RNA polymerases from Archaea and Eukaryotes consist of a core enzyme associated with a dimeric E’F (Rpb7/Rpb4) subcomplex but the functional contribution of the two subunit subcomplexes to the transcription process is poorly understood. Here we report the reconstitution of the 11-subunit RNA polymerase and of the core enzyme from the hyperthermophilic Archaeon *Pyrococcus furiosus*. The core enzyme showed significant activity between 70 and 80 °C but was almost inactive at 60 °C. E’ stimulated the activity of the core enzyme at 60 °C, dramatically suggesting an important role of this subunit at low growth temperatures. Subunit F did not contribute significantly to catalytic activity. Permanganate footprinting at low temperatures dissected the contributions of the core enzyme, subunit E’, and of archaeal TFE to open complex formation. Opening in the −2 and −4 region could be achieved by the core enzyme, subunit E’ stimulated bubble formation in general and opening at the upstream end of the transcription bubble was preferably stimulated by TFE. Analyses of the kinetic stabilities of open complexes revealed an unexpected E’-independent role of TFE in the stabilization of open complexes.

Transcription in cells of *Archaea* and *Bacteria* is catalyzed by a single RNA polymerase (RNAP), whereas eukaryotic cells contain three different types of RNAPs (I, II, and III) that carry out specialized functions. Despite their morphological similarity to *Bacteria*, *Archaea* have a transcriptional machinery that is more akin to the eukaryotic machinery (1–3).

Like eukaryotes, *Archaea* use extrinsic transcription factors for initiation. The process of transcription initiation in *Archaea* can be dissected in three steps. The general transcription factor TATA-binding protein (TBP) interacts with the archaeal TATA box, transcription factor B (TFB) stabilizes the binding of TBP to the promoter. The TBP-TFB promoter complex mediates recruitment of RNAP (4, 5). These extrinsic factors show the major structural features of eukaryotic TBP and TFIIB and interact with DNA and RNAP in a similar fashion as their eukaryotic counterparts (6, 7). This exceptional degree of similarity between the archaeal and eukaryotic transcriptional machineries extends also to the RNAP.

Archaeal RNAP consist of 11 or 12 different subunits (8–10) that display a high level of primary sequence similarity to the subunits present in eu karyotic RNAPII. With the exception of subunits RPB8 and RPB9, orthologs of other RNAPII subunits have been identified in all archaeal genomes studied so far. A recent in silico study revealed a high degree of conservation of subunits shared by pol II and *Archaea* in particular in the regions of RNAP comprising the catalytic center and protein-protein binding studies showed a similar pattern of subunit interactions between the subunits of pol II and of *Pyrococcus* RNAP (11). Although very similar to eukaryotic RNAP in subunit composition and transcription initiation factor requirement, the archaeal machinery is less complex than the eukaryotic machinery. Equivalents of the eukaryotic factors TFIIE and TFIIB are missing in archaeal genomes. Eukaryotic TFIIE is a heterodimer consisting of subunits α and β (12). The archaeal homolog of TFIIE, TFE, is monomeric and corresponds to the essential N-terminal half of TFIIEα, which is required for basal and activated transcription in vitro and interacts with TBP and RNAPII (13, 14). Archaeal TFE was reported to stimulate transcription on some weak promoters in vitro, in particular at suboptimal concentrations of TBP (15, 16). The crystal structure of eukaryotic RNAPII has been solved (17–19) but many key questions concerning structure-function relationships in eukaryotic RNAPs cannot be addressed because an active eukaryotic RNAP could not yet be reconstituted from single subunits or subcomplexes. Recently, the reconstitution of the archaeal RNAP from the methanogen *Methanocaldococcus jannaschii* has been reported (20) and was used as a tool to define the minimal set of subunits for an active pol II-like enzyme. The macromolecular complex consisting of subunits A’A”B’B”DLNP was the minimal active assembly in non specific assays and the RNAP reconstituted in the absence of subunit K or subunits F/E’ was still capable of specific transcription. The activity of the recombinant *Methanocaldococcus* enzyme was stimulated at weak promoters by archaeal TFE and this activation was dependent upon the presence of the E’/F subcomplex (Ref. 21; the orthologs of Rpb7/4) that is only loosely associated with the archaeal and/or the eukaryotic core enzyme (12, 22).

We are exploring the mechanism and regulation of transcription in the hyperthermophilic *Archaea* *Pyrococcus furiosus* (23–27). In this contribution we report the reconstitution of a
highly active RNAP from 11 bacterially expressed subunits. The reconstituted core enzyme lacking subunits E’ and F (ΔE’F) was used to investigate the effects of subunits E’, F, and TFE on RNAP activity. We found that promoter-dependent activity of the core enzyme was at low temperatures strictly dependent upon the presence of E’. Furthermore, we provide a model dissecting the roles of the core enzyme, E’, and TFE in open complex formation.

