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Temperature, template topology, and factor requirements of archaeal transcription

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ABSTRACT **Although Archaea are prokaryotic and resemble Bacteria morphologically, their transcription apparatus is remarkably similar to those of eukaryotic cell nuclei. Because some Archaea exist in environments with temperatures of around 100°C, they are likely to have evolved unique strategies for transcriptional control. Here, we investigate the effects of temperature and DNA template topology in a thermophilic archaeal transcription system. Significantly, and in marked contrast with characterized eucaryal systems, archaeal DNA template topology has negligible effect on transcription levels at physiological temperatures using highly purified polymerase and recombinant transcription factors. Furthermore, archaeal transcription does not require hydrolysis of the β-γ phosphoanhydride bond of ATP. However, at lower temperatures, negatively supercoiled templates are transcribed more highly than those that are positively supercoiled. Notably, the block to transcription on positively supercoiled templates at lowered temperatures is at the level of polymerase binding and promoter opening. These data imply that Archaea do not possess a functional homologue of transcription factor TFIIH, and that for the promoters studied, transcription is mediated by TATA box-binding protein, transcription factor TFB, and RNA polymerase alone. Furthermore, they suggest that the reduction of plasmid linking number by hyperthermophilic Archaea *in vivo* in response to cold shock is a mechanism to maintain gene expression under these adverse circumstances.**

In the past few years, it has become apparent that Archaea represent a considerable and important proportion of the biomass, with representatives being found in a wide range of environments (1). The hyperthermophilic Archaea are of particular interest because the inherent thermostability of their proteins has industrial relevance, and analysis of this property is likely to lead to important insights into our understanding of protein structure. The DNA of mesophilic Archaea appears to be essentially always negatively supercoiled, as is the case in Bacteria and Eucarya (2). In contrast, the DNA of hyperthermophilic Archaea ranges from relaxed to positively supercoiled under normal growth conditions (3, 4), suggesting that this regulation is an adaptation to living at high temperatures. Recent work has demonstrated that hyperthermophilic Archaea modulate DNA topology in response to certain environmental cues, raising the possibility that this modulation is used as a regulatory mechanism (5, 6). For example, subjecting *Sulfolobus* to a cold shock causes a rapid and dramatic reduction in plasmid linking number (6).

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Previous work has indicated that transcription in Archaea is fundamentally homologous to that in Eucarya (7–9). Thus, Archaea possess homologues of the eukaryotic TATA-binding protein (TBP) (10) and the basal transcription factor, TFIIB (known in Archaea as TFB or aTFA) (11, 12). Archaea also possess an RNA polymerase (RNAP) of similar complexity and subunit composition to eucaryal RNAPs (8). In fact, sequence comparisons indicate that the two largest subunits of the archaeal enzyme are more closely related to those of eucaryal RNAPII than the RNAPII subunits are to their homologues in either RNAPI or RNAPIII. Furthermore, the structure of the TBP/TFB/DNA ternary complex has been solved recently by x-ray crystallography (13) and demonstrates striking similarity to the eucaryal TBP/TFIIB/DNA structure (14). Recently, we and others (15–17) have shown that archaeal TBP, TFB, and RNAP are necessary and sufficient to mediate transcription from a range of archaeal promoters on negatively supercoiled plasmids *in vitro*.

Extensive studies of the effects of template topology on transcription have been performed in eucaryal and bacterial transcription systems and, despite there being some specific exceptions (e.g., ref. 18), a general rule has emerged that transcription levels increase with increasing negative supercoiling of the DNA template (reviewed in ref. 19). Furthermore, studies of eucaryal transcription have shown that, although certain negatively supercoiled templates are transcribed by TBP, the basal transcription factor TFIIB, and RNAP II alone, transcription of topologically relaxed or linearized templates by RNAPII requires the full complement of basal transcription factors in a reaction that depends on the hydrolysis of the β-γ phosphoanhydride bond of ATP (20–23). These data raise the possibility that analogues of other eucaryal basal transcription factors exist in hyperthermophilic Archaea to allow the transcription of DNA in certain topological states.

