Abstract
The recent success in reconstitution of RNAPs (RNA polymerases) from hyperthermophilic archaea from bacterially expressed purified subunits opens the way for detailed structure-function analyses of multisubunit RNAPs. The archaeal enzyme shows close structural similarity to eukaryotic RNAP, particularly to pol II, and can therefore be used as model for analyses of the eukaryotic transcriptional machinery. The cleft loops in the active centre of RNAP were deleted and modified to unravel their function in interaction with nucleic acids during transcription. The rudder, lid and fork 2 cleft loops were required for promoter-directed initiation and elongation. The rudder was essential for open complex formation. Analyses of transcripts from heteroduplex templates containing stable open complexes revealed that bubble reclosure is required for RNA displacement during elongation. Archaeal transcription systems contain, besides the orthologues of the eukaryotic transcription factors TBP (TATA-box-binding protein) and TF (transcription factor) IIB, an orthologue of the N-terminal part of the α subunit of eukaryotic TFIIE, called TFE, whose function is poorly understood. Recent analyses revealed that TFE is involved in open complex formation and, in striking contrast with eukaryotic TFIIE, is also present in elongation complexes. Recombinant archaeal RNAPs lacking specific subunits were used to investigate the functions of smaller subunits. These studies revealed that the subunits P and H, the orthologues of eukaryotic Rpb12 and Rpb5, were not required for RNAP assembly. Subunit P was essential for open complex formation, and the ΔH enzyme was greatly impaired in all assays, with the exception of promoter recruitment. Recent reconstitution studies indicate that Rpb12 and Rpb5 can be incorporated into archaeal RNAP and can complement for the function of the corresponding archaeal subunit in in vitro transcription assays.

Introduction
The multisubunit structure of RNA polymerase (RNA polymerase) was one of the first eukaryotic features of the archaeal transcriptional machinery to be discovered [1], and biochemical structural analyses have revealed a high similarity of essential parts of the archaeal RNAP and of pol II (RNA polymerase II) at the molecular level [2–5]. The two archaeal transcription factors TF (transcription factor) B and TBP (TATA-box-binding protein) are orthologues of the eukaryotic factors TFIIB and TBP [6–8]. A third archaeal transcription factor, TFE, is homologous with the N-terminal part of the largest eukaryotic heterotetrameric α2β2 factor TFIIE [9]. Archaeal TFE is involved in open complex formation and appears to stabilize the transcription bubble in early elongation complexes [10–12]. The recent success in reconstitution of archaeal RNAP from single subunits opened the way for detailed structure-function analyses of multisubunit RNAPs [11,13]. This system was used to investigate the function of the rudder, lid and fork 2 cleft loops, which are common to archaeal RNAP and pol II [14], and for an extensive analysis of amino acids forming the bridge helix in the cleft of RNAP [15].

The TBP not only is a component of the transcriptional machinery conserved between archaea and eukaryotes, but also can be functionally exchanged between the two transcription systems [16]. The striking similarity of archaeal and eukaryotic RNAP prompted us to investigate the potential interaction of archaeal and eukaryotic RNAP subunits. We showed recently that the two general eukaryotic RNAP subunits Rpb5 and Rpb12 can be incorporated into the archaeal RNAP (Ch. Reich, P. Milkereit, P. Cramer, H. Tschochner and M. Thomm, unpublished work). The hybrid archaeal enzyme containing the eukaryotic subunits was able to catalyse all essential steps of the transcription cycle in vitro. Our results elucidate the contribution of distinct domains and of conserved structural elements of Rpb5 and of Rpb12 in the transcription process [16a].