EXPERIMENTAL PROCEDURES

Cloning and Expression Procedures—The open reading frames encoding the P. furiosus RNAP subunits were amplified by PCR from genomic DNA using oligonucleotides corresponding to the 5’ and 3’ end of the open reading frames and introducing restriction sites. For the RNAP reconstitution experiments the PCR products were cloned into pET-30a-c(+) (Novagen) or pET151/D-TOPO (Invitrogen), and verified by sequencing (Geneart).

The resultant expression plasmids were transformed into BL21Star(DE3)Codon Plus (Stratagene) and expression of the subunits was induced during logarithmic growth of the cells by addition of isopropyl β-D-thiogalactopyranoside to 0.5 mM. The cultures were then incubated overnight at 20 °C.

Recombinant Subunits Purification—The His6-tagged subunits B, A’, A”, and K were highly insoluble and expressed in inclusion bodies (IB) in Escherichia coli cells. It was necessary to purify these subunits under denaturing conditions. The cell pastes were resuspended in lysis buffer A (20 mM Tris, pH 8, 1 mM PMSF, 5 mM β-mercaptoethanol, 0.3 mg/ml lysozyme) and sonicated. After centrifugation the pellets containing the IBs were extensively washed in IB buffer (20 mM Tris, pH 8, 0.5 M NaCl, 0.1% Tween 20 (Pierce), 1 mM PMSF, 5 mM β-mercaptoethanol). The IBs were solubilized in binding buffer A (20 mM Tris, pH 8, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM PMSF, 5 mM β-mercaptoethanol) for 1 h at room temperature. After centrifugation the supernatants were loaded onto a Ni-NTA column (HisTrapFF, GE Healthcare) and further washed with washing buffer (20 mM Tris, pH 8, 0.5 M NaCl, 5 mM imidazole, 6 mM urea, 1 mM PMSF, 5 mM β-mercaptoethanol). The refolding of the column-bound proteins was achieved by washing the column with decreasing linear urea gradient ranging from 6 to 0 M in refolding buffer (20 mM Tris, pH 8, 0.5 M NaCl, 10 mM imidazole, 1 mM PMSF, 5 mM β-mercaptoethanol). The refolded proteins were eluted with imidazole gradients ranging from 10 to 300 mM imidazole in refolding buffer. No further purification was required. The solubilized subunits were stored at −80 °C until they were used for reconstitution of wild type and core enzyme.

Cell pellets containing the recombinant RNAP subunits D, E’, H, F, N, L, and P were resuspended in lysis buffer B (20 mM Tris, pH 8, 0.5 M NaCl, 0.1% Tween 20, 15% glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol, 0.3 mg/ml lysozyme), sonicated, and centrifuged. The supernatants containing soluble His6-tagged D and E’ subunits were loaded on a Ni-NTA column (HisTrapFF, GE Healthcare) and the proteins were eluted with imidazole gradients from 10 to 200 mM in binding buffer B (20 mM NaHEPES, pH 7.8, 0.5 M NaCl, 10% glycerol, 0.01% Tween 20, 1 mM PMSF, 5 mM β-mercaptoethanol). To purify the thermostable subunits H, F, N, L, and P, the supernatants containing these subunits were heated for 20 min at 90 °C. The samples were centrifuged and the thermostable extract containing His6-tagged subunits F or H was loaded on Ni-NTA columns (HisTrapFF, GE Healthcare) and purified as described above. The thermostable extract containing the untagged N or L subunits were dialyzed against MonoQ binding buffer (20 mM Tris, pH 8, 10 mM NaCl, 10% glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol) and loaded on a MonoQ column (Mono Q 5/50 GL, GE Healthcare). The proteins were eluted with linear salt gradients in binding buffer ranging from 10 mM to 1 M NaCl. The thermostable extract containing the untagged P subunit was dialyzed against SP buffer (40 mM NaHEPES, pH 7, 10 mM NaCl, 10% glycerol, 1 mM PMSF, 5 mM β-mercaptoethanol) and loaded on a HiTrap SP HP column (1 ml, GE Healthcare). The proteins were eluted with salt gradients in SP buffer from 10 mM to 1 M NaCl. The untagged subunits (L, N, and P) were further purified by Superdex 75 chromatography. The concentrated proteins were loaded on a Superdex 75 column (HiLoad 16/60 Superdex 75 prep grade, GE Healthcare) equilibrated with a buffer containing 40 mM NaHEPES, pH 7.3, 250 mM NaCl, 2.5 mM MgCl2, 10% glycerol, 1 mM PMSF, and 5 mM β-mercaptoethanol.