MATERIALS AND METHODS

Proteins, Plasmids, and *in Vitro* Transcription. *Sulfolobus* RNAP was purified to essential homogeneity as described (16). *In vitro* transcription reactions were performed by using 300 fmol of TBP and TFB and 1 pmol of RNAP on 40 fmol (100 ng) of plasmid template as described (16). *Sulfolobus shibatae* extract was prepared as described (24). Transcription products were detected by primer extension using radiolabeled T7 sequencing primer (16).

Preparation of Positively Supercoiled Topoisomers. Negatively supercoiled plasmid (10 μg) was incubated at 75°C for 35 min with 1,750, 3,500 or 14,000 units of reverse gyrase purified from *S. shibatae* (25) in a 40-μl reaction mixture containing 50

Abbreviations: TBP, TATA box-binding protein; TF, transcription factor; RNAP, RNA polymerase.

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mM Tris·HCl (pH 8.0), 0.5 mM DTT, 0.55 mM Na_2EDTA, 10 mM $MgCl_2$, 1.25 mM ATP, 8% polyethylene glycol 6000, 2.5 mM NaH_2PO_4/Na_2HPO_4, 10 mM NaCl, 0.005% Triton X-100, and 30 μg/ml BSA. After incubation, preheated NaCl was added at a final concentration of 500 mM, and the incubation was continued for 2 min. SDS and Na_2EDTA were added at final concentrations of 0.9% and 9 mM, respectively. DNA was extracted three times with 1 vol of chloroform/isoamyl alchohol (24:1) and precipitated with ethanol. DNA was resuspended in Tris-EDTA buffer at a concentration of 0.1 mg/ml.

Generation of Negatively Supercoiled DNA. Negatively supercoiled plasmid (10 μg) was incubated at 37°C for 90 min with calf thymus topoisomerase I in 200-μl reaction mixtures containing 50 mM Tris·HCl (pH 8.0), 0.55 mM DTT, 0.55 mM Na_2EDTA, 2.5 mM NaH_2PO_4/Na_2HPO_4, 200 mM KCl, 30 μg/ml BSA, and 0.8, 2, 2.8 or 3.6 μg/ml of ethidium bromide to generate pools of increasing negative superhelicity. Reactions were stopped by addition of lithium dodecyl sulfate (1% final concentration), and ethidium bromide was removed by two extractions with butanol saturated with Tris-EDTA. DNA was recovered as described above.

Electrophoresis of Topoisomers. Positively supercoiled topoisomers were resolved by electrophoresis in the first dimension in TAE buffer (40 mM Tris·acetate/1 mM EDTA) in the presence of 5 mM Mg acetate and, in the second, in the same buffer containing 5 μg/ml netropsin. Negatively supercoiled topoisomers were resolved by electrophoresis in TEP buffer (36 mM Tris·HCl/30 mM NaH_2PO_4/1 mM EDTA, pH 7.8) containing, in the first dimension, 4 μg/ml of chloroquine and, in the second dimension, 20 μg/ml of chloroquine. Before staining, the gels were treated with 0.25 M HCl for 30 min.

Definition of Specific Linking Difference (σ). Plasmid relaxed by calf thymus topoisomerase at 37°C was taken as the zero reference point. The variation in σ as a function of temperature was calculated as described (26, 27). Accordingly, the σ values quoted in the figures are for the temperature at which the relevant assay was performed.

Permanganate Modification and DNaseI Footprinting. Reactions were assembled in a total volume of 40 μl, as above, by using 20 ng of template with nucleoside triphosphates as indicated in the Fig. 6 legend. Reactions were incubated for 10 min at 48°C before the addition of DNaseI or $KMnO_4$. DNaseI footprinting was performed by adding 10 milliunits of DNaseI (Boehringer Mannheim) for 30 sec, followed by the addition of EDTA to 50 mM and SDS to 0.5%. DNA was recovered by phenol/chloroform extraction followed by salt exchange on a G50 gel filtration column. DNaseI cleavage sites were detected by primer extension analysis on 10 ng of the purified DNA. Permanganate modification essentially followed the protocol of Jiang and Gralla (28). After 10-min incubation, potassium permanganate was added to 6 mM, and incubation was continued for 3 min before quenching the reaction with 1 μl of 14.2 M β-mercaptoethanol. SDS was added to 0.5%, and DNA was recovered and analyzed as for DNaseI-treated samples.

