability of some archaeal proteins, the supernatants containing subunits H, F, N, L and P were heated for 20 min at 90°C to precipitate most of *E. coli* proteins. The samples were centrifuged and the supernatants containing the thermostable his_6-tagged subunits F and H were loaded on Ni-NTA column (HisTrap Chelating HP**)** and purified as described above. The supernatants containing the untagged N and L subunits were purified with an anion-exchange (AE) column (Mono Q 5/50 GL) pre-equilibrated with AE-binding buffer. The proteins were eluted with salt gradient from 0 to 100% AE-elution buffer. The supernatant containing the untagged P subunit was purified with a cation-exchange (CE) column (HiTrap SP HP) pre-equilibrated with CE-binding buffer. The proteins were eluted with a salt gradient from 0 to 1000 mM NaCl in CE-elution buffer.
Further purification of the untagged subunits (L, N, and P) by size-exclusion chromatography was necessary. The concentrated proteins were loaded on a Superdex 75 column (HiLoad 16/60 Superdex 75 prep grade) and eluted with superdex buffer.

Lysis buffer: 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 0.1% Tween 20, 15% glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol, 0.3 mg/ml lysozyme

Ni-NTA binding buffer: 20 mM Na-Hepes pH 7.0.8, 0.5 M NaCl, 10 mM imidazole, 10% glycerol, 0.01% Tween 20, 1 mM PMSF, 5 mM β-mercaptoethanol

Ni-NTA elution buffer: 20 mM Na-Hepes pH 7.0.8, 0.5 M NaCl, 300 mM imidazole, 10% glycerol, 0.01% Tween 20, 1 mM PMSF, 5 mM β-mercaptoethanol

AE-binding buffer: 20 mM Tris-HCl pH 8.0, 10 mM NaCl, 10 % v/v glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol

AE-elution buffer: 20 mM Tris-HCl pH 8.0, 1 M NaCl, 10 % v/v glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol

CE-binding buffer: 40 mM Na-Hepes pH 7.0, 10 mM NaCl, 10 % v/v glycerol,1 mM PMSF, 5 mM β-mercaptoethanol

CE-elution buffer: 40 mM Na-Hepes pH 7.0, 1 M NaCl, 10 % v/v glycerol,1 mM PMSF, 5 mM β-mercaptoethanol

Superdex buffer 40 mM Na-Hepes pH 7.3, 250 mM NaCl, 2.5 mM $MgCl_2$, 10 % v/v glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol

6. *In vitro* reconstitution of recombinant RNAP and RNAP sub-complexes

The subunits were incubated together in equimolar ratios, to a total protein amount of about 500 µg in a final volume of 1 ml (unless otherwise indicated). The mixture was transferred to a dialysis frame and denaturated in reconstitution buffer (RB) containing 6 M urea, for 20 min at RT. The dialysis frame with the denaturated subunits was then transferred into TB containing 3 M urea for 20 min. Renaturation was achieved by dialysis against TB for one hour. The renatured RNAP or sub-complexes were heated for 10 min at 70°C, centrifuged in order to remove misfolded aggregates and loaded on an analytic Superdex 200 (Superdex 200 10/300 GL) or on an analytic Superose 6 (Superose 6 10/300 GL).

Reconstitution buffer: 40 mM Na-Hepes pH 7.3, 250 mM NaCl, 2.5 mM $MgCl_2$, 10 % v/v glycerol, 0.1 mM EDTA, 100 µM $ZnSO_4$; 1 mM PMSF, 5 mM β-mercaptoethanol

7. Promoter-Independent *in vitro* Transcription Assays

These assays take advantage of the fact that all RNAPs are capable of initiating transcription from 3' overhangs and nicks in double-stranded DNA templates independent of promoter sequences and in the absence of general transcription factors. Non-specific transcriptional activity was measured by the incorporation of α-^{32}P-UTP into trichloroacetic acid-insoluble material. Standard reactions were performed in transcription buffer (TB) in a total volume of 100 µl containing 100 ng *Pfu* RNAP (or mutant derivative), 900 µM ATP, 90 µM UTP and 5 µM [α-^{32}P] UTP (at 40 Ci/mmol) using 3 µg of poly (dA-dT) as template. Reactions were incubated for 30 min at 70°C and stopped by the addition of 1 ml 5% TCA, 160 mM NaCl. The trichloroacetic acid precipitates were collected on GF/F glass fiber filters and measured in a scintillation counter (TRI-CARB 2900TR, Packard).

Transcription buffer: 40 mM Na-Hepes, pH 7.3, 250 mM NaCl, 2.5 mM $MgCl_2$, 5 % v/v glycerol, 0.1 mM EDTA, 1 mM PMSF, 5 mM β-mercaptoethanol and 0.1 mg/ml bovine serum albumin (BSA)

8. Promoter-Directed *in vitro* Transcription Assays

In vitro transcription assays were performed as described (Hethke *et al*., 1996). 300 ng *gdh* promoter (*Xba*I-digested plasmid pUC19 containing the *Pfu glutamate dehydrogenase* promoter sequence from -95 to +163) was incubated with 42 nM TBP, 55 nM TFB and 9 to 45 nM endogenous or recombinant RNAP (or mutant derivative) in 25 µl TB. NTPs were added to 500 µM ATP, GTP, CTP and 10 µM [α-^{32}P] UTP at 40 Ci/mmol. The reaction mixtures were prepared at 4°C. Transcription was started by temperature shift to 70°C (unless indicated otherwise). After 30 min, transcription was stopped by the addition of an equal volume of deionized formamide containing bromophenol blue and xylene cyanol (each at 1 mg/ml). Transcription products were analyzed by electrophoresis in 6% polyacrylamide urea gels in 1 x TBE and visualized by phosphoimaging (FLA-500, Fuji, Japan).
To study the contribution of RNAP specific amino acids to the activity and to the maintenance of strand separation, different promoter DNA constructs were designed by Dr. Michela Bertero (Prof. Cramer's laboratory, University of Munich).
Transcription assays with these constructs as templates (Fig. 10) were performed as above described. The transcription products were separated on a 28% denaturing polyacrylamide gel and visualized by phosphoimaging.

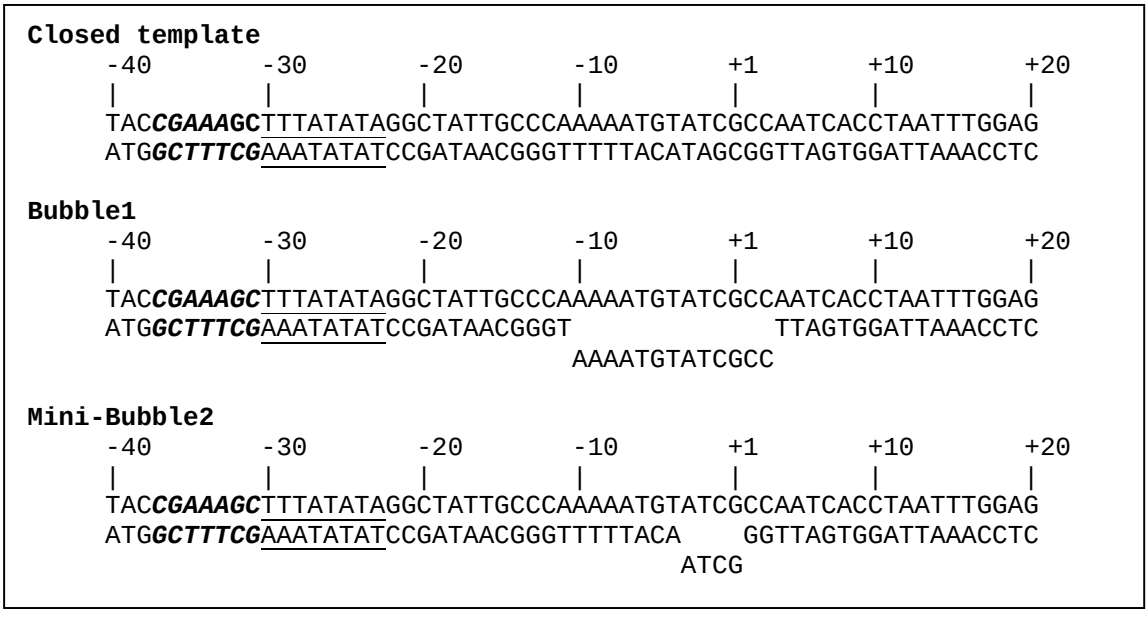


Figure 10. Sequences of heteroduplex template constructs based on the *gdh* promoter and containing a mismatched bubble. The TATA box is underlined, the B-recognition element is indicated by bold italic type and the transcription start site is indicated by +1

9. Electrophoretic Mobility Shift Assays (EMSAs)

The DNA template, spanning the *Pfu gdh* promoter and extending from bp -60 to +37, was obtained by PCR using Gdh-Fwd and Gdh-Rev primers, and genomic *Pfu* DNA as template. The PCR product was purified with PCR purification kit and ^{32}P end-labeled with [γ-^{32}P]ATP and T4-Polynukleotide kinase. The probe was purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. Additional purification of the probe with QIAquick Nucleotide Removal Kit (*QIAgen*) was necessary to remove non-incorporated [γ-^{32}P]ATP.
Protein-DNA complexes were assembled in a 25 µl EMSA-buffer, containing 1 µg poly[d(IC)] as nonspecific competitor DNA, with 42 nM TBP, 55 nM TFB and 0.5 nM *gdh* promoter. The samples were incubated at 70°C for 20 min in a thermocycler to allow the formation of DNA-TBP-TFB complex. 9 nM endogenous or 27 nM recombinant RNAP (or mutant derivative) was added and the samples were incubated at 70°C for 20 min. 10 µl aliquots were analyzed by 4% polyacrylamide native gel electrophoresis at RT for 2 h at 200 V and visualized by phosphoimaging.

EMSA-buffer: 40 mM Na-Hepes, pH 7.0.3, 250 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 5 mM β-mercaptoethanol, 5 % v/v glycerol, 0.1 mg/ml BSA
Running buffer: 0.5xTBE, 4% v/v glycerol and 0.5 mM β-mercaptoethanol

10. Western-blotting and immunodetection of proteins

Western-blotting technique involves transfer and immobilization of proteins, previously resolved by SDS-PAGE onto a membrane, and their detection with specific antibodies. Proteins were separated by gradient SDS-PAGE (8-20%) and transferred to a PVDF membrane, using a semidry blot apparatus (Bio-Rad). After SDS-PAGE, the gel, a PVDF membrane (pre-washed in methanol and water) and 4 pieces of Whatman 3MM filter paper were soaked in transfer buffer. A sandwich was assembled in the following order, from the anode to the cathode: two filter layers, membrane, gel and two filter layers. Transfer was achieved applying a current of 9V for 45 min. Following transfer, PVDF membrane was saturated overnight in blocking buffer, before incubation with primary antibody (diluted to 1/3000 in blocking buffer) for 1 h at RT. The membrane was then washed three times with blocking buffer to remove excess of primary antibody. A phosphatase-conjugated, secondary antibody, specific for the isotype of the primary antibody, was applied to the membrane, diluted in blocking buffer, for 1 h, at RT. Excess antibody was removed by washing three times in blocking buffer and three times in assay buffer. CDP-Star was used as substrate for the alkaline phosphatase enzymatic reaction, following the manufacturer's protocol. Protein-antibody complexes were detected on X-ray films.

Transfer buffer: 25 mM Tris-HCl pH 8.0, 192 mM glycine, 20% methanol
Blocking buffer: 1 x PBS, 0.1% Tween-20, 10% skim milk
Assay buffer: 100 mM Tris-HCl pH 8.0,1 mM $MgCl_2$

11. *Pfu* RNAP auto-phosphorylation

In order to study phosphorylation of *Pfu* RNAP subunits, the purified endogenous enzyme was incubated at 70°C in phosphorylation buffer containing 1 μCi [γ-^{32}P]ATP. The samples were incubated at 70°C for 30 seconds to 20 min in a thermocycler to allow the phosphorylation of RNAP subunits. The phosphorylated enzyme was analyzed by gradient SDS-PAGE (8-20%) and visualized by phosphoimaging.

Phosphorylation buffer: 20 mM K-Hepes, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 250 mM K-Acetat

IV Results

1. Purification of endogenous *Pfu* RNAP and its recombinant subunits

Endogenous *Pfu* RNAP was purified by four successive chromatographic steps: cation-exchanger (Biorex 70 column), affinity (Heparin column), anion exchanger (15Q Source column) and a second anion exchanger (Mono Q column), which resulted in the electrophoretically homogeneous enzyme.
In order to reconstitute *in vitro Pfu* RNAP, all its subunits were individually overexpressed in *E. coli*. The largest subunits B, A' and A'' and the small subunit K were found in inclusion bodies and therefore were purified under denaturing conditions. The other subunits were overexpressed in a soluble form in *E. coli* cells and purified under non-denaturing conditions. Subunits B, A', A", D, E', F, H, K and E" were overexpressed as fusion proteins carrying a His_6 tag at the N-terminus. They were purified by Ni-NTA agarose affinity chromatography. L, N and P subunits did not contain any fusion tag and were purified by ion-exchange chromatography. Purified recombinant subunits and endogenous RNAP were analyzed by SDS-PAGE. Figure 12 shows the homogeneity of these proteins.

Table 3. Properties of the ORFs that encode *Pfu* RNAP subunits

SU	ORF	Protein length (amino acids)	Protein mass (kDa)	pI
B	PF1564	1117	127.0	6.7
A'	PF1563	910	103.4	6.8
A"	PF1562	397	44.4	5.8
D	PF1647	261	29.8	4.7
E'	PF0256	189	21.7	7.7
F	PF1036	120	14.1	4.6
L	PF0050	95	11.1	5.1
H	PF1565	82	9.2	9.7
N	PF1643	70	8.2	6.5
E"	PF0255	61	6.7	7.2
K	PF1642	57	6.2	10.3
P	PF2009	49	5.8	10.7

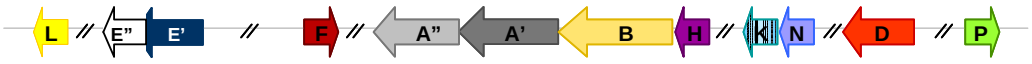


Figure 11. A schematic representation of ORFs location on the *Pfu* genome coding RNAP subunits. ORFs are indicated by arrows and are colored according to the color coding used in this work (Fig. 2). Arrows indicate the direction of genes in the genome. PF numbers for ORFs coding RNAP subunits are shown in table 3, as annotated by GenBank. Overlapping ORFs (PF0255 and PF256 coding E" and E', respectively) are indicated by the overlapping nature of the arrows. // indicate gaps between the ORFs.

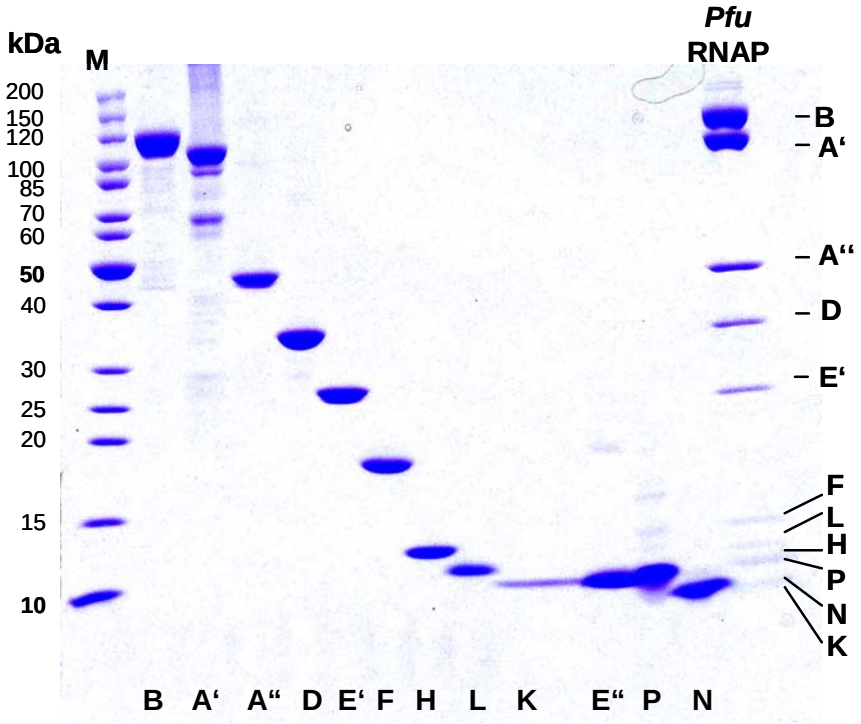


Figure 12. Cloned and bacterially expressed subunits of *P. furiosus* RNAP used for *in vitro* reconstitution of RNAP or RNAP sub-complexes. Endogenous *Pfu* RNAP and its recombinant purified subunits were separated on a 8-20% polyacrylamide gradient gel under denaturing conditions and stained with Coomassie Blue. Subunits B, A', A", D, E', F, H, K and E" contained a His_6 tag at the N-terminus. Therefore, the molecular mass of these subunits is higher than the molecular mass of subunits of the endogenous enzyme. L, N and P subunits did not contain any fusion tag. M: protein marker.