Mutational analysis of archaeal RNAPs
The subunit homologous with the second largest eukaryotic pol II subunit Rpb2 is the largest subunit in Pyrococcus furiosus RNAP, designated B. In Methanocaldococcus and all other methanogens, this subunit is spilt into two subunits, B’ and B” [5,17,18]. Hence, the Pyrococcus enzyme consists of 11 subunits and Methanocaldococcus jannaschii RNAP consists

Key words: archaean, RNA polymerase, transcription, transcription factor E (TFE).
Abbreviations used: pol II, RNA polymerase II; RNAP, RNA polymerase; TBP, TATA-box-binding protein; TF, transcription factor.
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of 12 subunits [19]. The cleft forming the active centre of archaeal and eukaryotic RNAPs is characterized by the rudder and lid cleft loops located in subunit A’ and the fork loops which are part of subunit B in Pyrococcus and of B” in Methanocaldococcus (Figure 1a). Structural analyses suggested that the rudder and lid are involved in stabilization of the upstream part of the DNA–RNA hybrid and that the lid additionally helps to separate RNA from DNA. Fork loop 2 was suggested to separate the DNA strands at the downstream edge of the transcription bubble by interaction with the non-coding strand. Structural analyses of yeast pol II suggested that two conserved basic residues in the switch 2 region are involved in pulling the template upwards in elongation complexes. These loops were deleted in the recombinant reconstituted Pyrococcus RNAP, and conserved residues in switch 2 and in loop 2 were replaced by alanine [11]. The bridge helix, which spans the active cleft near the active site seems to play a role by defining the position of the template strand and of the DNA–RNA hybrid. The crystal structure of an elongation complex of yeast pol II suggests that an invariant glycine residue is required for passage of the template strand into the active site. All other amino acids were predicted to decrease activity at this position owing to steric clashes of the larger side chains with the backbone of the template strand. The importance of this residue for RNAP activity was assayed by replacement of Gly825 with the other possible 19 amino acids.

These mutational studies allowed the following key conclusions. (i) The domains of RNAP contacting the nucleic acids in the active centre, such as rudder, lid, fork loop 1 and 2 and switch 2, are, with the exception of fork loop 1 essential for processive RNA synthesis. (ii) The rudder is essential for open complex formation. (iii) Lid, rudder and fork loop 2 and Arg313 in switch 2 are required for synthesis of run-off transcripts. (iv) Lid, rudder, fork 2 and the conserved Arg313 in switch 2 are required for abortive transcription.

Studies with heteroduplexes mimicking an open complex revealed that TFB formed a barrier inhibiting the synthesis of full-length RNA products by RNAP. The RNA synthesized on this template was degraded by RNase H which hydrolyses RNA in DNA–RNA hybrids. This finding indicates that bubble reclosure (which is not possible in a pre-formed heteroduplex) is required for RNA strand separation during elongation (Figure 2).

Gly825 is a key residue of the bridge helix which cannot be replaced without significant loss of function in non-specific assays.

The function of TFE in initiation and elongation

One striking similarity between archaeal RNAP and eukaryotic pol II is the existence of a loosely associated subcomplex consisting of subunits E’ and F (Figure 3) or their eukaryotic homologues Rpb7 and Rpb4 [9,20]. Previous studies revealed that this subcomplex is required for the activation of transcription by TFE [21]. Analysis of transcription initiation at 60°C, a temperature close to the lower growth temperature, using the reconstituted Pyrococcus core enzyme lacking the E’–F subcomplex, dissected the role of E’, F and TFE in initiation (Figure 3). The core enzyme is recruited by the TBP–TFB–promoter complex and melts the DNA region between positions –2 and –4. This complex is unable to initiate transcription at 60°C. Addition of E’ leads to an extension of the open region beyond the transcription start site, and this bubble opening is crucial for promoter activation. Addition of TFE increases bubble opening further, particularly in the upstream region, and this stimulates specific transcription by a factor of 14. The presence of subunit F stimulates...
transcription further, but this subunit is not required for recruitment of TFE to the pre-initiation complex [11].