Single-Round Transcription Reactions. Standard transcription reactions were assembled, omitting UTP. After a 7-min incubation, heparin was added to 100 μg/ml. This amount was determined empirically as a concentration that did not destabilize stalled elongation complexes, but which does inhibit reinitiation (S.D.B., unpublished data). After 30 sec, UTP was added, and elongation was allowed to proceed for an additional 5 min. RNA was recovered and analyzed as described (16).

RESULTS

At 75°C, Template Topology Does Not Affect Transcription by Archaeal TBP, TFB, and RNAP. As a first step to study the requirements for template topology in hyperthermophilic Archaea, the levels of transcription on equimolar amounts of negatively supercoiled and linearized templates containing the *S. shibatae* 16S rRNA promoter were compared. As shown in Fig. 1, these two templates are transcribed with similar efficiency to one another at 75°C, both by an unfractionated system and by a reconstituted system comprising recombinant TBP, TFB, and highly purified RNAP. However, if reactions are performed at 48°C, only the negatively supercoiled template is transcribed by the reconstituted system (Fig. 1*a*). Perhaps surprisingly, transcription of both negatively supercoiled and linear templates is virtually abolished at 48°C in the unfractionated system (Fig. 1*b*). This finding is attributable to an activity in the crude cell extract that converts the negatively supercoiled template rapidly to a relaxed or nicked form that cannot be transcribed at this temperature. Indeed, after 1 min of incubation with crude cell extract, the majority of the input negatively supercoiled template is relaxed (data not shown). Importantly, this relaxing activity is absent from the purified system, as templates recovered from reconstituted transcription reactions have unaltered template topology even after extended incubation periods (data not shown). These data indicate that, although the archaeal transcriptional apparatus is capable of transcribing negatively supercoiled DNA templates at 48°C, neither the reconstituted nor the unfractionated archaeal systems possess an activity that allows linear DNA templates to be transcribed under these conditions.

To extend these analyses, restricted ranges of plasmid topoisomers containing the promoter of the *S. shibatae* 16S rRNA and the T6 promoter of the *S. shibatae* virus SSV1 were prepared (Fig. 2 *a* and *b* and data not shown). These topoisomers, ranging from highly negatively supercoiled to highly positively supercoiled, then were used in reconstituted *in vitro* transcription reactions. Again, as shown in Fig. 3 *a* and *b*, template topology has little or no effect on transcription levels at 75°C. Indeed, even highly positively supercoiled templates are transcribed efficiently under such conditions. To rule out the possibility that transcription of relaxed and positively supercoiled templates at 75°C is made possible by the presence of an ATP-dependent helicase activity analogous to transcription factor TFIIH, we tested the effect of substituting the ATP analogue adenosine 5′-[β,γ-imido]triphosphate (AMP-PNP) for ATP in the reaction. Notably, over the range of concentrations tested, this analogue supports transcription to the same level as ATP on both positively and negatively supercoiled templates (Fig. 3 *c* and *d*). Thus, a TFIIH-like ATPase activity and hydrolysis of the β-γ bond of ATP are not required for transcription in the archaeal system.

Significantly, and in marked contrast to the results observed at 75°C, when reactions are conducted at the lower temperature of 48°C, a significant reduction is observed in transcription

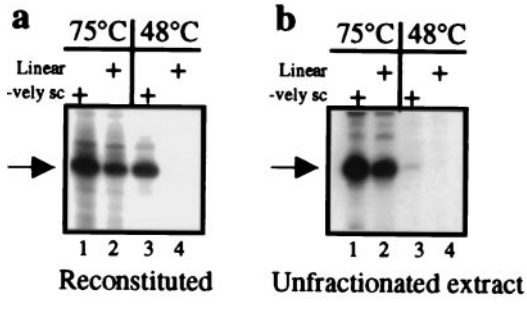

FIG. 1. Effect of template topology and temperature on *in vitro* transcription from the *S. shibatae* 16S rRNA promoter by using a reconstituted (*a*) or unfractionated (10 μg of *Sulfolobus* crude cell extract) system (*b*). Transcription of negatively supercoiled template (lanes 1 and 3) or linearized template (lanes 2 and 4) was as described in *Materials and Methods*. Transcription reactions were incubated at 75°C (lanes 1 and 2) or 48°C (lanes 3 and 4) and RNA detected by primer extension.