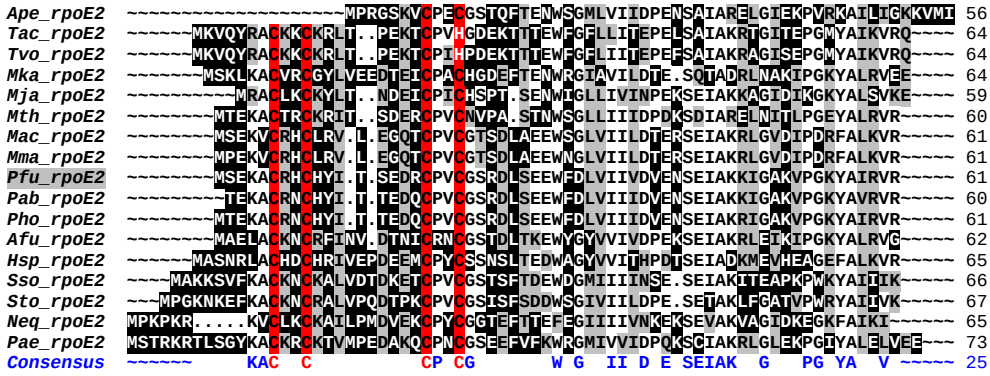
Table 4. Yield of purified endogenous *Pfu* RNAP and recombinant *Pfu* RNAP subunits

Protein	Endogenous RNAP	B	A'	A"	D	E'	F	H	L	K	P	N	E"
Yield (mg protein/g cells)	0.15-0.2	10	5	9	15	15	15	15	3	10	4	3	10

2. Is E" a subunit of *Pfu* RNAP?

In most archaeal genomes, the gene coding for the E subunit overlaps at its 3´-end with a different reading frame containing a second gene encoding a protein with a Zinc-finger motif (Fig. 11). To distinguish between the first gene, that is homologous to *rpb7*, and the second gene that has no homolog in yeast but is highly conserved among Archaea (Fig. 13A), the first one is designated in databases as *rpoE´* (PF0256) and the second one as *rpoE´´* (PF0255), and the corresponding proteins as E´ and E´´, respectively.
In order to determine whether E" is a subunit of *Pfu* RNAP, Western blot assay with antibodies directed against E" was performed. E" was not detected as a subunit of endogenous *Pfu* RNAP (Fig. 13B). This protein, however, was expressed in *Pfu* cells and was detected in *Pfu* cells harvested during exponential or stationary growth phase.

A



B

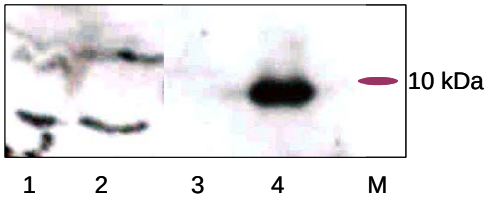


Figure 13. E" is not a subunit of *Pfu* RNAP. **(A)** Sequence alignment of E" within Archaea. The sequences of *Aeropyrum pernix*, *Thermoplasma acidophilum*, *Thermoplasma volcanium*, *Methanopyrus kandleri AV19*, *Methanocaldococcus jannaschii*, *Methanothermobacter thermautotrophicus*, *Methanosarcina acetivorans C2A*, *Methanosarcina mazei Goe1*, *Pyrococcus furiosus DSM 3638*, *Pyrococcus abyssi*, *Pyrococcus horikoshii*, *Archaeoglobus fulgidus*, *Halobacterium sp. NRC-1*, *Sulfolobus acidocaldarius*, *Sulfolobus solfataricus*, *Sulfolobus tokodaii*, *Nanoarchaeum equitans Kin4-M* and *Pyrobaculum aerophilum* are shown. The alignment was performed using Clustal W. The Zinc-finger motif is highlighted in red. Invariant, conserved and variant residues are highlighted in white, gray and black, respectively. Gaps introduced to maximize alignment are indicated by dashes. **(B)** Western blot analysis with specific antibodies directed against E" subunit. Lanes 1 and 2, Extracts of cells harvested during exponential or stationary growth phase, respectively; lane 3, endogenous purified *Pfu* RNAP; lane 4, recombinant purified E"; M, protein marker.

3. *In vitro* assembly of *Pfu* RNAP subunits

The 11 purified subunits were incubated together in equimolar ratios and subjected to a stepwise dialysis against transcription buffers containing decreasing urea concentrations (6 M, 3 M and 0 M). Subunits denaturation and renaturation was necessary to allow specific protein-protein contacts between different subunits. Exploiting the thermostablility of archaeal enzymes, misfolded assemblies were removed by heat treatment and centrifugation. The assembled complexes were separated by Superdex 200 (Fig. 14) or Superose 6 (Fig. 15) column chromatography.
Results from size-exclusion-chromatography indicates that the subunits indeed coeluted as a complex with a molecular weight of about 420 kDa, which corresponds to the MW of the recombinant *Pfu* RNAP (Fig. 14A and Fig. 15A). Superdex 200 size-exclusion-chromatography showed that large aggregates of RNAP with low specific activity were formed (Fig. 14A, B and E, fractions 13-17) overlapping in part the reconstituted enzyme with high specific activity (Fig. 14A, B and E, fractions 18-20). Superose 6 chromatography was used to separate the highly active RNAP (Fig. 15A and B, fractions 28-30) from aggregates (Fig. 15A and B, fractions 15-20).
The reconstituted enzyme contained the subunits B, A, A", D, L, H, K, P and N (Fig. 14B). Subunits E´ and F were not present in stoichiometric amounts. These subunits were visualized in active reconstituted RNAP fractions by Western blotting using polyclonal antisera raised against these two proteins (Fig. 14D). Additionally, gel filtration chromatogram showed that E' and F subunits eluted as a separate complex with an approximate MW of 40 kDa (Fig. 14C). This result supports the information obtained from a high resolution crystal structure of *S. cerevisiae* RNAPII that revealed the existence of a 10 subunits core and a dissociable Rbp7/Rpb4 complex, the eukaryotic homologs of E' and F (Armache et al., 2005).

4. Recombinant RNAP activity in promoter-directed *in vitro* transcription

To determine whether the assembled RNAP displayed any enzymatic activity, specific *in vitro* transcription assays were performed. Fractions containing reconstituted RNAP were diluted to 100 ng/µl final concentration, and 1 µl of each fraction was tested in an *in vitro* transcription assay in the presence of TBP, TFB and *gdh* promoter containing a TATA-box and a BRE element. The results indicated the presence of high RNAP activity in the fractions corresponding to the correctly refolded reconstituted RNAP (Fig. 14E and 15B). These results demonstrate that the recombinant *Pfu* RNAP can perform all the steps required for specific *in vitro* transcription, from promoter recruitment *via* TBP and TFB to promoter escape and elongation.
To quantify the specific activity of the reconstituted RNAP relative to the endogenous RNAP, 9.7 nM endogenous RNAP and reconstituted RNAP were tested in *in vitro* transcription assay in the presence of TBP, TFB and *gdh* promoter containing a TATA-box and a BRE element. The reconstituted RNAP had about 50% of the specific activity of the endogenous enzyme purified from *Pyrococcus* cells (Fig. 16).

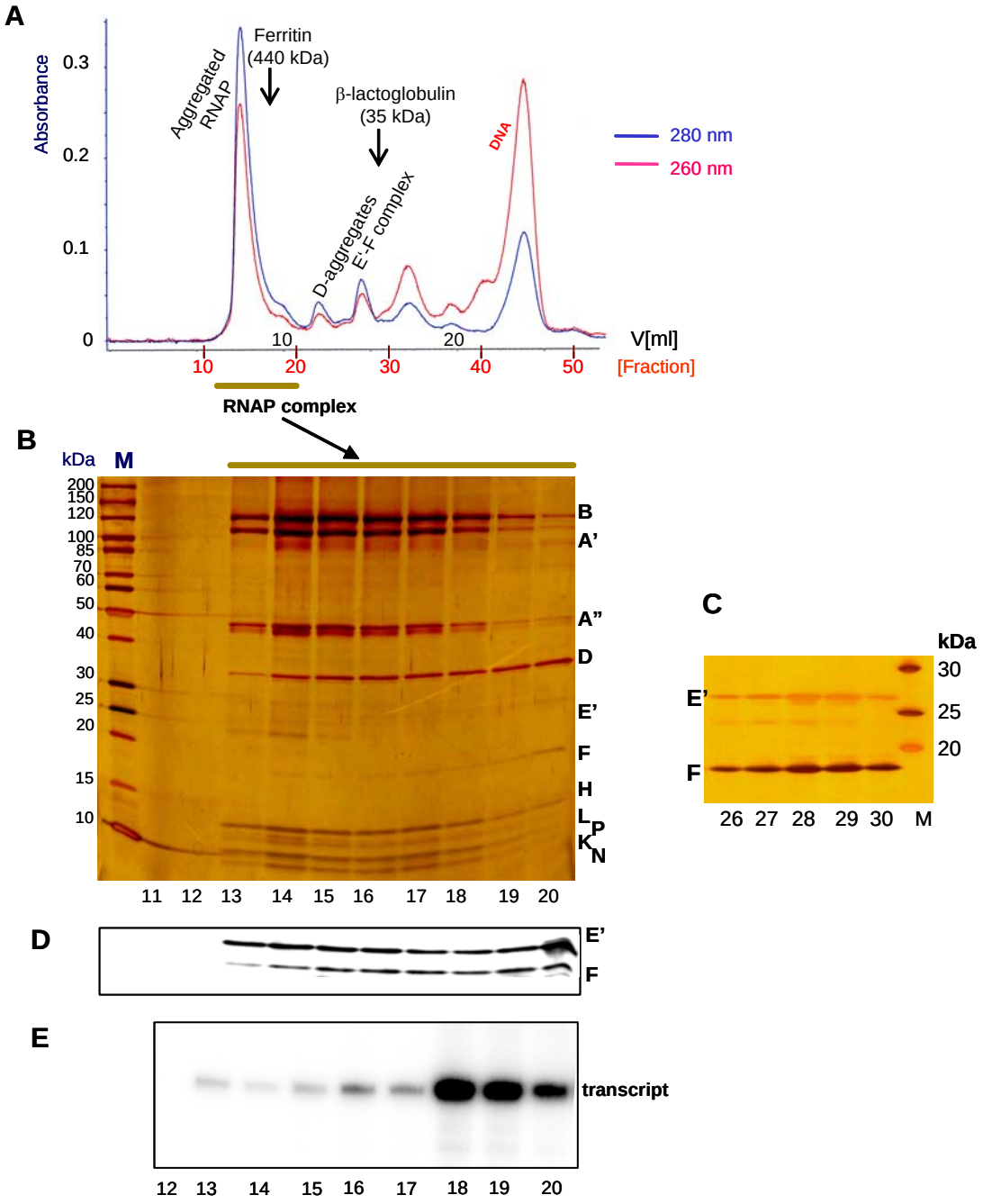


Figure 14. Assembly of *Pfu* RNAP from recombinant subunits using a Superdex 200 size exclusion chromatography. (A) Superdex 200 size-exclusion-chromatography profile of RNAP in *in vitro* reconstitution assay. **(B)** and **(C)** show silver-stained SDS 8-20%-PAGE analysis of Superdex 200 fractions 11 to 20 and 26 to 30, respectively. M, protein marker. **(D)** Western blot analysis with specific antibodies directed against E' and F subunits. **(E)** Promoter-directed *in vitro* transcription assay with 100 ng reconstituted RNAP in the presence of the archaeal transcription fators TBP and TFB.

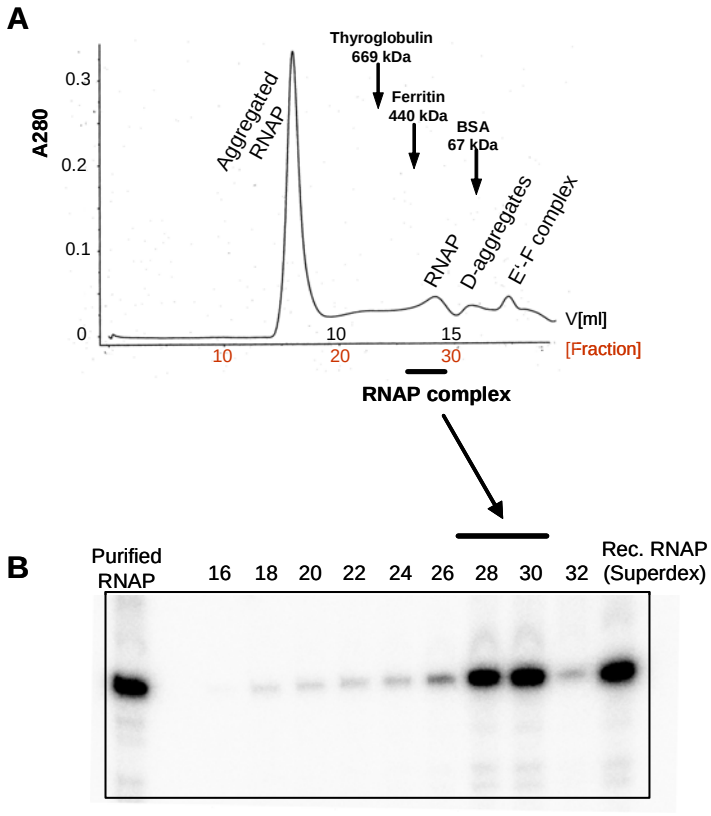


Figure 15. Assembly of *Pfu* RNAP from recombinant subunits using a Superose 6 size exclusion chromatography. (A) Size exclusion chromatography of the reconstituted mixture by a Superose 6 column. The apparent molecular weights of the RNAP and E'/F complex were estimated using gel filtration molecular weight markers, the position of which are indicated by arrows. A_{280}, Absorbance at 280 nm. **(B)** Promoter-directed *in vitro* transcription assay in the presence of the archaeal transcription fators TBP and TFB. 100 ng reconstituted RNAP and 50 ng endogenous RNAP were used in this assay. Activities of the endogenous RNAP and the RNAP reconstituted with a Superdex column are shown.

5. The importance of individual small subunits of *Pfu* RNAP

The availability of a procedure for *in vitro* reconstitution of *Pyrococcus* RNAP enabled to address questions regarding contributions of small subunits to the structure, the stability and the catalytic activity of the enzyme (Fig. 17A). DNA-dependent RNAPs are defined by a conserved core of five subunits homologous to the bacterial β'βα_2ω enzyme. However the archaeal enzyme, like the three eukaryotic RNAPs, contains 6 to 8 additional subunits (Woychik et al., 1990; Goede et al., 2006). These additional subunits bear no counterparts in the well-studied bacterial RNAP. The subunit configuration DLBA'A'' (or DLB'B''A'A'') is the closest possible approximation of an archaeal

RNAP to the minimal bacterial core enzyme α_2 ββ´ (Cramer et al., 2000; Werner and Weinzierl 2002). For these reasons subunits D, L, B, A' and A'' were not omitted when the RNAP was reconstituted from single subunits. To test the functional role of K, RNAP was assembled in the absence of the purified subunit. The RNAPΔK had almost the same levels of promoter specific activity as the complete enzyme. The recombinant RNAPΔN showed only very low activity in promoter-specific transcription assay when subunit N was omitted in reconstitution experiments. The activity of the RNAPΔN was significantly higher when the heat treatment step during the *in vitro* assembly strategy was omitted. This result suggests that N subunit has a function in the enzyme thermostability.

M. jannaschii ΔN enzyme showed a defect in the incorporation of subunit B into the enzyme when exposed to high temperature. Figure 17B shows that subunit B was present in stoichiometric amounts in the reconstituted *Pfu* ΔN enzyme.

RNAPΔP showed no activity. Leaving out H from the recombinant enzyme also abolished transcriptional activity. RNAP reconstituted in the absence of E´ and F showed only slightly reduced activity in standard transcription assay. These results indicate that subunits E', F and K are not essential and N, H and P are essential for the stability and the catalytic activity of the enzyme *in vitro*.