In Vitro Assembly of the Recombinant RNAP—500 μg of all 11 subunits were combined in a final volume of 0.5 ml. The mixture was transferred to a dialysis frame (Slide-A-Lyzer 3.5k, Pierce) and denatured in transcription buffer (TB) (40 mM NaHEPES, pH 7.3, 250 mM NaCl, 2.5 mM MgCl2, 10% (v/v) glycerol, 0.1 mM EDTA, 100 μM ZnSO4, 1 mM PMSF, 5 mM β-mercaptoethanol) containing 6 M urea for 20 min. The dialysis frame with the denatured subunits was then transferred into TB with 3 M urea for another 20 min. Renaturation was achieved by dialysis in TB for 1 h. The renatured RNAP assemblies were heated for 10 min at 70 °C to remove misfolded aggregates, and loaded on an analytic Superdex 200 or onto a Superose 6 column (200/10/300 GL, GE Healthcare).

Promoter-directed in Vitro Transcription Assay—in vitro transcription assays were performed as described (27). Standard transcription reactions contained 300 ng of gdh promoter, 42 nM TBP, 55 nM TFB, and 10 nM purified or recombinant RNAP in a total volume of 25 μl of transcription buffer (40 mM NaHEPES, pH 7.3, 250 mM NaCl, 2.5 mM MgCl2, 5% (v/v) glycerol, 0.1 mM EDTA, 5 mM β-mercaptoethanol, and 0.1 mg/ml bovine serum albumin). The amounts of DNA template, TBP, TFB, and purified RNAP, reconstituted RNAP, or ΔE’F RNAP used for the experiments described in Fig. 5 are indicated in the legend. NTPs were added to a concentration of 40 μM (ATP, GTP, and CTP) and 2 μM UTP and [α-32P]UTP at 0.15 MBq (110 TBq/mmol). The components were added at 4 °C and then incubated at 70 °C for 30 min. Labeled transcripts were analyzed by electrophoresis in 6% (w/v) polyacrylamide urea gels as described (27). The transcription products were visualized using an Image Plate and Image Analyzer (FLA-500, Fuji, Japan).

Immovlized in Vitro Transcription Assays and Isolation of Stalled Ternary Complexes—Biotinylated C-minus cassettes of the gdh promoter attached to streptavidin-coated magnetic beads were used as DNA templates for stalling of transcription
complexes at position +20 (23). Stalling of complexes was achieved by omitting CTP from the nucleotide mixture. A standard reaction contained 168 fmol of immobilized DNA template, 70 nM ΔE’F RNAP, 285 nM TBP, 60 nM TFB, 40 μM ATP and GTP, 2 μM UTP, and 0.15 MBq of [α-32P]UTP (110 TBq/mmol) in 25 μl of transcription buffer not containing glycerol. Ternary complexes were isolated as reported previously (23) and all washing and resuspending steps were carried out with preheated (60–70 °C) transcription buffer. Standard immobilized transcription reactions were incubated for 30 min at 60 or 70 °C and stopped by the addition of loading dye as described (23) followed by denaturation for 3 min at 95 °C.

KMnO4 Footprinting of Initiation Complexes—Footprinting experiments were performed using immobilized templates labeled with [γ-32P]ATP on the free 5’-end of the nontemplate DNA strand. DNA templates were attached to magnetic beads via the biotin-labeled 5’-end of the coding DNA strand as described previously (23). A footprinting reaction contained 168 fmol of DNA template, 70 nM purified RNAP or 70 nM RNAPΔE’F, 285 nM TBP, and 60 nM TFB in 25 μl of transcription buffer not containing β-mercaptoethanol. Complexes were incubated for 5 min at 60 or 70 °C, isolated, washed with transcription buffer (60 or 70 °C), and resuspended in transcription buffer preheated to 60 or 70 °C. KMnO4 was added to a final concentration of 23 mM. The samples were incubated for 5 min at 60 or 70 °C as indicated. The reaction was stopped and exposed to piperidin treatment as described previously (23).

Determination of Heparin Stability of Open Complexes—The stability of open complexes was measured using a modification of a heparin challenge assay (28). 26 nM RNAP or 58 nM ΔE’F RNAP were preincubated 5 min at 60 or 70 °C as indicated in Fig. 5C with 286 nM TBP, 60 nM TFB, and 200 ng of gdh promoter in transcription buffer in 50-μl reactions. Heparin was added to the reaction to a final concentration of 800 μg/ml. At this concentration of heparin single round conditions were established (data not shown). At various times 10-μl aliquots were transferred to tubes containing 5 μl of NTP mixture (40 μM ATP, GTP, CTP, 2 μM UTP, 0.09 MBq of [α-32P]UTP (110 TBq/mmol)) in transcription buffer and transcribed 10 min at 60 or 70 °C as indicated. Reactions were stopped by the addition of loading dye following denaturation for 3 min at 95 °C. For the 100% value, an aliquot was taken prior to heparin addition and incubated with NTPs as described above.

RESULTS

Purification and in Vitro Assembly of the P. furiosus RNAP Subunits—All P. furiosus RNAP subunits were expressed and purified in recombinant form in E. coli. The largest subunits B, A’, and A” and the small subunit K were insoluble and were purified under denaturing conditions. The other subunits were soluble. The purified subunits analyzed by SDS-PAGE and Coomassie staining are shown in Fig. 1.