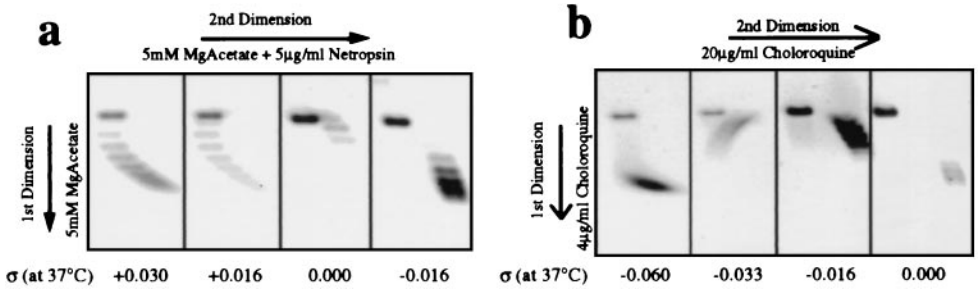

FIG. 2. Generation of topoisomer pools for use in the reconstituted archaeal transcription system. Negative image of ethidium bromide-stained two-dimensional gels resolving topoisomers of 16S promoter plasmid.

from positively supercoiled templates containing the T6 promoter (Fig. 3*e*). This effect is even more dramatic for the 16S promoter, where transcription is virtually abolished with positively supercoiled, relaxed, and even slightly negatively supercoiled ($\sigma \geq -0.026$) templates (Fig. 3*f*). Taken together, these data reveal that, at 48°C but not at 75°C, there is a specific block to transcription of non-negatively supercoiled DNA templates in the archaeal system.

The Block to Transcription Initiation on Nonpermissive Template Topologies at Reduced Temperature Is at the Level of Promoter Opening. To determine at which step transcription is inhibited on the nonpermissive templates at 48°C, a range of assays were used. First, the ability of the TBP/TFB ternary complex to form on both positively and negatively supercoiled templates was assayed by DNaseI footprinting. Conditions were used where DNaseI cut only once per template molecule and cleavage sites were detected by primer extension. No significant differences are detectable in the footprints obtained with negatively and positively supercoiled templates, indicating that ternary complex formation occurs with essentially equal affinity in each case (Fig. 4). To discriminate between events in transcriptional initiation and those in elongation, a single-round transcription assay was developed that exploited the observation that transcription initiation is abolished by addition of heparin, whereas RNAP in a stalled elongation complex is resistant to heparin addition (Fig. 5*a*). The single-round assay then was used to analyze transcription from the 16S promoter at 48°C and 75°C. Accordingly, transcription was initiated on negatively supercoiled or linear DNA at 48°C in the presence of ATP, CTP, and GTP, but in the absence of UTP, leading to an elongation complex stalled at position +6, where the first UMP would be incorporated. This preincubation was followed by the addition of heparin to prevent further initiation. Aliquots of the stalled complexes were next kept at 48°C or switched to 75°C, and then UTP was added to overcome the elongation block (Fig. 5*b*, lanes 1–4). Analogous experiments were performed with stalled complexes formed at 75°C (Fig. 5*b*, lanes 5–8). Notably, the results of these studies show that, whereas the polymerase is essentially equally processive at 75°C and 48°C in such assays, no detectable productive stalled complexes are formed on linear DNA at 48°C. This finding indicates that the inhibition of transcription on non-negatively supercoiled templates at 48°C occurs before the formation of the first five phosphodiester bonds in the nascent RNA.