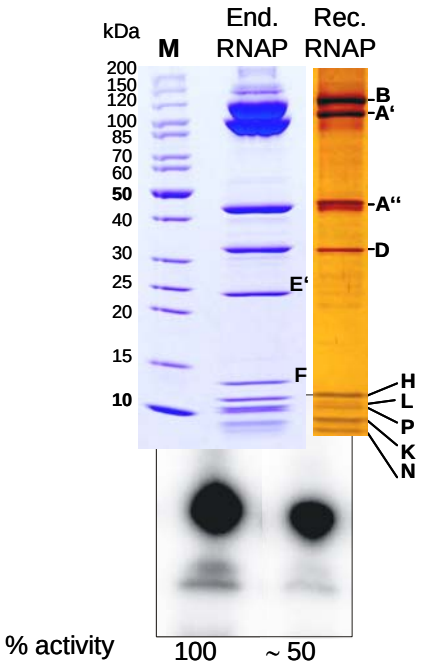


Figure 16. Transcriptional activity of the reconstituted RNAP. Top panel shows SDS-PAGE of coomassie stained endogenous RNAP (End. RNAP) and silver stained reconstituted RNAP (rec RNAP). M, protein marker. Lower panel show the transcriptional activity of both enzymes.

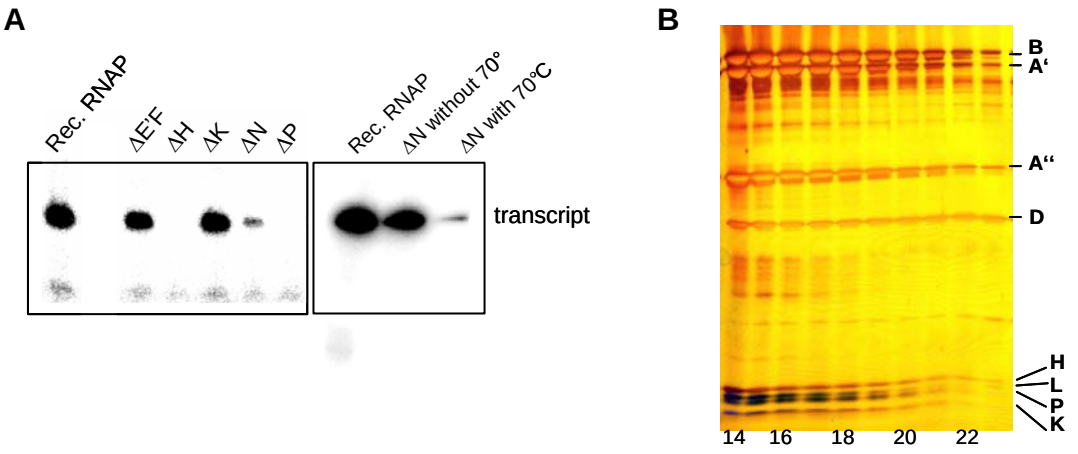


Figure 17. Subunits contribution to RNAP catalytic activity. **(A)** Promoter-directed transcription assays in the presence of different recombinant RNAPs lacking subunits E'-F, H, K, N or P. **(B)** Silver-stained SDS 8-20%-PAGE analysis of RNAPΔN (reconstituted without 70°C step).Superdex 200 fractions 14 to 23 are shown.

6. Functions of F and E' subunits of *Pfu* RNAP

6.1 *In vitro* transcription of RNAPΔE'F

To characterize the contribution of E' and F to the catalytic activity of the enzyme, specific *in vitro* transcription assays using a variety of archaeal promoters as templates were performed. Beside the *gdh* promoter analyzed in figure 17A, the reconstituted RNAPΔE'F was able to synthesize run-off transcripts from linearized templates containing *aaa*$^{+}$*atpase-*, *hsp20-*, *phr-* and *malE* promoter from *Pfu* and tRNA^{Val} promoter from *Methancoccus vannielii* (Fig. 18). These results indicated that the reconstituted RNAPΔE'F is able to initiate transcription at canonical archaeal promoters containing a TATA-box and a BRE element.

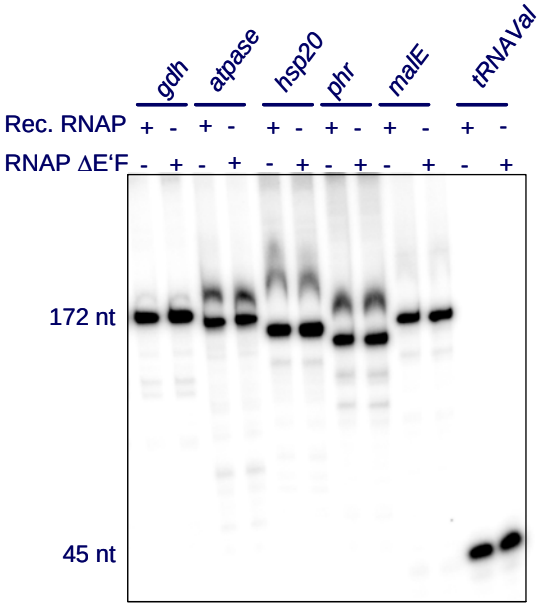


Figure 18. Promoter-directed *in vitro* transcription with reconstituted RNAP and RNAPΔE'F using different archaeal promoters. *Gdh*, *aaa+atpase*, *hsp20*, *phr* and *malE* promoters from *Pfu* and tRNA^{Val} promoter from *Methancoccus vannielii* were used as a template.

6.2 Interactions between E', F and TFE

Similar to the reconstituted *M. jannaschii* enzyme (Werner and Weinzierl, 2004), *Pfu* RNAP reconstituted in the absence of E´ and F showed only slightly reduced activity in standard transcription assay (Fig. 17A and 18). Since preparations of *M. jannaschii* enzyme lacking F/E failed to respond to TFE (Ouhammouch et al., 2004), interactions of *Pfu* E´, F and TFE were studied in more detail. Equimolar amounts of TFE, F and E' were reconstituted following the protocol described in chapter III.6. Superdex 75 chromatography and SDS-PAGE analysis showed the formation of a specific complex

between TFE and F (Fig. 19). Subunit E´ eluted in separate fractions with an apparent lower molecular mass. This finding shows the ability of TFE to form a complex with subunit F. When subunits E' and F were denatured and renatured in the absence of TFE, a specific E'/F complex was formed (Fig. 20). This finding demonstrates that TFE completely replaces E´ from the E´/F complex and has a high affinity to subunit F.

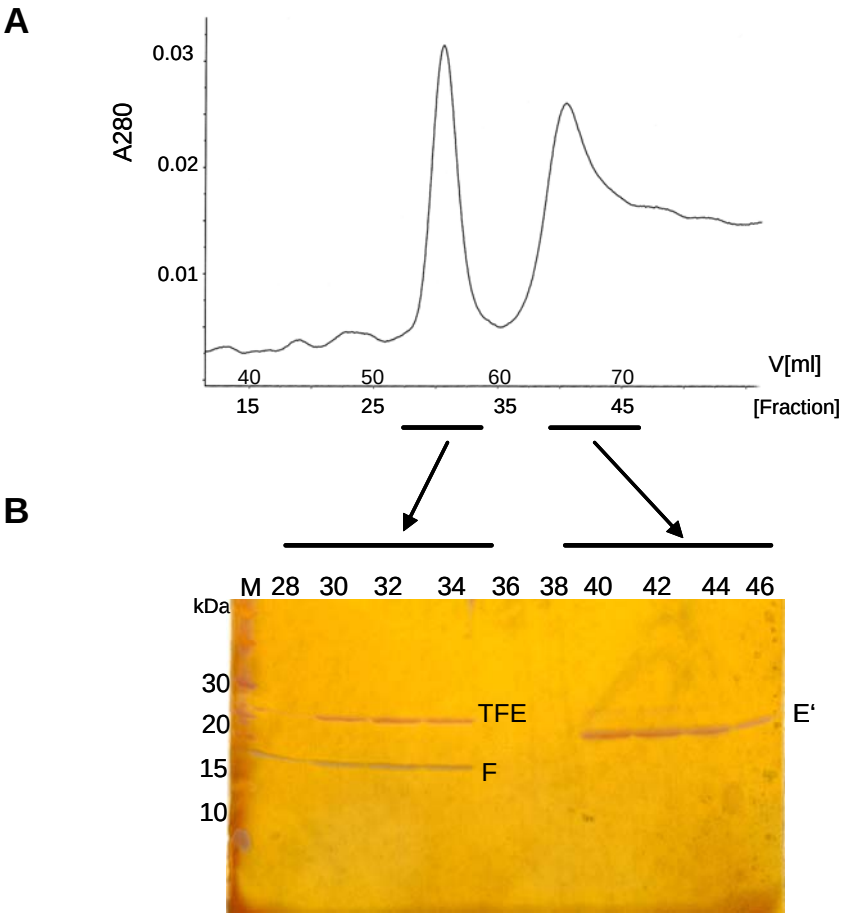


Figure 19. *In vitro* reconstitution of TFE, E' and F. (A) Superdex 75 size exclusion chromatography profile of assembled proteins. **(B)** Silver-stained SDS 8-20%-PAGE analysis of Superdex 75. Fractions 28 to 46 are shown. M, protein marker

6.3 Interactions between E', F and E"

To investigate whether E" binds to E'/F sub-complex, *in vitro* reconstitution of these proteins was performed following the protocol described in chapter III.6. Superdex 75 chromatography and SDS-PAGE analysis showed a specific complex consisting of E' and F (Fig. 20). Subunit E" eluted in separate fractions with an apparent lower molecular mass. This finding demonstrated that E" does not bind to E'/F sub-complex.

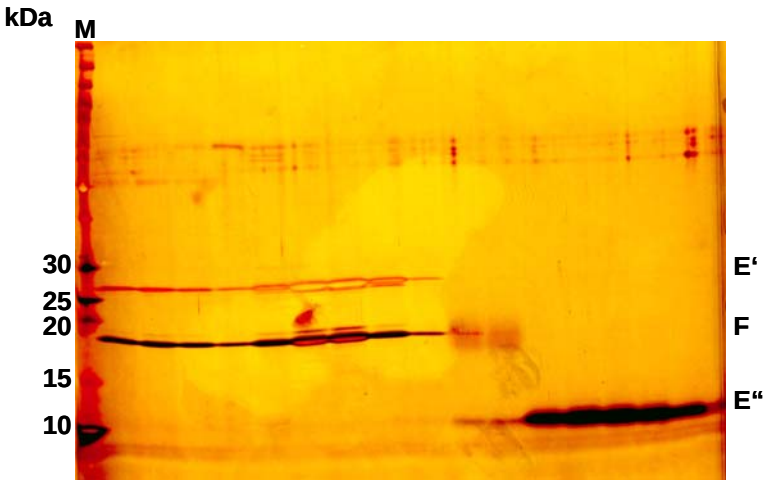


Figure 20. *In vitro* reconstitution of E", E' and F. Silver-stained SDS 8-20%-PAGE analysis of Superdex 75 Fractions. M, protein marker

6.4 Effects of E' and F on *in vitro* transcription at low temperatures

Previously described assays (Fig. 17A and 18) showed that subunits E' and F are not required for promoter-directed *in vitro* transcription assays conducted at 70°C. Recent proteomic results have shown that cellular levels of subunit E´ in the methanogenic *Methanococcoides burtonii* are higher at a low growth temperature (Goodchild et al., 2004) suggesting a specific role of this subunit in transcription at low temperatures. This finding prompted me to investigate the effect of subunit E´ on transcription of *Pfu* RNAP at low temperatures (Fig. 21). At 60°C, activity of the endogenous RNAP was low, but a strong transcription activation was observed upon addition of E´ (Fig. 21A). At 70°C and 65°C, the activity of endogenous RNAP was high and only moderately stimulated by E´. The reconstituted wild-type RNAP shows also a moderate stimulation of transcription at 65°C when E' was added to the reaction (Fig. 21A).
These results suggest a specific effect of subunit E´ on transcription at low temperatures. Therefore, transcription at different temperatures was analyzed in more detail using the RNAP lacking E' and F subunits.

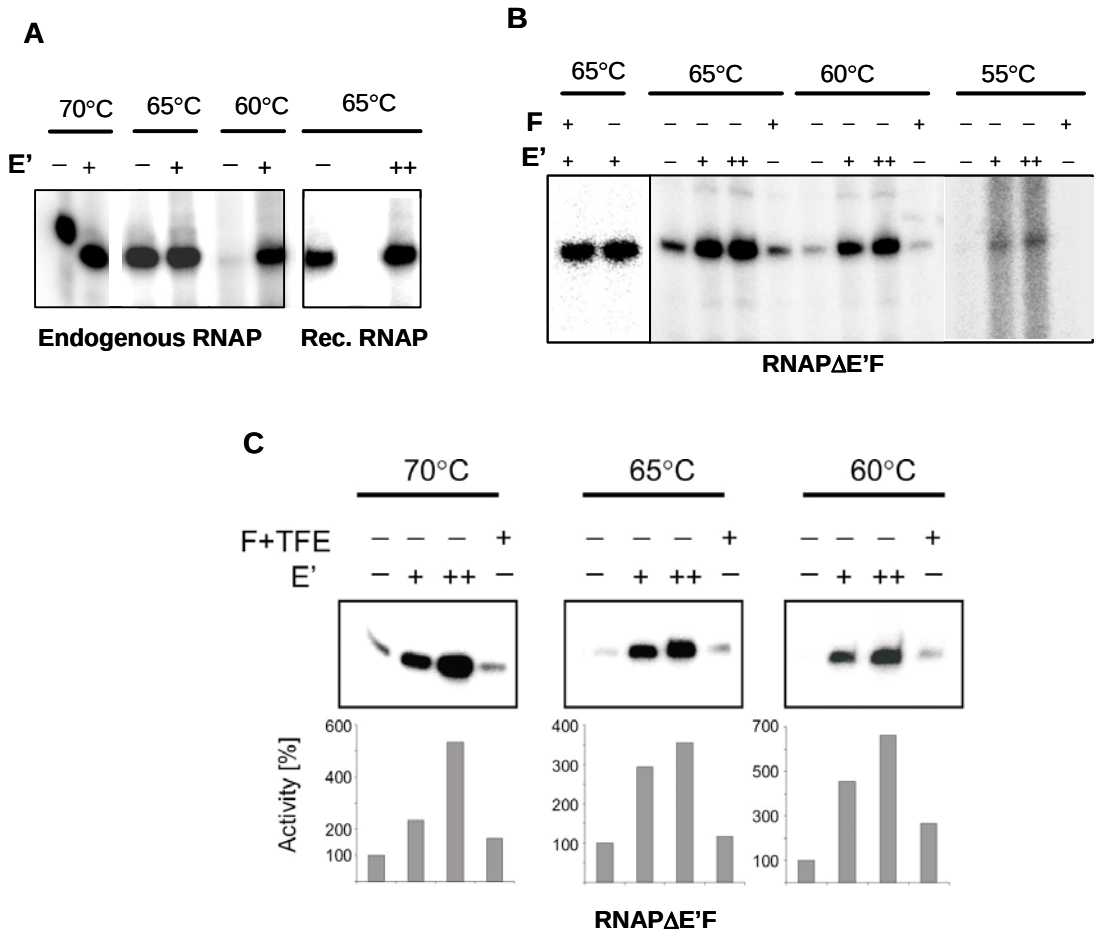


Figure 21. Effect of E', F and TFE on specific *in vitro* transcription at lower temperature. (A) Effect of E' on endogenous RNAP and reconstituted wild-type RNAP transcription (Rec RNAP). **(B)** Effect of E' and F subunits on RNAPΔE'F transcription. **(C)** Effect of TFE, F and E' on transcription. Quantifications of the transcripts are shown. Incubation temperatures are shown, +: 100 ng protein; ++: 200 ng protein.

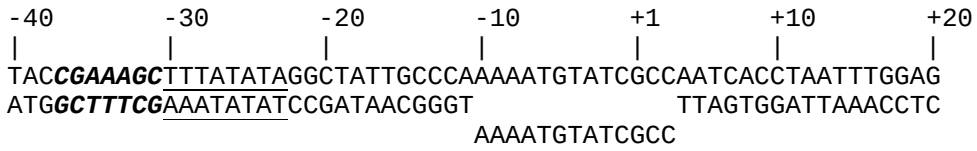
The activity of the core enzyme was stimulated by addition of increasing amount of E′ (Fig. 21B and C). In the presence of 200 ng E′, the activity of the core enzyme was stimulated threefold at 65°C, and sevenfold at 60°C (Fig. 21C). Because E′ can form complexes with F (Armache et al., 2005; Bernd et al., 2006) and F can form complexes with TFE (Fig. 19), effects of subunit F and transcription factor TFE on transcription at low temperatures were studied. When F alone was added to the core enzyme (RNAPΔE'F) and transcription was performed at different temperatures no stimulatory effect on transcription was observed (Fig. 21B). The stimulatory effect of E′ on the core enzyme was slightly decreased by addition of subunit F (Fig. 21B). Combination of subunit F and TFE showed only a two fold transcription stimulation at 60°C (Fig. 21C).