For the in vitro assembly assay all 11 subunits were combined in equimolar amounts. When this fraction was assayed in specific transcription assays in the presence of TBP and TFB using the homologous glutamate dehydrogenase (gdh) promoter as template a run-off transcript was detected (Fig. 2A, lane 1). This result provides evidence that an active Pyrococcus RNAP can be reconstituted simply by combining all purified subunits. This fraction was subjected to stepwise dialysis against transcription buffers containing decreasing urea concentrations (6, 3, and 0 m). After this step the specific activity of this fraction was greatly increased (Fig. 2A, lane 2). This finding indicates that the denaturation and renaturation procedure enhances the formation of specific protein-protein contact between RNAP subunits. When this fraction was heated for 10 min at 70 °C to remove misfolded aggregates as described (20) the specific RNAP was slightly reduced (Fig. 2A, lane 3) indicating that the heat treatment and centrifugation step leads also in part to denaturation of active RNAP molecules. The assembled complexes after the heat treatment step were purified by Superdex 200 column chromatography (Fig. 2B).

Superdex 200 size-exclusion chromatography showed that the RNAP eluted as a single homogenous peak. This RNAP eluted in the same fractions as endogenous RNAP and the marker protein ferritin (molecular mass 440,000). When the activity of RNAP was assayed in specific run-off transcription assays performed at 70 °C, fractions 18–24 of the column were capable of specific transcription (Fig. 2B). When the activity in the various fractions was analyzed the highest activity was found in later eluting fractions 22–24, which did not contain the highest amount of RNAP. The subunit composition of the various fractions did not differ significantly but the reconstituted RNAP contained substoichiometric amounts of subunits E’ and F (Fig. 3, lane 1). Subunits E’ and F eluted as a distinct nearly stoichiometric complex in fractions 33 and 34 (data not shown). When subunit E’ was added to the various fractions and the synthesis of run-off transcripts was studied at 60 °C, a clear E’-induced activation of transcription was observed in all fractions (Fig. 2B, lower panel). The E’-dependent stimulation of transcription was increased in the later eluting fractions rep-
represents the enzyme fractions with higher specific activity. The mechanism of E'-induced stimulation of transcription will be addressed in the following sections of this paper in more detail but these experiments indicate that two fractions of reconstituted RNAP exist, one fraction with lower specific activity (~2.2 nmol of UMP/mg) that was only slightly activated by E' and a second fraction of high specific activity (~6.9 nmol of UMP/mg) that was strongly activated by E'. The active fraction could be also separated from the less active fraction by Superose 6 gel filtration chromatography (data not shown). The reason for the different activities of these fractions is presently unclear. Probably, the first eluting and less active RNAP fraction contained still some aggregated subunits and subunits containing misfolded domains or helices that were inactive or less active.

To investigate the interaction of subunits E' and F with the active core of RNAP in more detail, the RNAP was reconstituted in the absence of these subunits. This ΔE'F RNAP showed a similar elution profile like the enzyme reconstituted from 11 subunits (Fig. 2C). An active RNAP was eluted in fractions 19–26. This finding indicates that the enzyme lacking these subunits was capable of synthesizing specific run-off transcripts. The ΔE'F RNAP is therefore also designated as core enzyme here. The most active fractions of the core enzyme were eluting later (fractions 23–26) indicating that the lower activity of fractions 19–21 containing higher amounts of RNAP (see SDS-PAGE of RNAP fractions in Fig. 2B) was not caused by a lower content of subunits E' and F in these fractions. Although both fractions appear to be suitable for characterization of the reconstituted ΔE'F enzyme we used the RNAP with higher specific activity for the more detailed analyses of the E'-core enzyme interaction described later in this paper. When the activities of the reconstituted and the core enzyme were compared under standard conditions (70 °C) with that of the endogenous RNAP, the less active fractions of reconstituted RNAP showed ~20%, the highly active fractions 50% of the activity of the endogenous RNAP (Fig. 2D). Fraction 25 of the core enzyme displayed ~40% of the activity of the endogenous RNAP. At 60 °C, the activity of the endogenous RNAP was reduced by a factor of ~2, that of the more active reconstituted RNAP by a factor of ~4, and that of the core enzyme by a factor of ~8 (Fig. 2D). Synthesis of the run-off transcripts was strictly dependent upon the presence of TBP and TFB. This finding indicates that the run-off products were synthesized as an Archaea RNAP dependent upon the presence of Archaea transcription factors. This result and our finding that no RNA synthesis was observed at temperatures <55 °C (data not shown) exclude the possibility that the activity observed is due to E. coli RNAP.