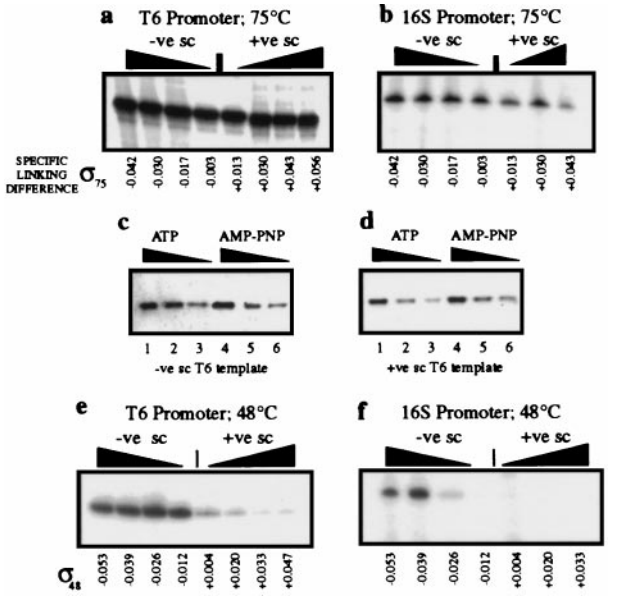

FIG. 3. Transcription reactions using discrete topoisomer pools in the reconstituted *Sulfolobus* system. Transcription reactions using T6 topoisomer pools, at 75°C (*a*) or 48°C (*e*). The specific linking difference (σ) values, calculated as described in *Materials and Methods*, are given under the appropriate lanes. (*b* and *f*) Transcription reactions using 16S pools at 75°C and 48°C as indicated. (*c* and *d*) Effect of substituting adenosine 5′-[β,γ-imido]triphosphate (AMP-PNP) for ATP in the reconstituted transcription reaction on negatively supercoiled ($\sigma = -0.042$) T6 template (*c*) and positively supercoiled ($\sigma = +0.056$) T6 template (*d*) at 75°C. Lanes 1–3 contain 100, 50, and 25 μM ATP, respectively, together with 100 μM GTP, CTP, and UTP. Lanes 4–6 contain 100, 50, and 25 μM AMP-PNP, respectively, together with 100 μM GTP, CTP, and UTP.

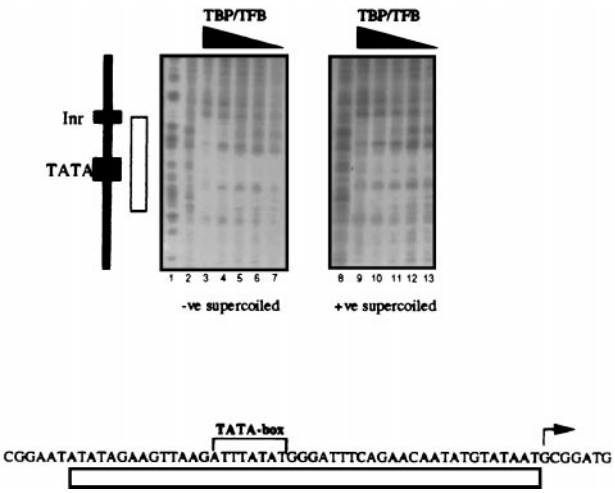

FIG. 4. DNaseI footprinting of archaeal TBP and TFB on the template strand of negatively supercoiled ($\sigma = -0.053$) 16S (lanes 1–7) and positively supercoiled ($\sigma = +0.047$) 16S templates (lanes 8–13). Lane 1 contains a ddA sequencing ladder. Reactions contain 0 pmol (lanes 2 and 8), 3 pmol (lanes 3 and 9), 1.5 pmol (lanes 4 and 10), 0.75 pmol (lanes 5 and 11), 0.38 pmol (lanes 6 and 12), and 0.19 pmol (lanes 7 and 13) of an equimolar mix of TBP and TFB. The region protected from cleavage by DNaseI is indicated by an open box; the TATA and initiator elements are shown as filled boxes.