6.5 Effect of E' on promoter opening during transcription initiation

E′ has a strong effect on transcription at low temperatures and only a moderate effect on transcription at 70°C. This finding suggests that E′ might act on RNAP at the stage of promoter opening. To investigate this effect, a DNA construct (bubble1, Fig. 22A) containing *gdh* promoter and a pre-opened synthetic bubble was used in *in vitro* transcription assays at 60°C This construct produced 20 nucleotides-long run-off transcripts. None of RNAPs showed a transcription stimulation by E' in the presence of bubble1 as a template (Fig. 22B). This finding provides evidence that subunit E′ plays a critical role in transcription at 60°C by stimulating open complex formation.

A

Bubble1



B

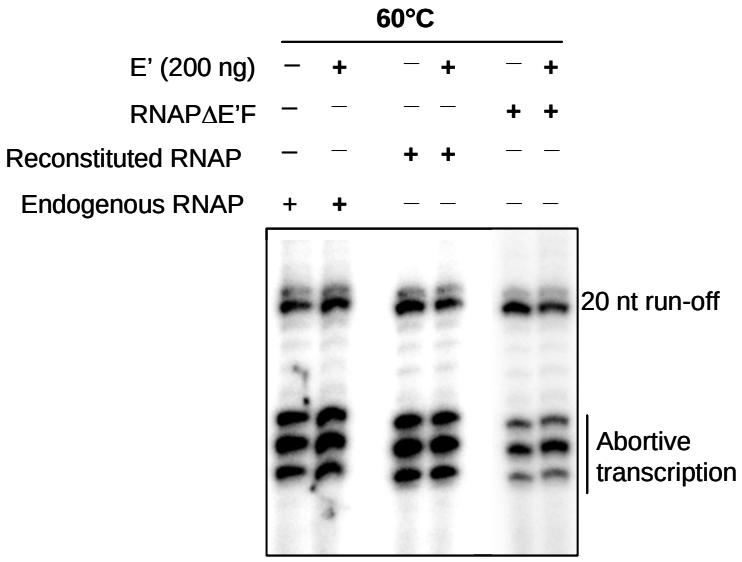


Figure 22. Effect of E' on promoter opening during transcription initiation. (A) Sequences of heteroduplex template construct based on the *gdh* promoter and containing a mismatched bubble (made by Dr. Michela Bertero, University of Munich). The TATA box is underlined, the B-recognition element is indicated by bold italic type and the transcription start site is indicated by +1. **(B)** *In vitro* transcription assays at 60°C using bubble1 and RNAP in the presence or absence of E' (200 ng).

7. The BDLNP sub-complex associates with promoter bound TBP-TFB

It has been shown in eukaryotic RNAPII transcription that the interactions of TFIIB with Rpb1 and Rpb2 are essential for RNAPII recruitment to promoters (Chen and Hahn, 2003; Chen and Hahn, 2004). In the archaeal RNAP system the binding of RNAP sub-complex BDLNP to promoter-bound TBP-TFB was investigated, in order to see if B subunit (homolog of Rpb2) was sufficient to recruit RNAP to the TBP-TFB complex.

7.1 *In vitro* assembly of *Pfu* RNAP sub-complex BDLNP

Equimolar amounts (2.5 nmol) of B and D subunits were combined, and small subunits L, N, and P were added in 2-fold molar excess. The sub-complex was reconstituted following the protocol described in chapter III.6 and analyzed by Superdex 200 chromatography. Gel filtration experiments showed that the subunits co-eluted as a stable complex (Fig. 23A). Analysis of the formed complex by SDS-PAGE indicated the apparent equimolar presence of all subunits (Fig. 23B, fractions 18-22).

7.2 Electrophoretic mobility shift assay of BDLNP with promoter-TBP-TFB complex

To investigate whether the isolated BDLNP sub-complex can associate with promoter-bound TBP-TFB platform, EMSAs were performed. The archaeal sub-complex BDLNP was incubated with a radiolabelled probe, in the presence or absence of TBP and TFB (Fig. 23C). The BDLNP sub-complex did not bind to a DNA fragment harbouring *Pfu gdh* promoter. This result indicates that this complex does not bind to DNA in a non-specific fashion. When the sub-complex was added to reactions containing TBP and TFB, a complex with lower electrophoretic mobility than the DNA-TBP-TFB complex was observed. The purified RNAP binds also DNA-TBP-TFB and forms a slower migrating complex, indicating the difference in molecular mass between BDLNP and the complete enzyme. This finding showed that *Pfu* RNAP is recruited to the promoter-TBP-TFB *via* BDLNP complex.

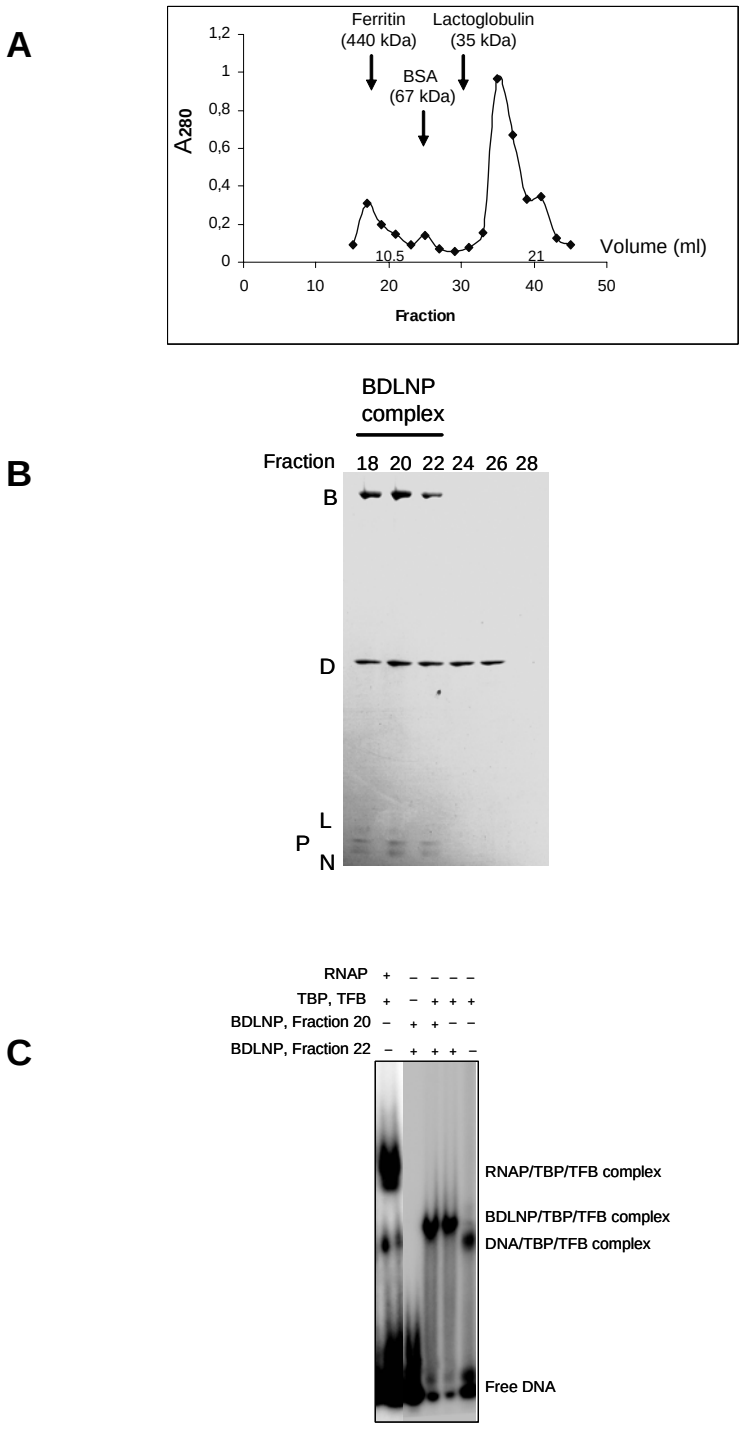


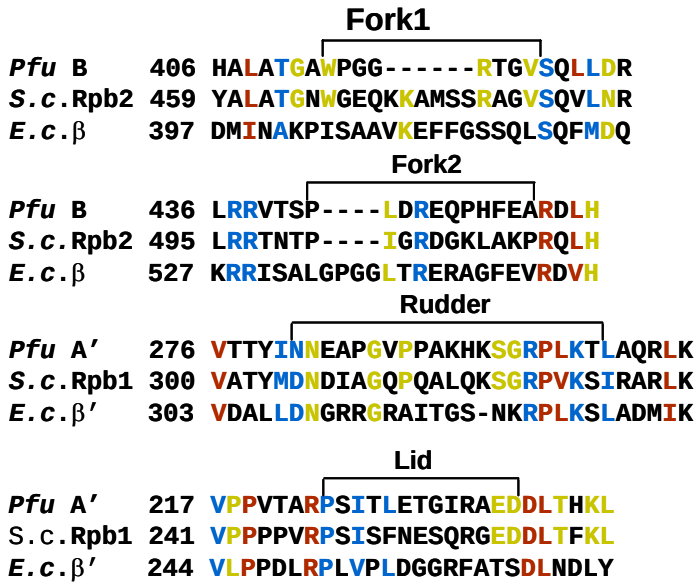
Figure 23. BDLNP sub-complex can associate with the promoter bound TBP-TFB. (A) Superdex 200 size exclusion chromatography profile of assembled subunits. **(B)** Silver-stained SDS 8-20%-PAGE analysis of Superdex 200 eluted fractions. Fractions 18 to 28 are shown. Peak elution fractions of standard proteins are indicated by arrows. The fractions containing the BDLNP subcomplex are indicated. M, protein marker. **(C)** Electrophoretic mobility shift assays with BDLNP on *Pfu gdh* promoter in the presence or absence of TBP and TFB.

8. Functional investigation of *Pfu* RNAP lid, rudder, fork 1 and fork 2

The high resolution structure of yeast RNAPII reveals the presence in the cleft of four conserved loops, so called rudder, lid, fork 1 and fork 2 (Kettenberger et al., 2004). However, functions of these loops are not yet investigated in RNAPII or in an RNAPII-like enzyme. The availability of *in vitro* reconstitution system for *Pfu* RNAP and the high degree of structural and functional similarity between archaeal and eukaryotic RNAPII core transcriptional machineries can be exploited to shed more light on the function of these loops. Sequence alignments between eukaryotic RNAP subunits and *Pfu* RNAP subunits revealed the presence of these loops within *Pfu* RNAP subunits A' and B (Fig. 24A). The sequences coding for these loops were individually deleted by mutagenesis. Mutant subunits lacking the corresponding loop were overexpressed in *E. coli* and purified under the same conditions as the wild-type subunits (Fig. 24B). Recombinant RNAP variants harbouring the mutated subunits were generated by *in vitro* assembly and were referred to as RNAPΔLid, RNAPΔRudder, RNAPΔFork1 and RNAPΔFork2. Structural studies of RNAPII revealed that fork 1 and rudder loops interact with each other and they might have a common function in stabilizing the transcription bubble. Therefore both structural elements were altered by mutagenesis to generate RNAPΔRudder/Fork1.

In vitro reconstitution of mutant enzymes did not show any differences from that of the wild-type enzyme. All subunits associated with each other to form a multi-protein complex, which had the same stoichiometry and behaved the same way for either wild-type or mutant enzyme reconstitutions.

A



B

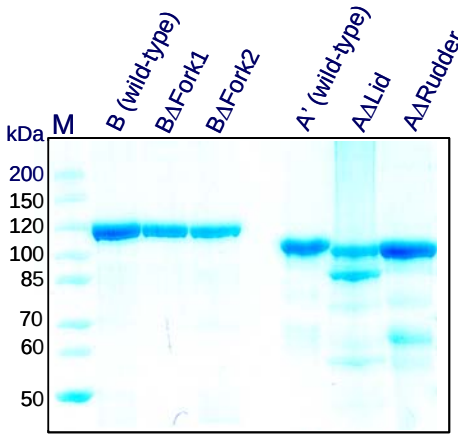


Figure 24. Internal deletion of B and A' subunits. (A) Sequence alignments of loops that partition the cleft. Amino acid alignments of the two fork loops in Rpb2 and the rudder and lid in Rpb1 are colored according to conservation between eukaryotic, archaeal, and bacterial RNA polymerases. Invariant, conserved, and weakly conserved residues are colored in red, blue and yellow, respectively. Sequences in subunits of *Pfu* RNAP are aligned with their homologs in *S. cerevisiae* (*S.c.*) RNAPII and *E. coli* (*E.c.*) RNAP. **(B)** Coomassie-stained SDS 10%-PAGE analysis of purified wild-type B subunit, BΔFork1, BΔFork2, wild-type A', A'ΔLid and A'ΔRudder. M, protein marker.

8.1 Promoter-independent *in vitro* transcription assays of RNAP mutants

To test whether the assembled mutated enzymes display transcriptional activity, non-specific transcription assays were performed. This assay is based on the incorporation of ^{32}P-UTP into trichloroacetic acid (TCA)-insoluble products in the presence of nicked double-stranded template DNA. The results of non-specific *in vitro* transcription showed very low activity of the mutated enzymes in the absence of transcription factors and promoter sequences (Fig. 25).

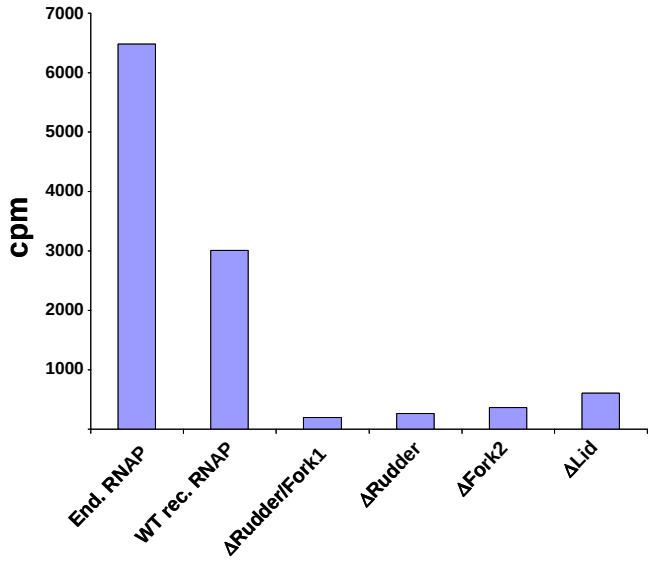


Figure 25. Non-specific transcription assays with RNAP mutants. Reconstituted RNAPs containing the mutated subunit were assembled strictly in parallel. 9 nM of each RNAP variant were used to compare the specific activities of the assemblies relative to the wild-type reconstituted enzyme (WT rec. RNAP) and the endogenous RNAP.

8.2 Promoter-directed *in vitro* transcription assays of RNAP mutants

10 µl Superdex chromatography fractions containing an RNAP mutant were tested in *in vitro* transcription assay in the presence of TBP, TFB and *gdh* promoter containing a TATA-box and a BRE element (Fig 26). RNAPΔLid, RNAPΔRudder, RNAPΔFork2 and RNAPΔRudder/Fork1 failed to transcribe, whereas RNAPΔFork1 was active and showed about 40% of the specific activity of reconstituted wild-type RNAP.

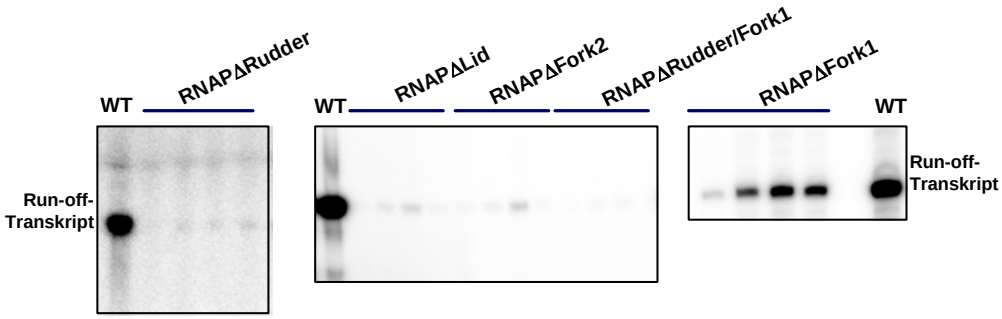


Figure 26. Promoter-directed *in vitro* transcription of RNAP mutants. Superdex 200 fractions of assembled RNAPΔRudder, RNAPΔLid, RNAPΔFork2, RNAPΔRudder/Fork1 and RNAPΔFork1 (45 nM) were assayed in the presence of *Pfu gdh* promoter, TBP and TFB. 45 nM of RNAP variant were used to compare the specific activities of the assemblies relative to the wild-type reconstituted enzyme (WT).