**Recombinant RNAP Activity in Promoter-directed in Vitro Transcription**—To characterize the enzymic catalytic properties of the recombinant enzyme, we performed specific in vitro transcription assays using a variety of archaeal promoters as templates. Beside the gdh promoter analyzed in Fig. 2, the reconstituted enzyme synthesized run-off transcript from linearized templates containing the aaaa” atpase, hsp20, phr, and malE promoters from P. furiosus and the tRNAVal promoter from Methanococcus vannielii (data not shown). The recombinant enzyme was able to transcribe the gdh promoter in a temperature range from 60 to 80 °C specifically (data not shown).

These results indicated that the reconstituted enzyme is able to initiate transcription at canonical archaeal promoters containing a TATA box and a BRE element. We conclude that the recombinant P. furiosus RNAP can perform all the steps
required for specific in vitro transcription, from RNAP promoter recruitment via TBP and TFB to promoter escape and elongation. The quantification of the reconstituted RNAP activity show that this enzyme has about 40–50% (Fig. 2D and Fig. 3) of the specific activity of the endogenous enzyme purified from Pyrococcus cells (Fig. 3, lanes 1 and 3). The ΔE’F RNAP showed a specific activity of 5.3 nmol of UMP/mg of protein.

To test the functional role of individual subunits in promoter-dependent transcription, RNAP was reconstituted in the absence of one of the small subunits, K, N, H, and K. Very similar results were obtained as reported by Werner and Weinzierl (20) for the M. jannaschii RNAP. The ΔK showed almost the same level of activity like the complete enzyme, the ΔN showed low activity and leaving out subunit H or P from reconstitution reactions almost completely abolished activity (data not shown).

Subunit E’ Stimulates Transcription at Low Temperatures—Pyrococcus enzyme reconstituted in the absence of E’ and F showed reduced but still significant activity in standard transcription assays at the gdh promoter (Fig. 2D). Therefore, the E’F subcomplex is not absolutely 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 methanogen Methanococcoides burtonii are higher at low growth temperature (29) suggesting a specific role of this subunit in transcription at low temperatures. This finding had prompted us to investigate the effect of subunit E’ on transcription of endogenous and reconstituted Pyrococcus RNAP at low temperatures (see also Fig. 2B and Fig. 4). At 55 °C, the activity of the endogenous RNAP was very low and only slightly stimulated by E’ (Fig. 4A). At 60 °C, the weak basal activity levels were strongly stimulated upon addition of E’. The activity was increased with the amount of E’ added and was maximal in the presence of 300 ng (553 nm) of E’ per reaction.

At 70 °C, the activity of RNAP was high and only moderately stimulated by E’ (Fig. 4A). As reported for some other archaean promoters a ~1.5 stimulation of specific transcription from the gdh promoter was observed after addition of TFE and this activation was further increased slightly when both E’ and TFE were added to transcription reactions (Fig. 4A, right panel). These findings suggest a specific effect of subunit E’ on transcription at low temperatures and therefore transcription at 60 °C was analyzed in more detail.

At 60 °C the activities of reconstituted and the core enzyme were stimulated significantly by the addition of increasing amounts of E’ (Fig. 4B). In the presence of 553 nm E’, the activity of the reconstituted enzyme was stimulated 3-fold, that of the core enzyme 7-fold (Fig. 4B). Compared with the endogenous RNAP the reconstituted RNAP was more susceptible to stimulation by E’. This can be explained by our finding that the reconstituted RNAP contains, in contrast to the endogenous enzyme, subunit E’ in substoichiometric amounts (see Fig. 3). When E’ was added to isolated stalled complexes, the extent of resumption of the stalled RNAP was not affected by the addition of E’ at neither 70 nor 60 °C (Fig. 4C). This finding suggests that E’ does not stimulate already initiated complexes but seems to act on the level of initiation of transcription. Because E’ can form a specific subcomplex with F also, the effect of subunit E’ on the stimulation of the core enzyme by subunit E’ was studied. The stimulatory effect of E’ on the core enzyme was not significantly increased by the addition of subunit F (Fig. 4D). This finding indicates that subunit F is not essential for the E’-mediated activation of core RNAP at 60 °C.

To investigate the effects of TFE on the activity of the core RNAP, TFE was added to reactions conducted at 60 and 70 °C. At 60 °C, TFE elicited a ~2-fold effect on the core enzyme. This TFE-dependent stimulation was optimal in the presence of 250 ng of TFE (500 nm) in the reaction and was not increased by the addition of higher amounts of TFE (Fig. 4E). At 70 °C only a very weak stimulatory effect of TFE was observed (Fig. 4E).