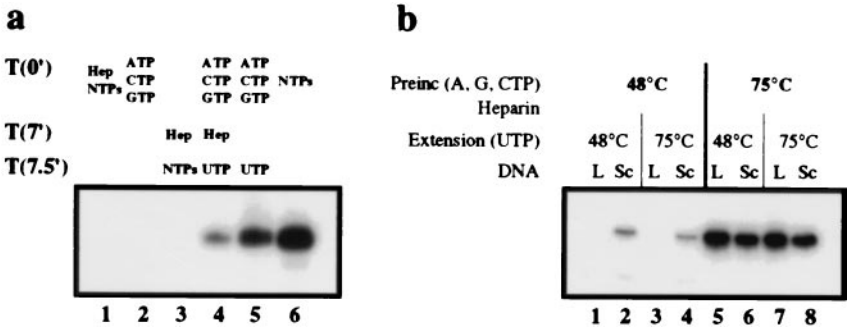

FIG. 5. Single-round transcription assays. (*a*) Establishment of single-round assay. Transcription of negatively supercoiled 16S promoter was conducted at 75°C with various orders of addition of heparin and NTPs, as indicated. (*b*) Linear (lanes 1, 3, 5, and 7) or supercoiled (lanes 2, 4, 6, and 8) template was preincubated with TBP, TFB, and RNAP in the presence of ATP, CTP, and GTP at 48°C (lanes 1–4) or 75°C (lanes 5–8). Heparin was added to prevent reinitiation, and reactions were incubated at 48°C (lanes 1, 2, 5, and 6) or 75°C (lanes 3, 4, 7, and 8) for an additional 5 min.

Finally, the ability of the preinitiation complex to isomerize to the open complex, which contains unwound DNA in the vicinity of the transcription initiation site, was assayed by testing the ability of RNAP to induce sensitivity to potassium permanganate modification of unpaired thymidine bases in single-stranded DNA. Initial studies using negatively supercoiled 16S rRNA promoter templates revealed that, in the presence of TBP, TFB, and RNAP, thymidine residues between position −1 and −12 on both coding and noncoding strands become sensitive to chemical modification by permanganate (Fig. 6 *a* and *c*). Thus, the archaeal RNAP brings about the opening of promoter DNA in the absence of ATP hydrolysis. This response is therefore similar to the situation for RNAPI, RNAPIII, and bacterial RNAP, but is in marked contrast to that for RNAPII. Addition of GTP and CTP allows the polymerase to synthesize RNA to position +5. This predicted movement of the polymerase results in an alteration of the pattern of modification by permanganate. Specifically, thymidines at positions −12, −11, −10, −9, and −8 become less sensitive to permanganate and thymidine at +7 becomes more sensitive. This observation is in agreement with the movement of the region of melted duplex with translocation of the RNAP during transcription.

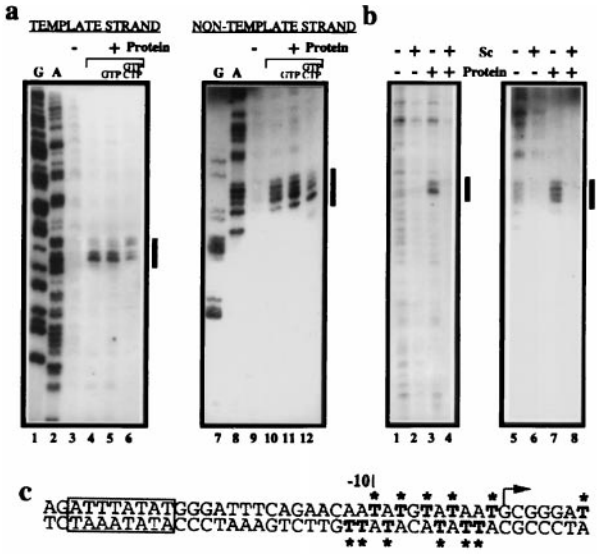

FIG. 6. Analysis of promoter opening by permanganate probing of the 16S promoter. (*a*) Open complex formation on negatively supercoiled 16S($\sigma = -0.053$) promoter at 48°C. Lanes 1 and 7 and lanes 2 and 8 contain ddG and ddA sequence ladders, respectively. Lanes contain modification pattern in the absence of TBP, TFB, and RNAP (−, lanes 3 and 9), modification with TBP, TFB, RNAP, and no nucleoside triphosphates (+, lanes 4 and 10), modification with TBP, TFB, RNAP, and GTP (+, GTP; lanes 5 and 11), and modification with protein with GTP and CTP (+, G, CTP; lanes 6 and 12). Modification is detected on both template (lanes 1–6) and nontemplate strand (lanes 7–12). The region of modified thymidines is indicated by a vertical bar on the right. (*b*) No open complex is detectable on positively supercoiled templates at 48°C. Permanganate modification assays were performed on negatively supercoiled 16S ($\sigma = -0.053$, lanes 1, 3, 5, and 7) or positively supercoiled 16S ($\sigma = +.047$, lanes 2, 4, 6, and 8) DNA at 48°C in the presence (lanes 3, 4, 7, and 8) or absence (lanes 1, 2, 5, and 6) of TBP, TFB, and RNAP. (*c*) Summary of permanganate sensitivity data. The sequence of the 16S promoter is shown with the position of modified Ts indicated by bold type and *. The TATA element is boxed, and the site of transcription initiation is indicated by an arrow.