8.3 At which steps of the transcription pathway are lid, rudder and fork2 important for the activity?

To investigate which steps of the transcription cycle are affected by the different deletions, a panel of discriminative *in vitro* transcription assays were designed and performed.
In the present work, I investigated the transcription pathways: closed complex formation, open complex formation, abortive transcription and promoter-escape.

8.3.1 Closed complex formation

To determine whether RNAP mutants could be recruited to promoter/TBP/TFB complex and form a closed complex, electrophoretic mobility shift assays were performed (Fig 27). RNAP mutants were incubated with a radiolabelled probe containing the *gdh* promoter, TBP and TFB as described in chapter III.9. Native gel electrophoresis analysis showed that all RNAP mutants were capable of shifting promoter/TBP/TFB complex, indicating that all mutants can form a closed promoter complex.

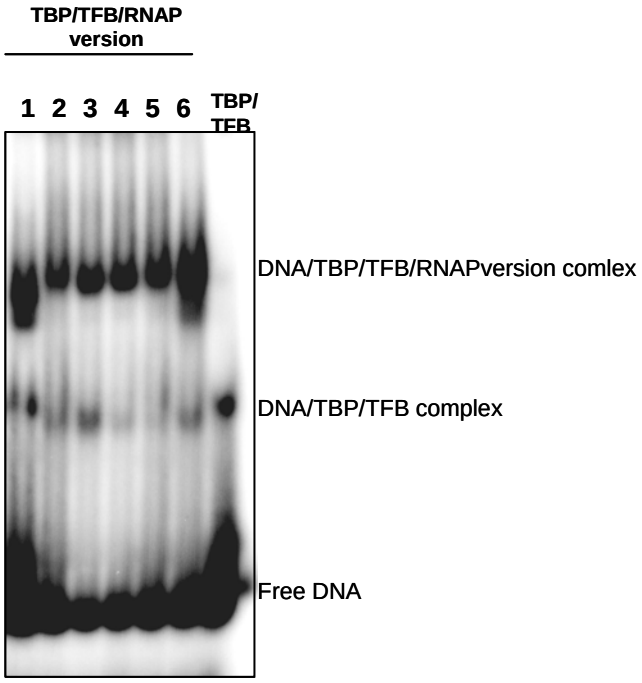


Figure 27. Assembly of RNAP variant on TBP/TFB/promoter. Electrophoretic mobility shift assays were used to monitor the recruitment of RNAP variant to the promoter/TBP/TFB complex RNAPΔRudder, RNAPΔLid, RNAPΔFork2, RNAPΔRudder/Fork1. 1, Endogenous RNAP; 2, Reconstituted wild-type RNAP; 3, RNAPΔRudder; 4, RNAPΔLid; 5, RNAPΔFork2; RNAPΔRudder/Fork1. Formed complexes are indicated.

8.3.2 Open complex formation

In order to test whether RNAP mutants are able to form open complexes, *in vitro* transcription assays were performed. A *gdh* template construct containing a mini-bubble at the transcription start site (mini-bubble2; Fig. 28A) was designed. *In vitro* transcription was performed with mini-bubble2 in the presence or absence of TBP and TFB (Fig. 28B). Results of these assays show that, in contrast to the wild-type enzyme, none of the mutant RNAPs was able to transcribe from the mini- bubble2 template when transcription factors were not added to the reactions. In the presence of TBP and TFB, abortive transcription products were formed by endogenous RNAP, recombinant RNAP and RNAPΔFork1. RNAPΔLid showed very low activity, while RNAPΔRudder, RNAPΔFork2 and RNAPΔRudder/Fork1 were completely inactive in the synthesis of abortive products.

A

Mini-Bubble2

```
-40       -30       -20       -10       +1        +10       +20
|         |         |         |         |         |         |
TAC***CGAAAGC***TTTATATAGGCTATTGCCCAAAAATGTATCGCCAATCACCTAATTTGGAG
ATG***GCTTTCG***AAATATATCCGATAACGGGTTTTTACA    GGTTAGTGGATTAAACCTC
                                          ATCG
```

B

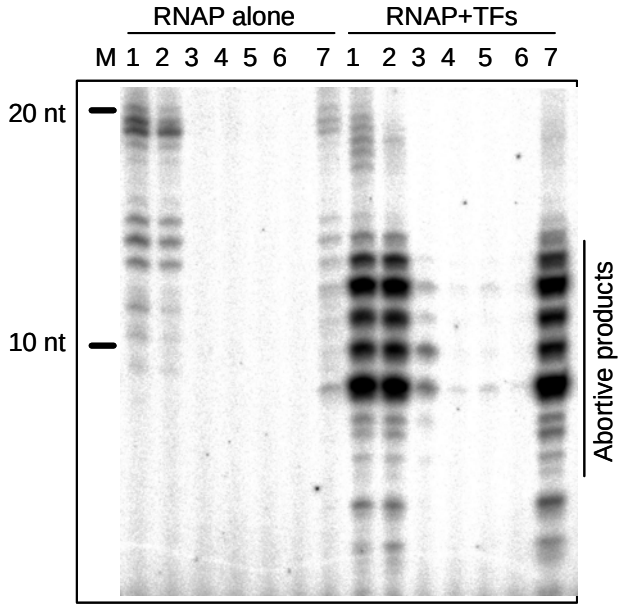


Figure 28. Open complex formation with *Pfu* RNAP. (A) Sequence of Mini-Bubble2 template construct based on the *gdh* promoter. The construct contained a mismatched bubble extending form -3 to +1. The TATA box is underlined, the B-recognition element is indicated by bold italic type and the transcription start site is indicated by +1. **(B)** Specific *in vitro* transcription assays with RNAP mutants in the presence or absence of TBP and TFB. Mini-Bubble2 construct was used as a template for these assays. RNAPΔRudder, RNAPΔLid, RNAPΔFork2, RNAPΔRudder/Fork1. 1, Endogenous RNAP; 2, Reconstituted wild-type RNAP; 3, RNAPΔLid; 4, RNAPΔRudder; 5, RNAPΔFork2; RNAPΔRudder/Fork1; 7, RNAPΔFork1. M, RNA marker.

8.3.3 Abortive transcription and promoter escape

To study the abortive transcription and promoter escape, *in vitro* transcription assays were performed as described above (chapter III.8.2.2). In this case, the DNA construct contained a larger bubble, extending from -10 to +3 (bubble1; Fig. 29A).

A

Bubble1

```
-40       -30       -20       -10       +1        +10       +20
|         |         |         |         |         |         |
TAC***CGAAAGC***TTTATATAGGCTATTGCCCAAAAATGTATCGCCAATCACCTAATTTGGAG
ATG***GCTTTCG***AAATATATCCGATAACGGGT              TTAGTGGATTAAACCTC
                                  AAAATGTATCGCC
```

B

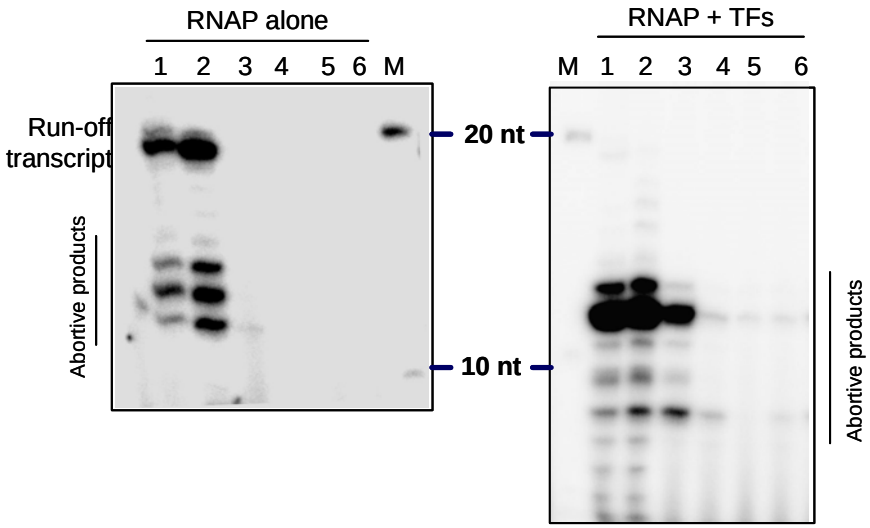


Figure 29. Initial transcription and promoter escape with *Pfu* RNAP mutants. (A) Sequence of Bubble1 template construct based on the *gdh* promoter. The construct contained a mismatched bubble extending form -10 to +3. The TATA box is underlined, the B-recognition element is indicated by bold italic type and the transcription start site is indicated by +1. **(B)** Specific *in vitro* transcription assays with RNAP mutants in the presence or absence of TBP and TFB. Bubble1 construct was used as a template for studying inial transcription and promoter escape. 1, Endogenous RNAP; 2, Reconstituted wild-type RNAP; 3, RNAPΔLid; 4, RNAPΔRudder; 5, RNAPΔFork2; RNAPΔRudder/Fork1; M, RNA marker.

In the absence of transcription factors, only endogenous and recombinant wild-type RNAPs were able to synthesize run-off products (Fig. 29). Surprisingly, these two RNAPs could synthesize only abortive products when the template bubble1 was pre-incubated with TBP and TFB. Likewise, RNAPΔLid was the only active mutant enzyme in the presence of transcription factors and synthesized abortive products. RNAPs could not synthesize run-off but rather abortive products may be because TBP and TFB could not be displaced in this complex after synthesis of the first 10-12 nt of RNA and the bubble cannot collapse upstream.

9. Autophosphorylation of *Pfu* RNAP

To test whether *Pfu* RNAP autophosphorylates, the purified endogenous enzyme was incubated at 70°C for 30 seconds to 20 min in phosphorylation buffer containing 1 µCi [γ-^{32}P]ATP. SDS-PAGE results show a phosphorylated protein with MW of about 100 kDA, which is very close to the molecular mass of A' subunit (103 kDa). After only 30 seconds the protein was phophorylated, this phosphorylation increased with incubation time and after 30 min the proteins were immoblized in the wells of the gel (Fig. 30).

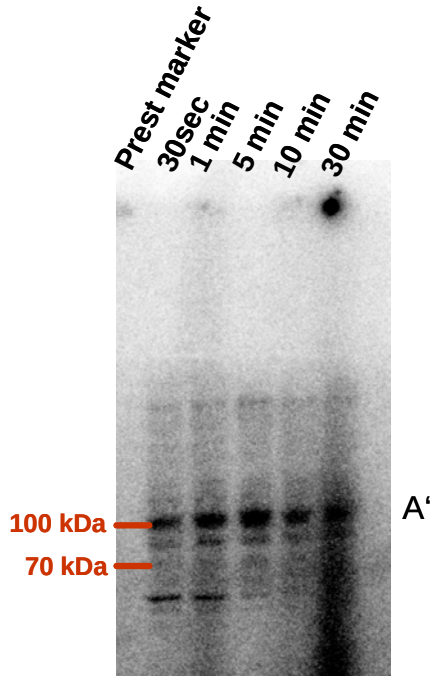


Figure 30. SDS-Page of autophosphorylated *Pfu* RNAP. Prestained marker is shown. The gel was visualized by phosphoimaging.

10. Function of *Pfu* RNAP phosphorylation in the transcriptional activity

To investigate whether RNAP phosphorylation is required for the transcriptional activity of the enzyme, *Pfu* RNAP was dephosphorylated with λ Protein Phosphatase (*New England Biolabs, USA*) or with Calf Intestine Alkaline Phosphatase immobilized on agarose beads (*Sigma, USA*) according to the instructions of the manufacturer. Dephosphorylated RNAPs were then assayed in *in vitro* transcription assay in the presence of NTPs mix containing AMP-PNP (Fig. 31) and GMP-PNP, which are non-hydrolysable analogues of ATP and GTP, respectively.

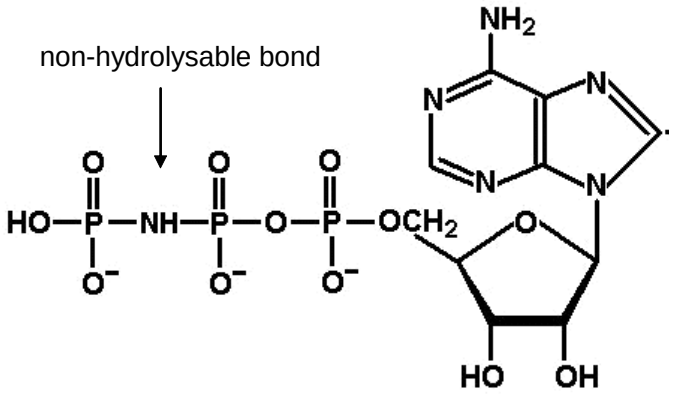


Figure 31. AMP-PNP (Adenylylimidodiphosphate). The non-hydrolysable β-γ bond of AMP-PNP is shown

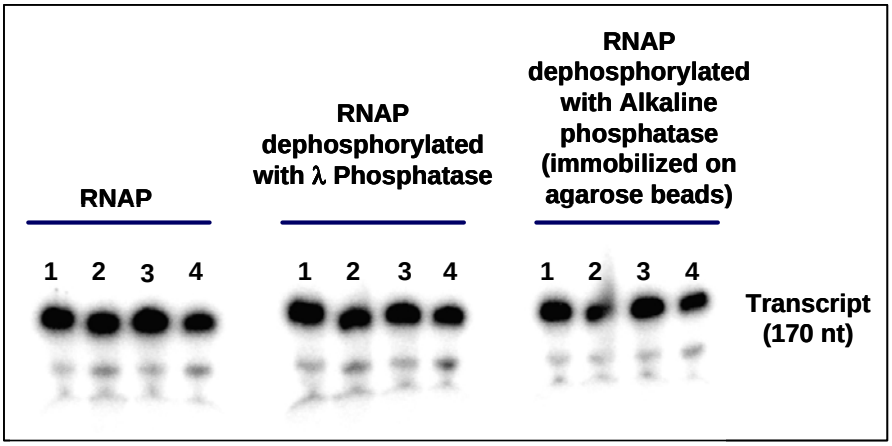


Figure 32. Promoter-directed *in vitro* transcription with dephosphorylated *Pfu* RNAP. 1: NTP mix containing ATP, GTP, CTP and UTP. 2: NTP mix containing AMP-PNP, GTP, CTP and UTP. 3: NTP mix containing ATP, GMP-PNP, CTP and UTP. 4: NTP mix containing AMP-PNP, GMP-PNP, CTP and UTP.

Promoter-directed *in vitro* transcription assays in the presence of TBP, TFB and *gdh* promoter show that *Pfu* RNAP does not require hydrolysis of the β-γ bond of ATP and GTP for the transcriptional activity. In addition a phosphorylated form of the RNAP is not required for accomplishing the transcription.

V Discussion

In the present work, the PolII-like RNAP form the hyperthermophilic *Pyrococcus furiosus* was reconstituted *in vitro* from its 11 recombinant subunits. The reconstituted RNAP was capable of specific transcription initiation at various archaeal standard promoters at temperatures between 60 and 80°C. At 60°C, the activity of the reconstituted core enzyme (assembled in the absence of E´ and F) was sevenfold stimulated by addition of subunit E´. This subunit has a role in transcription initiation by catalyzing open complex formation.
The availability of a procedure for *in vitro* reconstitution of *Pfu* RNAP enabled us to study the contribution of the small subunits to the function and the structure of this enzyme. *Pfu* RNAP structural elements homologous to RNAPII lid, rudder, fork1 and fork2 were also investigated in functional studies
In addition, endogenous RNAP was purified to homogeneity and in large scale in order to study the structural and functional properties of this enzyme.

1. Purification of endogenous *Pfu* RNAP

Pfu RNAP was purified from cells harvested during exponential growth phase. The homogeneity of the enzyme is shown in Figure 12.