To investigate the effect of TFE on the E’-mediated activation of transcription at 60 °C, TFE was added to reactions containing core enzyme, E’, or F or a combination of E’ and F (Fig. 4, F and G). When TFE was added at optimal concentrations (500 nm) to reactions containing E’ transcription was increased by a factor of 14 (Fig. 4F, lane 3). The activity of core enzyme was increased by a factor of 25 when F, E’, and TFE were added in combination (Fig. 4F, lanes 2 and 9). This 25-fold stimulation was observed in the presence of 100 and 250 ng of TFE and was reduced to a 20-fold effect when more (500 ng) TFE was added (Fig. 4G). This finding indicates that TFE was contained in optimal concentrations in the reactions shown in Fig. 4F. The ~2-fold stimulatory effect of TFE was not increased by the addition of subunit F (Fig. 4F, lanes 7 and 4). Subunit F when added separately did not activate transcription (Fig. 4F, lanes 5 and 6). These findings indicate that the stimulatory effect of TFE is primarily mediated by subunit E’ but further increased...
in the presence of the heterodimeric E’F complex. Subunit F has only a minor effect on the stimulatory activity of E’ but the core enzyme combined with E’ and F is much more susceptible to stimulation by TFE than the core enzyme-E’ combination. This finding indicates an important role of subunit F in mediating the stimulatory activity of TFE.

At 70 °C, the effects of TFE on the E’-mediated activation of the core enzyme were moderate and similar as described for the M. jannaschii enzyme (21). The 1.5-fold stimulation of the core enzyme by E’ (Fig. 4H, lanes 8–10) was slightly increased by the addition of TFE (lane 3) or both TFE and F (lane 2).

Subunit E’ and TFE Stimulate Formation of the Open Complex—The findings that E’ has a strong effect on transcription at low temperatures, only a moderate effect on transcription at 70 °C, and no effect on the elongation of stalled complexes suggest that E’ acts at the level of promoter opening. To investigate this, open complex formation by the endogenous and ΔE’F RNAP was studied at 70 and 60 °C in the presence and absence of E’ by permanganate footprinting. At 70 °C, the T residues at positions −2, −4, and −6 of the nontemplate DNA strand of the gdh promoter showed high and the T residues at positions +2 and +3 showed somewhat weaker reactivity to KMnO₄ (23) (Fig. 5A, lane 2). The addition of E’ affected promoter opening by the endogenous RNAP marginally (Fig. 5A, lane 3). In the presence of both E’ and TFE in the permanganate footprinting reactions, the sensitivity of all T residues in the bubble was clearly enhanced, particularly at position −6 (Fig. 5A, lane 4; see also a quantitative analysis of the data below the figure). This finding demonstrates that TFE stimulates open complex formation in the presence of E’.

When the endogenous RNAP was incubated with the gdh promoter at 60 °C the T residue at −2 displayed significant reactivity toward KMnO₄, the T residues at positions −6, −4 and +2 and +3 were only weakly modified (Fig. 5B, lane 2). Addition of isolated subunit E’ to the endogenous RNAP increased the KMnO₄ sensitivity of the T residues between −6 and +3 considerably, indicating that E’ is involved in open complex formation too. The reactivity of all T residues was further increased upon addition of TFE to the endogenous RNAP.

The endogenous RNAP contains E’ and therefore the specific role of E’ in open complex formation can be best addressed in experiments with the core enzyme lacking this subunit. When the ΔE’F RNAP was incubated with this template at 60 °C, only a weak modification of the T residues at −2 and −4 was found (Fig. 5B, lane 5), but no modifications at positions +3, +2, or −6 could be detected. Upon addition of subunit E’, a stimulation of the reactivity of all T residues was observed. The T residues at +2 and +3 showed the highest reactivity, but the T residue at position −6 was still not modified (Fig. 5B, lane 6). This finding suggests an E’-induced opening of the bubble mainly at the transcription start site. When TFE was added in addition to E’ the sensitivity of T residues at positions −4, −2, +2, and +3 was further increased and, in addition, also the T residue at −6 was modified (Fig. 5B, lane 7). The quantitative analysis of the data below the figure corroborates that TFE further stimulates E’-induced promoter opening and in particular contributes to bubble opening at the 5’ end of the open complex. Taken together the results presented here support the conclusion that subunit E’ stimulates transcription at 60 °C by catalyzing open complex formation and that TFE stimulates bubble opening further at the upstream end of the bubble.

**TFE Stabilizes Open Complexes in the Absence of Subunit E’**—To characterize the effect of TFE on initiation by the archaean RNAP in more detail the kinetic stability of open complexes of the endogenous RNAP and of the ΔE’F enzyme was studied in the presence and absence of TFE. To measure the stability of open complexes, RNAP was incubated with the gdh promoter-TBP-TFB complex in DNA-binding complexes at 70 and 60 °C. Then, open complexes were challenged by the addition of heparin (the experimental design is outlined in Fig. 6). The fraction of remaining open complexes at various times after heparin addition was quantified by transcription assays. In these assays...
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Both at 70 and 60 °C indicates that TFE is able to interact with RNAP in the absence of subunit E' and that the TFE-mediated stabilization of open complexes can occur in the absence of subunit E'.