In addition to providing insights into the mechanism of archaeal transcription, our results reveal that, despite being fundamentally homologous, there are important differences between the eukaryotic RNAPII system and the transcriptional apparatus of hyperthermophilic Archaea. Thus, whereas RNAPII requires ATP hydrolysis to mediate promoter opening, there is no such requirement in the archaeal system. Related to this point, unlike the situation with RNAPII, which requires the action of basal transcription factors such as TFIIH and TFIIE to transcribe relaxed and positively supercoiled templates, archaeal TBP, TFB, and RNAP alone can mediate transcription of even highly positively supercoiled DNA at the physiological temperature of 75°C. The available evidence, therefore, strongly suggests that transcription in hyperthermophilic Archaea does not require functional homologues of eukaryotic TFIIE and TFIIH. However, both the crude and reconstituted archaeal transcription systems display an inability to transcribe relaxed or positively supercoiled templates at lower temperatures. The block to initiation on positively supercoiled templates at reduced temperature appears to be at the level of promoter melting. We currently assume that this effect indicates that thermal energy is required to facilitate melting. Thus, at higher temperatures, the localized melting of DNA by RNAP is favored on all templates. In contrast, at reduced temperature, the reduced thermodynamic input from

The permanganate sensitivity assay then was used to examine open complex formation on negatively supercoiled and positively supercoiled 16S template DNA at 48°C (Fig. 6*b*). Significantly, open complex formation was detectable on the negatively supercoiled but not on positively supercoiled DNA at this temperature. Taken together with the other data, these results reveal that the block at 48°C to transcription on relaxed and positively supercoiled DNA templates in the archaeal transcription system lies at the level of polymerase recruitment and promoter opening.

DISCUSSION

the environment is now insufficient to assist the RNAP in overcoming the resistance to melting in the overwound, positively supercoiled DNA.

The analyses of template topology herein are likely to be relevant physiologically because the natural state of hyperthermophilic archaeal DNA ranges from relaxed to positively supercoiled, depending on the archaeal species and the environmental conditions. Indeed, it is noteworthy that, in response to cold shock, hyperthermophiles rapidly reduce their plasmid linking number (5, 6). In light of our observations, it is tempting to speculate that this global regulation of DNA superhelical state *in vivo* represents an effective mechanism for ensuring continued gene expression after drastic changes in environmental temperature. In this regard, it will clearly be of great interest to determine how such regulation is achieved. The reduction in plasmid linking number on cold shock is, however, simply an initial response. If cold shock is prolonged, supercoiling returns to near initial levels (6). There are two possible explanations for this event. First, this new level of supercoiling may represent a maximal linking number at which gene expression can be mediated by the transcription machinery. A second possibility is that during the initial phase of the cold-shock response, the sharp reduction of linking number, a factor is induced that allows the block to promoter opening on positively supercoiled templates to be overcome, thereby allowing the subsequent increase in linking number. It is interesting to note that the recently completed archaeal genome sequences of *Archaeoglobus fulgidus* and *Methanococcus jannaschii* possess weak homologues of the N terminus of the alpha subunit of TFIIE (29, 30). Our data indicate that this putative TFIIE homologue is not required for transcription at the physiological temperature on the templates we have studied. It is possible that this protein may be required on a limited subset of promoters or it could conceivably be involved in the second stage of the cold-shock response. It therefore will be of interest to analyze its effects on transcription on a range of templates, particularly under cold-shock conditions.

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