1.1 Transmission electron microscopy of *Pfu* RNAP

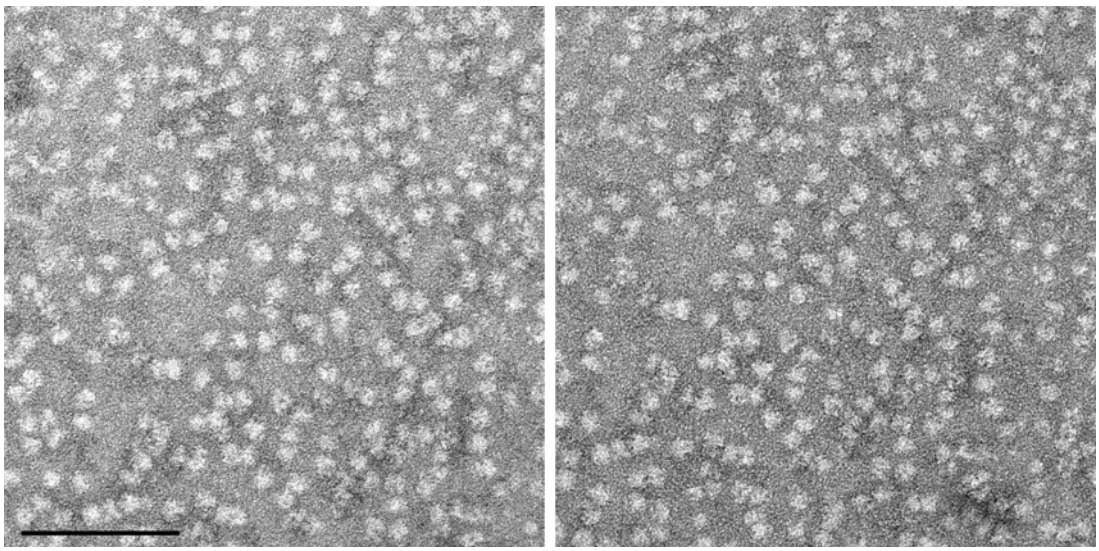


Figure 33: Two transmission electron micrographs of RNA-Polymerase molecules isolated from *Pfu* cells. RNAP was purified as described in Chapter II.1. The enzyme was negatively stained with Uranyl acetate (2%). Micrographs were taken on a CM12 transmission electron microscope at 120 keV and magnification of 35.000x. The bar represents 100 nm. This image was kindly provided by Dr. R. Rachel.

Pfu Polymerase molecules were imaged after negative staining by TEM (Fig. 33). In projection, they have a diameter of about 13 to 15 nm, i.e. they are slightly elongated in shape. This is in line with data obtained by x-ray crystallography from the eukaryotic RNAP II complexes (Cramer et al., 2001) and the bacterial RNAP (Zhang et al., 1999). The various projections visible in the micrographs are not identical, showing that the molecules can adopt different orientations on the carbon support film, resulting in different projection images.

1.2 E" is not a subunit of *Pfu* RNAP

In most archaeal genomes the gene encoding RNAP subunit E'overlaps at its 3'-end with a gene encoding the protein E". E" has no homolog in eukarya but it is highly conserved among archaea (Fig. 13A). This protein contains a conserved Zinc-finger motif, which is characteristic of DNA binding proteins. It was unclear whether E" is a subunit of RNAP. To determine whether this protein is a subunit of the endogenous RNAP, Western-blot assay with anti-E" was performed. E" was not detected as a subunit of endogenous *Pfu* RNAP (Fig. 13B). *In vitro* reconstitution assays of E", E' and F show that E" did not bind to E'/F sub-complex (Fig. 20). E" was, however, expressed in *Pfu* cells (Fig. 13B). To confirm that E" is not a subunit of RNAP, additional Western-blot analysis should be performed after each purification step of the endogenous RNAP.

2. Reconstitution of active recombinant *Pfu* RNAP

A procedure for the reconstitution of a PolII-like RNA polymerase from individually expressed subunits was described (Fig. 12). This approach, based on assembly controlled by stepwise renaturation of urea-denatured subunits, essentially mirrors the previously used procedures for assembly of *E. coli* RNAP (Zalenskaya et al., 1989) and *M. jannaschii* RNAP (Werner and Weinzierl, 2002). The reconstituted *Pfu* RNAP containing subunits B´A´ A´´DHLPKN eluted as a distinct macromolecular complex after size exclusion chromatography (Fig. 14). This complex contained all RNAP subunits, with the exception of E´ and F, in nearly stoichiometric amounts. Separation of the highly active enzyme fractions from an excess of large aggregates with low specific activity by size exclusion chromatography turned out as crucial step for the isolation of a stable reconstituted RNAP with high activity in promoter-dependent assays (Fig. 15). Promoter-directed *in vitro* transcription assays showed that the reconstituted RNAP was capable of synthesizing run-off transcripts from linearized templates containing the *aaa*$^{+}$*atpase-*, the *hsp20-*, the *phr-* and the *malE* promoter from *Pfu* and the tRNA^{Val} promoter from *Methanococcus vannielii* (Fig. 18). These results indicated that the reconstituted enzyme was able to initiate transcription at canonical archaeal promoters containing a TATA-box and a BRE element. The recombinant *Pfu* RNAP could perform most of the steps required for specific *in vitro* transcription, from RNAP promoter recruitment *via* TBP and TFB to promoter escape and elongation. The specific activity of the reconstituted RNAP in promoter-dependent transcription assays had about 50% of the specific activity of the endogenous enzyme purified from *Pfu* cells (Fig. 16). However, transcription termination has not yet been assayed.

The bacterial core enzyme was assembled sequentially in the order: $2\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$ (premature core) $\rightarrow$ active core, where α_2 formed the assembly platform for the incorporation of the large subunits β and β' which contain the catalytic site (Ishihama, 1981). The *M. jannaschii* enzyme was reconstituted with 4-fold excess of the small subunits L, N, D, P, K, H, F and E. In addition, subunits D/L and E´/F were coexpressed in *E. coli* (Werner and Weinzierl, 2002). In the assembly procedure described in the present work, equimolar amounts of individually expressed subunits were used and the subunits renaturation procedure was faster than the procedure used for the reconstitution of *M. jannashii*. A recombinant enzyme with higher activity was obtained indicating that a sequential order for reconstitution and an excess of small subunits was not required for successful reconstitution of *Pfu* RNAP (Fig. 14 and Fig. 15).
In *S. cerevisiae*, deletion of the gene encoding any of the subunits, with the exception of the genes encoding Rpb4 and Rpb9 subunits, results in cell death, implying that these 10 subunits are essential for the viability of yeast cells (Woychik and Young, 1989). Thus far, genetic studies in archaea have not yet been developed except for the hyperthermophile Thermococcus kodakaraensis. This transformation system has been very recently developed in the laboratory of Dr. H. Atomi in Kyoto, Japan (Sato et al., 2005). Therefore, nothing is known about the viability of archaeal cells in the absence of RNAP subunits. However, the function of subunits for the structure and transcriptional activity of the RNAP can be studied in a fully reconstituted *in vitro* transcription system.
DNA-dependent RNAPs are defined by a conserved core of five subunits homologous to the bacterial $\beta'\beta\alpha 2\omega$ core enzyme. The subunit configuration DLBA'A'' (or DLB'B''A'A'') is the closest possible approximation of an archaeal RNAP to the minimal bacterial core enzyme $\alpha 2\beta\beta'$ (Cramer et al., 2001; Werner and Weinzierl, 2002). However, the archaeal enzyme, like the three eukaryotic RNAPs, contains 6 to 7 additional subunits (Woychik et al., 1990; Goede et al., 2006). To address questions regarding the importance of these additional subunits (K, H, N, P, E' and F) for structure, stability and catalytic activity of the enzyme, mutant enzymes lacking these subunits were reconstituted and assayed *in vitro* in promoter-directed transcription.
Analysis of the catalytic properties of reconstituted *Pfu* RNAP lacking K, H, P, N or E'/F revealed similar results as in the case of the M. jannaschii enzyme (Werner and Weinzierl, 2002).

3. The role of individual subunits in the catalytic activity of RNAP

3.1 Subunit K

To test the functional role of K, the homolog of bacterial ω, *Pfu* RNAP was assembled in the absence of this subunit. The ΔK RNAP had almost the same levels of promoter specific activity as the complete enzyme (Fig. 17A). This result is in agreement with the results obtained with the reconstituted *M. jannaschii* enzyme lacking subunit K (Werner and Weinzierl, 2002). In bacteria, the ω subunit is not required for bacterial viability or for transcription, either *in vivo* or *in vitro*, but promotes RNAP stability and assembly by reducing the configurational entropy of the largest subunit β' (Minakhin et al., 2001).

Rpb6, the eukaryotic homolog of K, and ω occupy comparable positions in RNAP, as revealed by analysis of crystal structures of both enzymes (Cramer et al., 2001; Murakami et al., 2002). Rpb6 and ω wrap around portions of the enzymes' largest subunit, Rpb1 and β', respectively, to promote subunit assembly and stability, in agreement with the work indicating a role of Rpb6 in assembly (Nouraini et al., 1996). Rpb6 is essential for cell viability and is also involved in the functional interaction between RNAPII and TFIIS (Ishiguro et al., 2000). It is thus possible that the archaeal K subunit might recruit the archaeal TFS to the polymerase.

3.2 Subunit H

Subunit H is the archaeal homolog of eukaryotic Rpb5, which contributes to the formation of the lower jaw of the enzyme and also contacts DNA upstream of the transcription bubble (Armache et al., 2005). Leaving out H from the recombinant enzyme abolished *in vitro* transcriptional activity (Fig. 17A), implying that this subunit is essential for the catalytic activity of the enzyme. However, *M. jannaschii* RNAP ΔH was active in promoter-directed *in vitro* transcription assay at the rubredoxin 2 (*rb2*) promoter (Ouhammouch et al., 2004). Previous photochemical protein-DNA crosslinks studies showed that the *Pfu* H subunit crosslinked to DNA in transcription initiation complexes between bp +12 and +20 on the template strand, and bp +14 to +20 on the nontemplate strand. These crosslinks were not observed with the *Methanocaldococcus jannaschii* and *Methanothermococcus thermolithotrophicus* RNAPs (Bartlett et al, 2004). It might be that H subunit is located at different position in *Pfu* and *M. jannaschii* enzyme.

3.3 Subunits P and N

The subunits N and P are homologs of the eukaryotic subunits Rpb10 and Rpb12. These two subunits, as shown in the RNAPII crystal structure, belong to the so-called α-domain of RNA polymerases and probably consolidate the overall architecture of this enzyme (Cramer et al., 2000).

Like *M. jannaschii* enzyme (Werner and Weinzierl, 2002), *Pfu* RNAP ΔP showed no transcriptional activity in promoter specific *in vitro* transcription assay (Fig. 17A). P might be a part of the assembly platform since this subunit interacts with subunits D, N and B as shown in Far-Western blotting experiments (Goede et al., 2006).

ΔN enzyme showed reduced but still detectable specific activity (Fig. 17A). The activity was completely restored when the heat treatment step after RNAP assembly was omitted, indicating that ΔN enzyme is thermolabile. *M. jannaschii* ΔN enzyme showed a defect in the incorporation of subunit B into the enzyme when exposed to high temperature and was, therefore, inactive both in non-specific and specific assays. B was present in stoichiometric amounts in the reconstituted *Pfu* ΔN enzyme (Fig. 17B).

Subunit N interacted with subunits D, P and B in Far-Western blotting experiments (Goede et al., 2006). P and N subunits might have a role in stabilizing the BDLNP sub-complex and N might be involved in stabilizing the interaction of subunit B with the DLNP sub-complex of RNAP at normal growth temperatures of *Pfu* (70-103°C). The finding that the thermolabile ΔN enzyme is active in specific *in vitro* transcription assay conducted at 70°C indicates that interactions of this enzyme with DNA and transcription

factors stabilize the otherwise thermolabile ΔN assembly and demonstrate that subunit N is not required for recruitment of RNAP to the promoter and the catalytic activity of the enzyme.

3.4 Subunits E' and F

The reconstituted enzyme did not contain subunits E´ and F in stoichiometric amounts. These subunits were detected in active RNAP fractions only by Western blotting, using polyclonal antisera raised against these two proteins (Fig. 14D). Gel filtration chromatography showed that E' and F subunits eluted as a separate complex (Fig. 14A and C) with the predicted molecular mass. This result confirms the information obtained from a high resolution crystal structure of *S. cerevisiae* RNAPII that revealed the existence of a 10 subunits core and a dissociable Rbp7/Rpb4 sub-complex, the eukaryotic homolog of E'/F sub-complex (Armache et al., 2005). In addition, it has been shown that the Rpb7/Rpb4 sub-complex is easily dissociable and fails to copurify with the core RNA polymerase II under mild denaturing conditions (Edwards et al., 1991).
The E´/F sub-complex and its eukaryotic orthologs Rpb7/Rpb4 (RNAPII), Rpc25/Rpc17 (RNAPIII) and Rpa43/Rpa14 (RNAPI) form heterodimers that are a landmark of all non-bacterial RNA polymerases (Sadhale and Woychik, 1994; Langer et al., 1995; Shpakovski and Shematorova, 1999; Werner et al., 2000; Peyroche et al., 2002; Siaut et al., 2003). The sequences of the eukaryotic Rpb7 subunits and the homologous archaeal E' subunits are well conserved (Fig. 34), while the sequence similarity between the eukaryotic Rpb4 subunit and the archaeal F subunits is much lower. Despite these differences in primary sequence similarity, there is a structural similarity between the Rpb7/Rpb4 complex and the archaeal E/F heterodimer (Werner et al., 2000; Armache et al., 2005; Fig. 35). This also applies to the RNAPIII counterpart of these subunits, C17/C25 (Siaut et al., 2003; Jasiak et al., 2006). However, the similarity of their structures strongly argues for their functional equivalence. Functional studies revealed that the Rpb7/Rpb4 and C25/C17 sub-assemblies are required for promoter-directed transcription initiation but dispensable for transcription elongation (Edwards et al., 1991; Orlicky et al., 2001; Zaros et al., 2005), while the reconstituted archaeal RNAP lacking E' and F subunits is still able to initiate transcription at 70°C at canonical archaeal promoters containing a TATA-box and a BRE element (Fig. 17Aand 18). These results are in agreement with the results obtained with the reconstituted *M. jannaschii* enzyme lacking the E'/F sub-complex (Werner and Weinzierl, 2002).

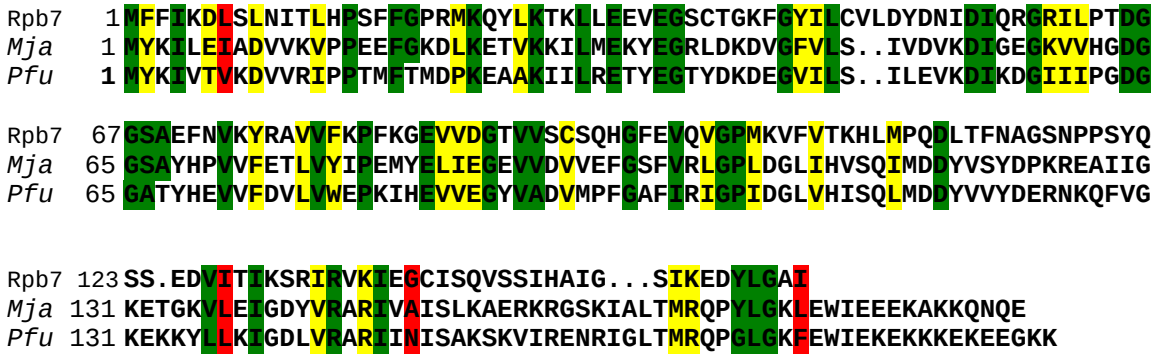


Figure 34. Amino acid alignment for E' homologs. An alignment of *Pfu* E' subunit with *Methanocaldococcus jannaschii (Mja*) subunit E and *Saccharomyces cerevisiae* Rpb7. Conserved residues are highlighted in dark green, conservative exchanges in red and less conserved residues are indicated in yellow.