The position of TFE in initiation and stalled elongation complexes was studied by photochemical cross-linking. In pre-initiation complexes, TFE is cross-linked specifically to the non-coding strand in the region upstream of the open complex, at positions −9 and −11. To investigate the fate of TFE after initiation cross-linking, permanganate footprinting was performed with complexes stalled at position +20. In eukaryotic systems, TFE is released from early elongation complexes at position +10 [22]. Surprisingly, archaeal TFE was present in stalled complexes and cross-links to positions +9, +11 and +16 of the non-template strand [12]. Moreover, the presence of TFE stimulated resumption of stalled RNAP activity and increased the potassium permanganate sensitivity of T residues in the transcription bubble. This indicates that TFE translocates with RNAP following initiation and that it acts by stabilizing the transcription bubble in elongation complexes (Figure 3E). Recent result indicate that archaeal TFE is also present in elongation complexes stalled at position +45 (S. Grünberg and M. Thomm, unpublished work).

The contribution of small subunits to RNAP function and analysis of archaeal eukaryotic hybrid RNAPs

Mutational analyses of the prominent structural elements in the cleft of the RNAP revealed some important functions of the subdomains of the two large subunits of RNAP, but the function of the smaller subunits in the catalytic function of RNAP remain obscure. Studies with the reconstituted *M. jannaschii* RNAP revealed that the reconstituted complex consisting of subunits A′-A″-B′-B″-D-L-N-P was the minimal assembly required for basic catalytic activity of RNAP [13]. The ΔN enzyme still assembled to a multisubunit RNAP complex, but contained substoichiometric amounts of subunit B′. Accordingly, the activity of this assembly was significantly impaired. The ΔP and ΔH enzyme (P and H are the orthologues of Rpb12 and Rpb5 respectively) assembled as wild-type RNAP, but showed only residual activity in non-specific and specific transcription assays [13]. Our analyses with *Pyrococcus* ΔP polymerase revealed that this enzyme is not capable of open complex formation, but
showed almost wild-type levels of activity in elongation assays (Ch. Reich, M. Zeller, P. Milkereit, W. Hausner, P. Cramer, H. Tschochner and M. Thomm, unpublished work). The addition of a C-terminal peptide containing 17 amino acids of subunit P rescued the activity of the ΔP enzyme in all assays, indicating that the C-terminal part of P is essential for the structural integrity of RNAP and contributes more to the catalytic activity than the N-terminal part containing a zinc ribbon. The ΔH enzyme was recruited by the TBP–TFB–promoter complex in a gel-shift assay, but was greatly impaired in all transcription assays (Ch. Reich, M. Thomm, P. Cramer and H. Tschochner, unpublished work).

These findings indicate that subunit H is not required for RNAP assembly and binding to the promoter, but is essential for all catalytic steps in the transcription cycle.

The great structural similarity of archaeal and eukaryotic RNAP prompted us to investigate the interaction of eukaryotic subunits Rbp12 and Rpb5 with the subunit of the *Pyrococcus* RNAP. The eukaryotic subunits show an additional domain at the N-terminus, but the C-terminal domain is conserved between the archaeal and eukaryotic RNAPs (Figure 1b, and summarized in [2]). The purified eukaryotic subunits Rpb5 and Rpb12 added to reconstitution reactions with archaeal subunits were incorporated into the RNAP complex (Ch. Reich, M. Zeller, P. Milkereit, W. Hausner, P. Cramer, H. Tschochner and M. Thomm, unpublished work). The activity of the archaeal ΔP and ΔH enzyme which was low in most assays was rescued by the addition of the orthologous eukaryotic subunits. In addition, the eukaryotic subunits rescued the activity of the archaeal delta enzymes when added to transcription reaction mixtures, indicating that these two subunits are not required for RNAP assembly. The archaeal subunits P and H could not complement the growth defects caused by deletion of subunits Rpb12 and Rpb5 in yeast, even when linked to the N-terminal domain of the corresponding yeast subunit. This finding indicates that the archaeal
enzyme subunits RNAPs cannot fulfill the more sophisticated functions in the eukaryotic cell, but incorporation of subunits H into pol II could be shown in vitro, and the purified eukaryotic enzyme containing the archaeal enzyme showed wild-type levels of activity in most in vitro assays (Ch. Reich, M. Thomm, P. Cramer and H. Tschochner, unpublished work). These results provide biochemical evidence for the similarity of archaeal RNAPs and eukaryotic pol II and for the archaeal evolutionary origin of eukaryotic RNAPs.

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References


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