DISCUSSION

Here we describe the reconstitution of a pol II-like RNA polymerase from individually expressed subunits. Surprisingly, simple incubation of all purified subunits lead to the formation of an RNAP capable of promoter-specific initiation and elongation (Fig. 2), but the efficiency of RNAP reconstitution was greatly increased by denaturation of the 11 subunits in the presence of 6 M urea and stepwise renaturation at 3 M urea and finally, in the absence of urea as described for the reconstitution of the M. jannaschii enzyme (20). The reconstituted subunits B’A’A’DHLPKN eluted as a distinct macromolecular complex after size exclusion chromatography (Fig. 2).

This complex contained all RNAP subunits with the exception of E’. The strong stimulation of initiation observed was mediated by TFE when exposed to heparin (Fig. 6). Open complexes formed by both enzymes were significantly stabilized by TFE when exposed to heparin for 5–15 min. In the presence of TFE the residual activities of the endogenous enzyme and the core enzyme were ~60 and ~40%, respectively. Open complexes of the core enzyme containing E’ and TFE displayed the same sensitivity against heparin challenge as complexes containing only the core enzyme and TFE (Fig. 6). When the core enzyme was subjected to heparin challenge in the presence of E’, no stabilization was observed (Fig. 6). These findings indicate that open complexes are stabilized by TFE and not by E’.

At 60 °C, open complexes formed by the ΔE’F enzyme displayed a higher stability than at 70 °C. The remaining activity of the core enzyme after 60 min exposure to heparin was ~80%. When TFE was contained in addition in binding reactions at 60 °C no significant inactivation of open complexes was observed (Fig. 6). The addition of subunit E’ to the core enzyme had no stabilizing effect (data not shown). These results corroborate our conclusion that open complexes are stabilized by TFE. The finding that open complexes formed by the core RNAP not containing subunits E’ and F were stabilized by TFE and F in nearly stoichiometric amounts and showed a specific activity in promoter-dependent transcription assays that was comparable with that of the purified enzyme (Fig. 3). Functional studies revealed that the Rpb4/Rpb7 and C17/C25 subassemblies are required for promoter-directed initiation of eukaryotic transcription but dispensable for elongation (30–32). In contrast to the eukaryotic core enzyme the reconstituted M. jannaschii (20) and P. furiosusus 9 subunit assemblies (Fig. 2B) showed significant catalytic activity in promoter-dependent standard assays conducted at 70 °C. Our finding that the Pyrococcus core enzyme is almost inactive at 60 °C and can be dramatically stimulated by subunit E’ provides a functional definition of an archaeal core enzyme. In addition, the property of E’ in concert with TFE and (to lower extent) with subunit F to stimulate transcription at low temperatures is a useful tool to investigate the interplay of the core enzyme, E’, TFE, and F to catalytic activity in various functional assays.

The Rpb7 Ortholog E’ Stimulates Transcription at Low Temperatures by Catalyzing Open Complex Formation—The E’-F complex and its eukaryotic orthologs Rpb7/Rpb4 (RNAPII), RpC25/RpC17 (RNAPII), and Rpa43/Rpa14 (RNAPI) form heterodimers that are a landmark of all non-bacterial RNA polymerases (33–37). The structure of the Rpb7/Rpb4 complex is very similar to the archaeal E’F dimer structure (22, 17) and to the RNAPII counterpart of these subunits, C17/C25 (37, 38). Their similar structure strongly argues for their functional equivalence. E’ had no effect on the elongation of stalled complexes (Fig. 4C) indicating that it works on the level of initiation. The strong stimulation of initiation observed was mediated by...
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E' alone (Fig. 4, A and B). This finding demonstrates that complex formation of E' with F is not a prerequisite for its activating properties. This is a further similarity to the eukaryotic system. In Saccharomyces cerevisiae Rpb7 is an essential protein and Rpb4 is dispensable under optimal growth conditions (reviewed in Ref. 39). The major interaction of Rpb4/Rpb7 with the core enzyme occurs via the tip loops of Rpb7 and this interaction does not involve Rpb4 (39, 40). Our finding that E' stimulates transcription in the absence of E' suggests that E', like Rpb7, can interact as monomer with the core enzyme. The slight stimulation of E'-dependent activation of the core enzyme by the addition of F (Fig. 4D) suggests that subunit F may facilitate the association of E' with the core enzyme. Hence, weak not yet identified contacts of F with the core enzyme may stabilize the interaction of E' with the core enzyme. The finding that the presence of F further stimulates transcription of the core enzyme in reactions containing E' and TFE (Fig. 4E, lane 2) suggests that F facilitates TFE recruitment.