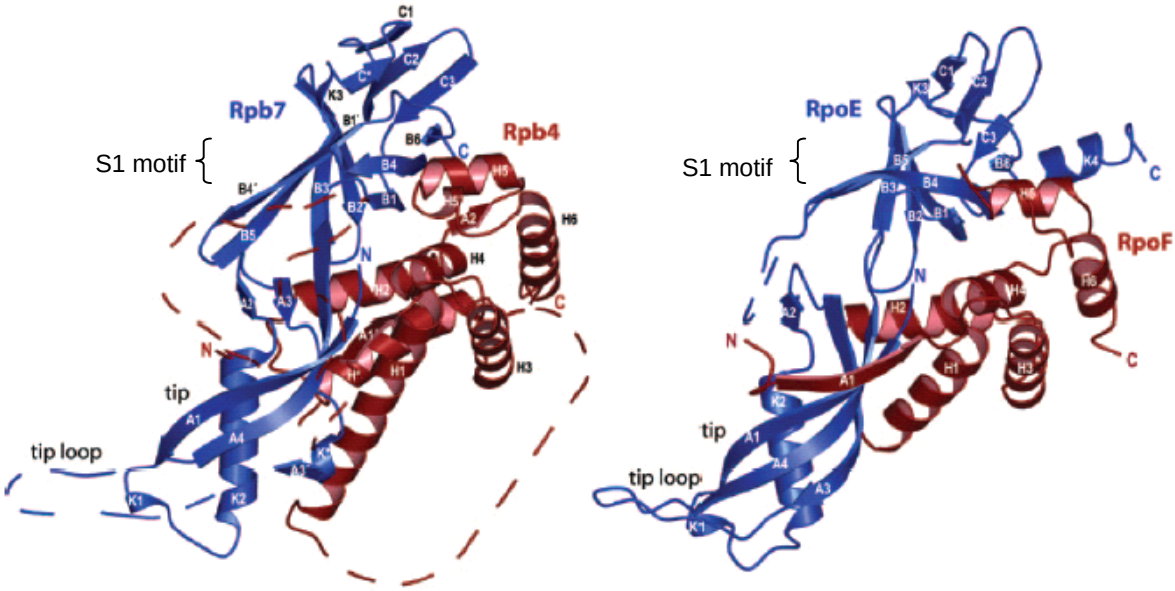


Figure 35. Structure of eukaryotic Rpb7/4 sub-complex at 2.3 Å and of the archaeal E/F sub-complex at 1.75 Å resolution. Ribbon diagram of *S. cerevisiae* Rpb7/4 (Armache et al., 2005; PDB entry 1 wcm) and of archaeal RpoE/F (*Todone et al., 2001;* PDB entry 1go3*)* structures. Rpb7 and E are shown in blue, Rpb4 and F are shown in red. The S1 motif of Rpb7 and E is indicated. The S1 motif folds into an antiparallel barrel with an OB-fold topology and it is present in the C-terminal half domain of Rpb7 and E. Dashed lines represent disordered loops. Disordered in the Rpb7/4structure are the Rpb7 tip loop (residues 57–68) and Rpb4 residues 35–46, 77–81, and 101–118. These figures were prepared with PYMOL software (DeLano Scientific). The overall structure of the E'/F complex is similar to that of its eukaryotic counterpart (Armache et al., 2005; Todone et al., 2001). F binds between two E domains, the amino-terminal "tip" domain and a carboxyl-terminal domain like their eukaryotic homologs do (Fig. 31). Compared to the archaeal protein, Rpb7 lacks the carboxyl-terminal helix K4 and has an additional helix inserted into the A3 strand. About half of the Rpb4 residues fold as in the archaeal counterpart. Additionally, Rpb4 contains a non-conserved amino-terminal extension (residues 1–46), a longer helix H1, and an insertion between helices H1 and H2 , which comprises a long disordered loop. The N- and C-terminal conserved regions of Rpb4 and F are involved in interactions with Rpb7 and E, respectively. The Rpb4 C-terminal conserved region may be also involved in the stress-responsive role of Rpb4 and in activated transcription from a subset of genes (Sampath et al., 2003). The nonconserved regions of Rpb4 seem to be required to maintain the conserved N- and C-terminal regions in an appropriate conformation (Sampath et al., 2003). Rpb7 and its archaeal counterpart E or E' contain an S1 motif domain that is evolutionarily conserved (Todone et al., 2001). The presence of S1 motif strongly suggested that their role is also to bind single-stranded nucleic acids. Indeed, it has been shown that *M jannaschii* RNAP E/F sub-complex binds RNA (Meka et al., 2005), and its eukaryotic counterpart Rpb7/Rpb4 binds to single stranded DNA (Orlicky et al., 2001) and to RNA (Meka et al., 2005). Recently, it was reported that the RNA exiting from RNAPII crosslinks to Rpb7 (Ujvari and Luse, 2006). It was also suggested that C25/C17 may bind RNAPIII transcripts emerging from the adjacent exit pore, since this sub-complex binds to tRNA *in vitro* (Jasiak et al, 2006). These interactions of Rpb7 with the nascent RNA suggest a role of Rpb7 in elongation or recycling.

4. E' stimulates transcription at lower temperatures

Recent proteomic experiments have shown that cellular levels of subunit E′ in the methanogen *Methanococcoides burtonii* are higher at low growth temperature (Goodchild et al., 2004), suggesting a specific role of this subunit in transcription at low temperatures. The availability of *Pfu* RNAP *in vitro* reconstitution system offered a possibility to investigate the effect of subunit E′ on transcription at low temperatures (Fig. 21). First, the transcriptional activity of the endogenous *Pfu* RNAP was tested at 70°C, 65°C and 60°C. At 70°C and 65°C the activity of RNAP was high and only moderately stimulated by E′. At 60°C, the activity of the purified endogenous RNAP was lower and a strong activation of transcription was observed upon addition of E′ (Fig. 21A). Likewise, the activity of the complete reconstituted enzyme was stimulated at 65°C by addition of E′ (Fig. 21A). These findings suggest a specific effect of subunit E′ on transcription at low temperatures and therefore transcription at these temperatures was analyzed in more detail using *in vitro* reconstituted RNAP lacking subunits E' and F. Indeed, in the presence of E', the activity of the core enzyme was stimulated 3.5 fold at 65°C and 7 fold at 60°C (Fig. 21C). Because E′ can form complexes with F, the effect of subunit F on transcription at lower temperatures was studied. The addition of F subunit alone did not affect the activity of the core enzyme at 65, 60 or 55°C (Fig. 21B). Surprisingly, the stimulatory effect of E′ on the core enzyme was slightly decreased by the addition of subunit F (Fig. 21B, first and second lanes). The explanation for this result might be that the core enzyme and F subunit compete to bind to E'. Furthermore, F has a high affinity to E' and tends to form the E'/F sub-complex, which makes E' less available for the core enzyme. When TFE was added to reactions containing F, the transcriptional activity at 60°C was increased by a factor of 2 (Fig. 21C). Like E', TFE has a similar effect on transcription at 60°C. However, the stimulatory effect of E' on transcriptional activity at low temperatures is much stronger than the effect of TFE.

5. E' catalyzes open complex formation during transcription initiation

The finding that transcription at 60°C becomes dependent upon the addition of E′ (Fig. 21) enabled us to determine which step of the transcription cycle is affected by E'. Rpb7/Rpb4, the eukaryotic homolog of E'/F subcomplex, is known to play an important role in transcription, mRNA transport and DNA repair (Choder, 2004). The Rpb7/Rpb4 complex is required for promoter-directed initiation *in vitro* but dispensable for elongation or promoter-independent initiation (Chung et al., 2003). A previous study showed that the Rpb7/Rpb4 heterodimer is not required for stable recruitment of polymerases to pre-initiation complexes (Orlicky et al., 2001). Another study showed that Rpc25, the RNAPIII homolog of E', is important for transcription initiation but not for the elongation by RNAPIII. These reports suggested that Rpb7/Rpb4 and their homologs might mediate an essential step after promoter binding but before transcription elongation. The step that follows promoter binding in the transcription cycle is open complex formation. Therefore, the function of E′ was investigated in open complex formation by using the completely recombinant *in vitro* reconstituted system. A

discriminative *in vitro* transcription assay at 60°C was performed using a template construct containing the *gdh* promoter and a synthetic bubble (Fig. 22), in the presence and absence of E'. In this assay the RNAP specifically binds to the opened bubble and starts transcription independently of transcription factors. The results show that the endogenous RNAP, the reconstituted wild-type and even the core enzyme were not stimulated by E' at 60°C when a template construct containing a pre-opened bubble was used in *in vitro* transcription assay. This finding provides evidence that subunit E´ stimulates transcription at 60°C by stimulating open complex formation. Another way to study open complex formation is the permanganate footprinting technique. This assay was performed by my colleague Sebastian Gruenberg by following the permanganate footprinting protocol previously described (Spitalny and Thomm, 2003). Results of this assay show that the transcription bubble opening in the -2 to -4 region can be achieved by the core enzyme, and that subunit E' extends the bubble to position +3.

The strong stimulation of initiation observed was mediated by E´ alone. This indicates that the formation of the E´/F sub-complex is not a prerequisite for its activating properties. This shows a further similarity to the eukaryotic system. Structural studies of RNAPII indicated that the major interaction of Rpb7/Rpb4 with the core enzyme occurs via the tip loops of Rpb7 (Fig. 36). However, this interaction does not involve Rpb4 (Fig. 31; Armache et al., 2003). Therefore E´, like Rpb7, can interact as a monomer with the core enzyme regardless of the presence of the Rpb4 ortholog.

The report that the intracellular levels of RNAP subunit E´ increase during growth at low temperatures (Goodchild et al., 2004) suggests that E´ facilitates transcription at these temperatures. Open complex formation assays at the *gdh* promoter at 60°C give an explanation of the stimulatory roles of E' and identify a major function of E´ in open complex formation during initiation of transcription.

Saccharomyces cerevisiae Rpb7 is an essential protein and Rpb4 is dispensable under optimal growth conditions (Choder, 2004). It is unclear whether E´ is essential for growth of archaeal cells but cell-free transcription experiments demonstrate that E´ is not required for cell-free transcription of the *gdh* promoter at 70°C. However, the presence of E´ is crucial at 60 °C (Fig. 21).

Although E´ catalyzed promoter opening may not be required under normal growth conditions of the hyperthermophilic Archaeon *Pyrococcus*, the finding reported here has important implications for the mechanism of transcription by a RNAPII-like polymerase. Structural studies of RNAPII indicated that Rpb7/Rpb4 sub-complex protrudes from the polymerase upstream face (Fig. 36), where initiation factors might bind to the RNAP. Rpb7 formed a wedge underneath the mobile RNAPII clamp, apparently restricting the clamp to a closed position. This tightens the interacton of the polymerase with the RNA-DNA hybrid and presumably enhances processivity (Meisenheimer et al., 2000; Bushnell and Kornberg, 2003). This observation further supports the conclusion that the association of E´ with the archaeal core enzyme induces a major conformational change of the core enzyme, stabilizes clamp closure and catalyzes open complex formation.

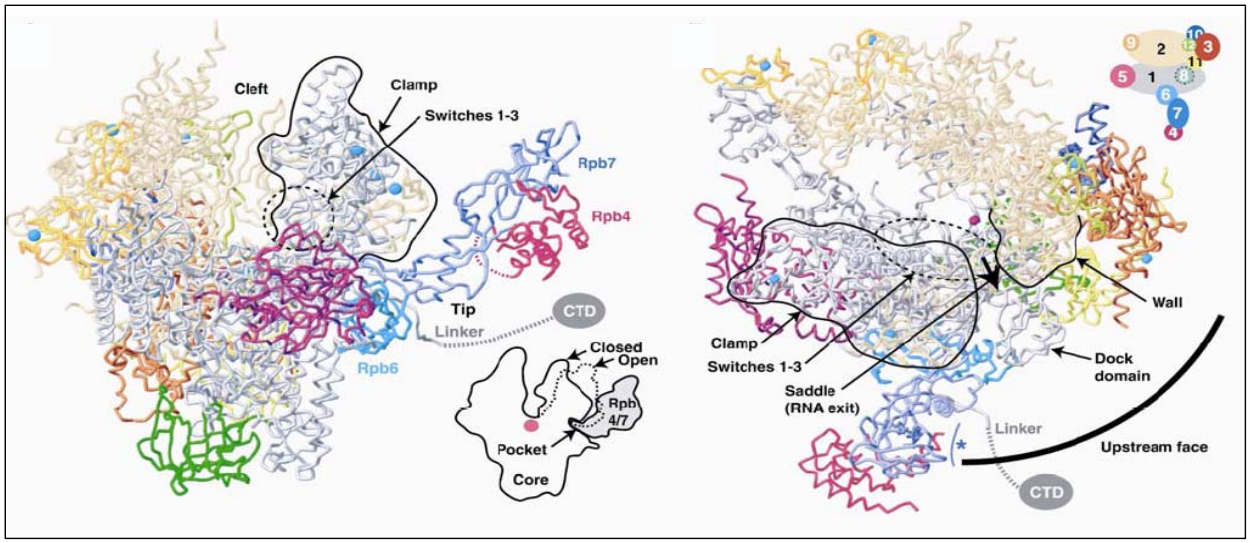


Figure 36. Structure of the 12-subunits initiation-competent RNAPII. The structure was resolved at 3.8 Å resolution and is shown as a ribbon model (Armache et al., 2003). The 12 subunits, Rpb1 to Rpb12, are colored according to the key above the views. Cyan spheres and the pink sphere depict zinc ions and an active-site magnesium ion, respectively. A black line encircles the clamp. The linker to the CTD is indicated as a dashed line. A view of the model from the top is shown on the left. The tip loops of Rpb7 are indicated (Tip) and the interaction of Rpb7/Rpb4 with the core enzyme is shown. Only Rpb 7 is involved in this interaction. The N-terminal half of Rpb7 contacts the main body of RNAPII adjacent to the linker of the C-terminal domain (CTD) of Rpb1 (Bushnell and Kornberg, 2003; Armache et al., 2003). The C-terminal half is projected away from the enzyme and is wrapped by its Rpb4 partner.The saddle between the wall and the clamp and the assumed direction of RNA exit are indicated. A schematic cut-away view is shown in the middle. A dashed like indicates the open clamp position observed in RNAPII core structure (Cramer et al., 2001). The presence of Rpb7/Rpb4 complex locks the mobile clamp in a closed conformation, which was suggested to be required for the transition from initiation to elongation. This figure was a very kind gift from Dr. Karim Armache.

6. F interacts with the transcription factor TFE

Pfu RNAP subunits E´ and F and *Pfu* transcription factor TFE, the homolog of the eukaryotic TFIIEα, were incubated together *in vitro* following the assembly procedure described in this work (Fig. 19). The results indicated a tight and strong interaction between TFE and F. When subunits E' and F were assembled *in vitro* in the absence of TFE, a specific E'/F complex was formed. TFE completely replaces E´ from the E´/F complex. This finding demonstrates that F subunit has a higher affinity for TFE than for E', and suggests that F is the subunit that mediates recruitment of TFE to RNAP. A twofold stimulation of weak promoters by TFE was shown to be dependent on the presence of the E/F complex in the purified *M. jannaschii* system (Ouhammouch et al., 2004). The data here confirm this result and, furthermore, demonstrate the contribution of

F subunit in TFE-mediated stimulation of transcription by recruitment of TFE to the RNAP.

In the eukaryotic transcription system, Rpb7/Rpb4 heterodimer contacts the main body of RNAPII adjacent to the linker of the C-terminal domain (CTD) of Rpb1 (Fig. 36; Bushnell and Kornberg, 2003; Armache et al., 2003). The CTD is known to have a role in recruitment of factors involved in transcription regulation, RNA capping, splicing, polyadenylation and termination (Proudfoot et al., 2002; Maniatis and Reed, 2002; Howe, 2002). It has been shown that Rpb4 interacts with Fcp1, a CTD phosphatase, and that *in vitro* interaction of Fcp1 with RNAPII is dependent on the presence of Rpb7/Rpb4 complex (Kamenski et al., 2004). This might suggest that the recruitment of regulating proteins to the RNAP is mediated by Rpb4, the eukaryotic homolog of F subunit. On the other hand, Rpb4 is not essential under optimal growth conditions, but *rpb4*Δ yeast cells are heat and cold sensitive (Woychik and Young, 1989; Rosenheck and Choder, 1998) and are unable to enter the stationary phase when encountering nutrient depletion (Choder, 1993). This further supports that Rpb4 is involved in recruitment of regulating proteins to RNAP in response to stress conditions.

Microarrays of yeast genome-wide expression profile of RNAPII mutants of yeast showed that Rpb4 has a hitherto unsuspected repressive effect on a very large number of genes. Specifically, genes related to sugar metabolism are severely down-regulated in its absence, which may explain the slow growth defect seen in *rpb4*Δ mutants (Pillai et al., 2001). These data might suggest that Rpb4 is not only involved in recruitment of regulators to the RNAP, but may itself be a regulator. This might be true also for its archaeal homolog, subunit F.

The availability of a reconstituted RNAP will allow future studies to determine whether F subunit is a regulator. The most straightforward approach to study the function of F in regulation of sugar metabolism is to use promoters of genes for sugar metabolism as templates in *in vitro* transcription assays in the presence of reconstituted RNAP lacking subunit F. Another line of studies can focus on analyzing the common structural and functional features of promoters affected by the F subunit. In addition, regions of subunit F could be manipulated with mutations to understand the finer details of how it mediates interactions with TFE and E' and how it achieves transcriptional regulation.