A Dual Role of TFE in Initiation—First studies suggested that archaeal TFE stabilizes interactions between TBP and the TATA box (5, 16). Recent work showed that TFE increases RNA polymerase elongation complex formation in electrophoretic mobility shift assays (41) and functionally interacts with the E'F subcomplex (21). Our KdMnO4 footprints at 70 °C (Fig. 5A) demonstrated that TFE leads to an increased complex stability by promoting DNA melting similar to the properties reported for eukaryotic TFIIE (12, 24, 42). The roles of E' and TFE in open complex formation were better resolved when initiation by the Pyrococcus core enzyme was analyzed at 60 °C. The 7-fold activation of initiation by E' was further increased to 14-fold by TFE and the stimulation of initiation was 25-fold in the presence of both E'F and TFE (Fig. 4, D and G). This finding demonstrates that E' and TFE work synergistically during activation. Analyses of kinetic stabilities of open complexes (Fig. 6) show a clear E'-independent stabilizing effect of TFE on the core enzyme. This finding indicates that the presence of E' is not required for TFE recruitment to the preinitiation complex. Although TFE stabilizes open complexes by the core enzyme significantly in the absence of E' (Fig. 6), the major stimulatory effect of TFE on transcription at 60 °C strictly requires the presence of subunit E' (Fig. 4F, lane 3). The data shown here demonstrate that TFE stabilizes open complexes formed by the endogenous RNA polymerase and the core enzyme. Furthermore, at low temperatures TFE converts a partially active to a fully open complex (Figs. 4F, lane 3; 5B, lanes 4 and 7; and 6). Our data also identify subunit E' as the major target for the TFE-induced stimulation of transcription.

The Interplay of Core Enzyme, E', and TFE during Initiation—Structural analyses revealed the presence of a deep cleft in pol II comprising the DNA-binding site and active center (17). One side of the cleft is formed by a mobile clamp that is open in the structure of the 10-subunit core enzyme and closes the cleft in elongation complexes that traps the template DNA inside. Although the role of Rpb4/7 in closure of the clamp during elongation is not known, Rpb7 forms a wedge between the mobile clamp and the linker to the C-terminal repeat and restricts the clamp to the closed position even in the absence of DNA (18, 19). Hence, an interaction of Rpb7 with the clamp during initiation is very likely but the role of this interaction and the exact function of Rpb7 in initiation are unclear. The observation that the intracellular levels of E' increase during growth at low temperatures (29) suggest that this E' facilitates transcription at low temperatures and our present data show a role of E' in open-complex formation. It is unclear whether E' is essential for growth of archael cells but cell-free transcription data demonstrate that E' is dispensable for cell-free transcription of the gdh promoter at temperatures between 70 (Fig. 4A) and 80 °C, but the presence of E' is crucial at 60 °C (Fig. 4). Analyses of open complex formation at the gdh promoter provide an explanation of the stimulatory roles of E' and TFE in initiation and identify a major function of E' during initiation of transcription. E' also facilitates open complex formation by the purified RNA polymer core enzyme by the addition of TFE containing E'/F but the distinct roles of E' and TFE during bubble opening were elucidated by permanganate footprinting experiments with the core enzyme at 60 °C. The presence of the core enzyme leads to a weak modification of T residues at positions −2 and −4. This melting in this small region is not sufficient to support initiation but shows that the core enzyme interacts with promoter-bound transcription factors and promoter DNA. Therefore, E' is not required for stable recruitment of the archaeal RNA polymer core enzyme, like Rpb4/7 is not required for recruitment of pol II (31). Addition of E' stimulates promoter opening in general and leads to an extension of the open region beyond the transcription start site. Most likely bubble opening in this region is crucial for promoter activation, because addition of E' to the core enzyme stimulates initiation dramatically (see Fig. 4, B and F). The addition of TFE further increased bubble opening particularly in the upstream region at position −6 (Fig. 5, A, lane 4, and B, lane 7) and this leads to a further dramatic stimulation of the initiation rate (compare in Fig. 4F, lanes 3 and 10). Recently, cross-linking experiments have revealed that TFIIEα approaches promoter DNA at position −10 and that artificial premelting of the DNA in the −11/−1 and in particular in the −7/−8 region increased the stimulating effect of TFIIEα on abortive transcription (42). Although the E' catalyzed promoter opening may not be required under normal growth conditions of the hyperthermophilic Archaea Pyrococcus, the findings reported here have important implications for the mechanism of transcription by a pol II-like polymerase. The pol II core enzyme can be recruited to the TBP-TFIIIB-promoter complex and the presence of Rpb4/7 restricts the mobile clamp of RNA polymerase to the closed position. The findings that association of E' with the promoter-bound archael core enzyme induces further opening of the region slightly melted at −2 by the core enzyme beyond the transcription start site suggest that binding of E' to the core enzyme induces clamp closure. The presence of TFE seems to stabilize clamp closure further and E'-mediated contacts of TFE with the core enzyme catalyze further opening of the bubble at the upstream end. Considering the structural similarities between E'F and Rpb7/4 the novel role of subunit E' elucidated in the present work is likely to be also of general

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significance for the mechanism of initiation by eukaryotic RNAPs.

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