7. The BDLNP sub-complex associates with promoter bound TBP-TFB

Protein interactions studies of *Pfu* RNAP showed that the architecture of this enzyme is very similar to the structure of yeast RNAPII (Goede et al., 2006). To determine which part of the RNAP binds to the promoter-TBP-TFB complex, the BDLNP sub-complex of *Pfu* RNAP was reconstituted *in vitro* and analyzed in electrophoretic mobility shift assays (Fig. 23). The sub-complex was added to reactions containing preformed DNA-TBP-TFB complex and DNA alone (Fig. 23C). In the presence of promoter and transcription factors, BDLNP showed a lower electrophoretic mobility than the DNA-TBP-TFB complex. The archaeal RNAP formed a larger complex of lower electrophoretic mobility in EMSA with promoter bound TBP-TFB. TBP-TFB formed a distinct complex with the *gdh* promoter. These results demonstrate that the BDLNP sub-complex is able to form a

stable complex with promoter bound transcription factors TBP and TFB. This suggests that BDLNP sub-complex is sufficient to recruit the RNAP to the promoter bound TBP and TFB to form preinitiation complex assembly. Apparently, A´, A´´, H, K, E´ and F are not required for RNAP recruitment.
It was proposed from structural studies that the dock domain (Fig. 36) formed by Rpb1 (homologous of archeal RNAP subunits A' and A") is the target binding site of TFIIB in the preinitiation complex (Cramer et al., 2001; Armache et al., 2003). Based on functional studies, Hahn et al. suggested that in the PIC, the TFIIB ribbon domain interacts with a surface of the RNAPII dock domain of Rpb1 (Chen and Hahn, 2003; Chen and Hahn, 2004), which is highly conserved in archaeal RNAP subunit A'. Kornberg et al. located the TFIIB B-finger deep within the active site cleft and near the Rpb2 fork loops, consistent with RNAP-TFIIB co-crystal structural data (Bushnell et al., 2004). The sequences immediately surrounding fork loop 1 in Rpb2 are well conserved in archaeal RNAP subunit B.
This novel finding suggests that the dock domain is not essential for promoter and transcription factors recognition 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) sub-complex. EMSA assay established in this work for binding B-D-L-N-P sub-complex to promoter bound TBP-TFB (Fig. 23C) might be a useful tool to identify the sites involved in recruitment of a PolII-like RNAP by mutational analyses.
Studies on bacterial transcription showed that a flexible structural element, the flap domain of β subunit, is involved in recruiting sigma factors to the bacterial promoters (Kuznedelov et al., 2002). The β flap is present and highly conserved in the RNAPII subunit Rpb2 (Cramer et al., 2001) and also in the archaeal RNAP subunit B, supporting to some extend the results presented in this work.

8. Function of RNAP loop structural elements

The relative simplicity of the archaeal transcriptional machinery, combined with its close structural similarity to the core components of the eukaryotic RNAPII system, and the availability of *in vitro* reconstituted RNAP offer an opportunity for mutational studies to investigate the functional role of specific structural elements revealed by the high resolution structures of RNAPII, regardless of compatibility with cell viability.
The structure of the RNAPII elongation complex was recently resolved at 4 Å resolution (Kettenberger et al., 2004). This structure revealed four prominent loops, located in the cleft, that interact with both ends of the transcription bubble and with the RNA-DNA hybrid. These loops were thought to perform important functions of the enzyme.
During transcription, DNA strands are separated around the active center of RNAP to form the transcription bubble. The 3'end-proximal portion of the nascent RNA forms a hybrid with the DNA template strand within the transcription bubble. The nascent RNA is displaced from the DNA template upstream and the DNA strands re-anneal to form double-stranded upstream DNA. Simultaneously, RNAP and the transcription bubble move downstream along the DNA. The RNA-DNA hybrid size is 9-12 bp in an early archaeal elongation complex (Spitalny and Thomm, 2003) and 8-9 bp in bacterial elongation complex (Nudler et al., 1997; Korzheva et al., 1998). The structure of RNAPII

elongation complex reveals interactions between four prominent RNAP loops, lid, rudder, fork1 and fork2, and the transcription bubble close to the catalytic site (Fig. 7; Kettenberger et al., 2004). The lid loop appears to act as a wedge to separate the upstream end of the hybrid. The rudder is located at the upstream edge of the DNA-RNA hybrid, where the DNA template strand separates from the RNA transcript and re-anneals with the nontemplate strand. Fork1 loop interacts with rudder loop, and therefore is supposed to have a stabilizing function. Fork2, based on its location, seems to interfere with the non template strand, and it might prevent reassociation of separated strands and stabilize the transcription bubble. Fork2 was proposed to be the site of many replacements that negatively regulate the polymerization rate (Trinh et al., 2006). These four loops are well conserved in yeast RNAPII, archaeal and bacterial RNAPs (Fig. 24A).

In collaboration with Prof. Dr. P. Cramer's group, the functions of *Pfu* RNAP structural elements homologous to RNAPII lid, rudder, fork1 and fork2 were investigated. Recombinant *Pfu* RNAP variants, harbouring the mutated subunits, were produced by *in vitro* assembly and were assayed to evaluate the contribution of these loops to the enzyme transcriptional activity.

Promoter-directed *in vitro* transcription assays of RNAP mutants showed that RNAPΔLid, RNAPΔRudder, RNAPΔFork2 and RNAPΔRudder/Fork1 were completely inactive, whereas RNAPΔFork1 was the least impaired (Fig. 26). These results indicate that lid, rudder, fork2 loops are necessary for the RNAP transcriptional activity. The deletion of fork1 loop had only a mild effect on transcription. As suggested from structural studies, these prominent loops indeed participate in the transcriptional activity of RNAP.

To determine which steps of the transcription cycle are affected by the different deletions, specific assays were designed. Results form gel mobility shift assays demonstrated that wild-type and mutant enzymes exhibited comparable activities for binding DNA/TBP/TFB complex and forming a closed pre-initiation complex (Fig. 27). To study open complex formation, $KMnO_4$ footprinting assays were performed by my colleague Patrizia Spitalny. $KMnO_4$ probing detects thymine bases in single-stranded (melted) DNA (Hayatsu and Ukita, 1967). This assay showed that open complex formation is not severely impaired by the loop mutants. Additional assays were necessary to analyze which step of the transcription cycle these RNAP loops are involved in nucleic acids separation and/or maintenance. Hence, artificial templates containing a pre-formed transcription bubble were used in *in vitro* transcription assays. Promoter templates containing the entire transcription bubble length (-10 to +3) or a shorter bubble at the start site (-3 to +1) were used in these assays in the presence or absence of TBP and TFB (Fig. 28 and 29).

In the absence of transcription factors, endogenous RNAP and wild-type reconstituted enzyme produced run-off transcripts from promoters containing the entire or the short bubble. ΔFork1 enzyme mimics the wild-type RNAP in these assays, whereas the other mutant enzymes were severely impaired.

In the presence of TBP and TFB, endogenous RNAP and wild-type reconstituted enzyme failed to produce run-off transcripts, but instead they formed substantial amounts of9-12 nt abortive products, which have the same size as the RNA-DNA hybrid in the archaeal elongation complex (Spitalny and Thomm, 2003). ΔRudder and ΔFork2 enzymes did not show any activity (Fig. 28 and 29). ΔLid enzyme was less impaired, it showed increased

abortive initiation comparable to the wild-type when a complete bubble (-10 to +3) was used (Fig. 29). However, the ΔLid activity in producing abortive products decreased strongly when the downstream short bubble (-3 to +1) was used in transcription assays. These results show that the ΔLid enzyme is catalytically proficient in the presence of the entire transcription bubble and the A' lid loop might be involved in DNA melting downstream of the transcription bubble. To support these results, complementary transcription assays with templates containing a short bubble near the upstream point of melting (-12 to -2) will be performed in the next future.
RNAP mutants could not synthesize run-off products but rather abortive products, because probably TBP and TFB could not be displaced in this complex after synthesis of the first 10-12 nt of RNA (Fig. 28B and 29B). Consequently, the RNAP could not clear the promoter and the bubble could not collapse upstream, leading to increased abortive initiation and impairment of promoter escape. Superposition of the crystallographic structures of the core RNAPII/TFIIB complex and the core elongation complex (Gnatt et al., 2001) shows that TFIIB finger domain and the DNA-RNA hybrid occupy the same location and that the RNA would clash with the TFIIB finger domain in the active site beyond synthesis of the fifth residue. When the RNA grows beyond 9 residues, a clash with TFIIB is unavoidable. RNA and TFIIB must compete for space on the polymerase saddle. If RNA wins the competition, TFIIB is ejected and the polymerase is released from the promoter to complete transcription of the gene. If TFIIB wins, initiation is abortive and must be started again. When the assay is performed in the absence of TBP and TFB, the RNA has no competitor for the saddle in the active site and run-off products were synthesized (Fig. 28B and 29B).
Rudder and lid loops were previously investigated in the bacterial transcription system in Dr. K. Severinov's laboratory. The bacterial RNAPΔRudder is unable to initiate transcription unless the template is pre-melted in the -7 to +1 region. This mutant showed substantial destabilization of transcription complexes during elongation. Length of RNA-DNA hybrid and transcription termination were not affected by RNAPΔRudder (Kuznedelov et al., 2002). It has been shown that the bacterial β' lid prevents formation of extended RNA-DNA hybrid and is not required for open promoter complex formation (Naryshkina et al., 2006). This finding is not consistent with the results obtained in this work, showing that the archaeal A' lid could be involved in DNA melting downstream the transcription bubble. The bacterial lid is not required for transcription elongation and termination (Naryshkina et al., 2006).

These are preliminary results on the function of fork1, fork2, lid and rudder loops in the catalytic activity of RNAP. More studies on elongation complex maintenance will be performed in the next future. Assays with elongation complex scaffold containing an RNA transcript will lead to understanding the function of these loops.
Future experiments involving point mutations of conserved and positive residues of these loops will shed more light on our understanding of the interactions of these loops with parts of the transcription bubble.

9. Autophosphorylation of *Pfu* RNAP

Phosphorylation analyzis of the endogenous *Pfu* RNAP shows that A' subunit is autophosphorylated (Fig. 30).
Unlike the eukaryotic RNAPII, the archaeal *Pfu* RNAP did not require hydrolysis of the β-γ bond of ATP and GTP for *in vitro* transcription at 70°C (Fig. 32). This finding supports the result obtained with *Methanococcus* RNAP, which did not require energie in form of ATP or GTP for initiation of transcription and open complex formation (Hausner and Thomm, 2001).

VI Summary

The main focus of the present work was the functional and structural characterization of *Pyrococcus furiosus* RNAP.
A protocol was developed for the purification of the endogenous RNAP to homogeneity and in a large scale. Transmission electron microscope images of this purified denzyme show that the archaeal *Pfu* RNAP can adopt different forms and it is slightly elongated in shape with a diameter of about 13-15 nm. This is in agreement with data obtained by x-ray crystallography from the eukaryotic RNAPII and the bacterial RNAP.
It was unclear whether E" protein, which is highly conserved in archaea, was a subunit of RNAP. E" was detected in cell extract of *Pfu* but it was not detected as a subunit of endogenous *Pfu* RNAP. In addition, E" did not bind to the E'/F sub-complex.
Autophosphorylation experiments of the purified endogenous RNAP show that a 100 kDa protein was phosphorylated, this protein could be the subunit A'. It has been also shown in this work that *Pfu* RNAP does not require hydrolysis of the β-γ bond of ATP or GTP for its transcriptional activity at 70°C.
The PolII-like RNAP from the hyperthemophilic archaeon *Pfu* was reconstituted *in vitro* from 11 recombinant subunits. The reconstituted *Pfu* RNAP could perform most of the steps required for *in vitro* transcription, from RNAP promoter recruitment *via* TBP and TFB to promoter escape and elongation. The specific activity of the reconstituted RNAP in promoter-dependent *in vitro* transcription assays had about 50% of the specific activity of the endogenous enzyme purified from *Pfu* cells.
The availability of the RNAP reconstitution system allowed the analysis of the contributions of various subunits to *Pfu* RNAP activity, structure and stability. K, E' and F subunits were not required, whereas, N, P and H subunits were necessary for the transcriptional activity of the enzyme at 70°C. Subunit N is important for the stability of the enzyme at high temperatures. At 60°C, the activity of the reconstituted core enzyme (assembled in the absence of E´ and F) was sevenfold stimulated by addition of subunit E´, which was shown to catalyze open complex formation during transcription initiation at low temperatures.
Gel filtration chromatography showed that E' and F were not present in stoichiometric amounts in the reconstituted RNAP and that E' and F co-eluted as a separate heterodimer complex. This explain that *Pfu* RNAP can exist as a 12-subunit holoenzyme complex or as a 10-subunit core with a dissociable E'/F sub-complex. F subunit was shown to interact strongly with the transcription factor TFE, and it might be involved in recruitment of this factor to the core enzyme.
A sub-complex of RNAP consisting of subunits B, D, L, N and P was also reconstituted *in vitro*. The BDLNP sub-complex was shown to be able to form a stable complex with promoter bound transcription factors TBP and TFB.
The availability of *in vitro* reconstituted *Pfu* RNAP system offers an opportunity for mutational studies of structural elements identified in the high resolution structure of RNAPII. The functions of *Pfu* RNAP structural elements homologous to RNAPII lid, rudder, fork1 and fork2 were investigated. Recombinant *Pfu* RNAP variants, harbouring the mutated subunits, were produced by *in vitro* assembly and were assayed to evaluate the contribution of these loops to the transcriptional activity of the enzyme.

The RNAP structural elements Lid, Rudder, Fork2 loops are necessary for the transcriptional activity of RNAP. The deletion of Fork1 loop has only a mild effect on the transcription. As suggested from structural studies, these prominent loops participate indeed in the transcriptional activity of RNAP.

The ΔLid enzyme showed increased abortive initiation comparable to the wild-type when a complete pre-opened bubble (-10 to +3) was used as a template. However, the ΔLid activity in producing abortive products decreased strongly when the downstream short bubble (-3 to +1) was used as a template in transcription assays. These results show that the ΔLid enzyme is catalytically proficient in the presence of the entire transcription bubble and the A' lid loop might be involved in DNA melting downstream of the transcription bubble.

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VIII Appendix

1. List of abbreviations

A	adenosine
Å	Ångstrøm (10^{-10} m)
A_{280}	Absorbance at 280 nm
aa	amino acid
ATP	adenosine triphosphate
Bp	base pair
BLAST	Basic Local Alignment Search Tool
BRE	TFIIB or TFB Recognition Element
BSA	bovine serum albumin
C	cytosine
CTD	carboxy terminal domain of RBP1
CTP	cytosine triphosphate
Da	Dalton(g mol^{-1})
DNA	2'-deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	3'-deoxyribonucleoside-5'-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetic acid
EM	electron microscopy
EMSA	electrophoretic gel mobility shift assay
G	guanosine
GTP	guanosine triphosphate
H	hour
IPTG	isopropyl-β-thiogalactopyranoside
kD	kilo Dalton
LB	Luria-Broth medium
M	mol l^{-1}
Min	minute
mRNA	messenger RNA
Mc. jannaschii	*Methanococcus jannaschii*
Mc. thermolithotrophicus	*Methanococcus thermolithotrophicus*
MW	molecular weight
NiNTA	nickel-nitrilotriacetic acid
Nt	nucleotide
NTP	nucleotide triphosphate
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiosus*
P. furiosus	*Pyrococcus furiosus*
pI	isoelectric points
PIC	pre-initiation complex
Pol I/II/III	DNA dependent RNA-polymerase I/II/III
Poly[d(AT)]	poly-desoxy-Adenin-desoxy-Thymin
Poly[d(I-C)]	poly-desoxy-Cytosin-desoxy-Inosin
PMSF	phenylmethyl-sulphonyl fluoride
PVDF	polyvinylidenfluorid
RNA	ribonucleic acid
RNAP	DNA dependent RNA-polymerase
RNase	ribonuclease
rRNA	ribosomal RNA
RT	room temperature
RPB1-12	RNA polymerase B: subunit 1-12 of RNAP
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecylsulphate
Sec	second
SnRNA	small nuclear RNA
T	thymidine
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA buffer
TBP	TATA-box binding protein
TE	Tris-chloride/EDTA (10:1)
TEMED	N,N,N',N'-tetramethylethylenediamine
TFB	transcription faktor B
TFIIB	transcription factor B of PolII
TFIID	transcription factor D of PolII
TFIIE	transcription factor E of PolII
TFIIF	transcriptionsfaktor F of PolII
TFE	transcription faktor E
TFS	transcription faktor S
tRNA	transfer RNA
$tRNA^{Val}$	tRNA for valin
TTP	thymidine triphosphate
U	units
UTP	uridine triphosphate
UV	ultraviolet
v/v	percentage volume to volume
w/v	percentage weight to volume
wt	wild type

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3. Publications

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