Phage N4 RNA polymerase II recruitment to DNA by a single-stranded DNA-binding protein

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Transcription of bacteriophage N4 middle genes is carried out by a phage-coded, heterodimeric RNA polymerase [N4 RNAPII], which belongs to the family of T7-like RNA polymerases. In contrast to phage T7-RNAP, N4 RNAPII displays no activity on double-stranded templates and low activity on single-stranded templates. In vivo, at least one additional N4-coded protein (p17) is required for N4 middle transcription. We show that N4 ORF2 encodes p17 (gp2). Characterization of purified gp2 revealed that it is a single-stranded DNA-binding protein that activates N4 RNAPII transcription on single-stranded DNA templates through specific interaction with N4 RNAPII. On the basis of the properties of the proteins involved in N4 RNAPII transcription and of middle promoters, we propose a model for N4 RNAPII promoter recognition, in which gp2 plays two roles, stabilization of a single-stranded region at the promoter and recruitment of N4 RNAPII through gp2–N4 RNAPII interactions. Furthermore, we discuss our results in the context of transcription initiation by mitochondrial RNA polymerases.

Keywords: N4 RNAPII; single-stranded DNA-binding protein; transcriptional activation

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Transcription of the 70.6-kb linear double-stranded DNA (dsDNA) N4 genome is regulated through the sequential activity of three distinct RNA polymerases [RNAPs; Zivin et al. 1981]. Early transcription is carried out by an N4-coded, rifampicin-resistant, virion-encapsulated RNAP (N4 vRNAP), which is injected into the host with the N4 genome (Falco et al. 1977) and requires the Escherichia coli single-stranded DNA-binding protein for transcription [Markiewicz et al. 1992; Glucksman-Kuis et al. 1996; Davydova and Rothman-Denes 2003]. Transcription of middle genes is regulated through the synthesis of three N4 early proteins, p4, p7, and p17 [Falco and Rothman-Denes 1979a,b; Zehring et al. 1983]. Two of these proteins, p4 and p7, comprise a heterodimeric, rifampicin-resistant RNAP [N4 RNAPII; Zehring and Rothman-Denes 1983]. Middle genes encode proteins that include N4 replication functions. One of these, the N4 single-stranded DNA-binding protein [N4 SSB], activates the E. coli σ70-holoenzyme at late gene promoters [Cho et al. 1995; Choi et al. 1995; Miller et al. 1997].

Sequence analysis identified N4 RNAPII as belonging to the T7 RNAP family, which includes phage-encoded, mitochondrial, and some chloroplast nuclear-encoded, and linear plasmid-encoded enzymes [Willis et al. 2002]. In contrast to the T7-RNAP, N4 RNAPII is inactive on double-stranded, promoter-containing templates, and transcribes single-stranded DNAs (ssDNA), albeit non-specifically and inefficiently [Zehring and Rothman-Denes 1983]. A bipartite consensus promoter sequence was derived from comparison of upstream sequences of six in vivo transcription initiation sites [Abravaya and Rothman-Denes 1989b]. These sequences are characterized by an AT-rich element, 5’-t/aTTTAa/t-3’, located at the site of transcript initiation. The second element, 5’-At/aGACCTGt/a-3’, is found 12–20 bp upstream of the AT-rich element. At present, no functional significance has been ascribed to these two regions.

N4 p17 is a 14.7-kD protein required for RNAPII transcription in vitro and in vivo [Zehring et al. 1983; Abravaya and Rothman-Denes 1989a]. In wild-type N4-infected cells, both RNAPII and p17 are found tightly associated with an N4 DNA/inner membrane complex [Falco and Rothman-Denes 1979b; Zehring et al. 1983]; however, N4 RNAPII is found in the soluble fraction when an P17 is absent [Zehring et al. 1983]. The roles that p17 plays in localization and activation of RNAPII and whether p17 is sufficient for specific transcription...
initiation are unknown. To elucidate the role of p17 in middle transcription, its gene was identified, sequenced, and cloned. P17, encoded by ORF2, shows no similarities to sequences in the database. The ORF2 product (gp2) was purified to homogeneity and characterized. We show that gp2 is a ssDNA-binding protein that activates transcription through recruitment of N4 RNAPII to ssDNAs, propose a model for N4 RNAPII promoter recognition, and discuss our results in the context of transcription initiation by mitochondrial RNA polymerases.

Results

Identification of ORF2, the gene encoding p17, and purification of the protein

N4 vRNAP transcription of the early gene segment of the N4 genome initiates at three promoters, Pe1, Pe2, and Pe3, which direct the transcription of the four N4 early genes. ORF15 and ORF16, encoding the subunits of N4 RNAPII, are transcribed from Pe3 (Willis et al. 2002). Therefore, ORF1 or ORF2, transcribed from Pe1 and Pe2, respectively, must encode p17. N4am98 phage infection displays a middle transcription-defective phenotype. Only three major proteins, corresponding in size to those encoded by ORF1 (12.3 kD), ORF15 (31.7 kD), and ORF16 (46.4 kD), are produced in N4am98-infected cells, whereas p17 is absent (Fig. 2A, lanes 4, 5). To identify the gene encoding p17, ORF1 or ORF2 were PCR amplified from both N4 wild-type and N4am98 DNA templates, and the amplicons were sequenced. A single mutation, a C–T transition at nucleotide position 172 in ORF2 generating an amber codon, was detected in N4am98 DNA. Therefore, ORF2 encodes p17 (hereafter named gp2), a 128-amino acid protein of calculated MW 14,284, close to the 14.7 estimated size of p17. The sequence of this protein displays no similarities to sequences in the database (Fig. 1).

ORF2 was cloned into plasmid pET11a to create pSH2, and the pattern of protein expression of cells bearing plasmid pSH2 was analyzed after pulse labeling with [35S]methionine and SDS-PAGE [Fig. 2A]. An abundant polypeptide of ~15 kD, absent in uninduced cells [Fig. 2A, lane 1], is produced 30 min after induction with IPTG in cells carrying pSH2 [Fig. 2A, lane 2]. The 15-kD protein is the major species produced in cells pretreated with rifampicin to inhibit E. coli RNA polymerase-dependent RNA synthesis, as ORF2 is under the control of a T7 RNAP promoter [Fig. 2A, lane 3]. These results, in conjunction with the finding that the N4am98 mutation maps to ORF2, confirm that ORF2 encodes p17 (gp2).

We tested whether recombinant gp2 can complement N4am98 phage for middle transcription by assaying for phage DNA synthesis, which requires middle gene products [Fig. 2B]. A decrease in DNA synthesis is observed after wild-type N4 infection due to shut-off of host DNA synthesis (Guinata et al. 1986). The rate of DNA synthesis increases ~10 min post-infection as wild-type phage replication begins [Fig. 2B, open circles; Guinata et al. 1986]. Infection with N4am98 phage results in a decrease in the rate of DNA synthesis, but no subsequent increase is observed because phage DNA replication cannot occur in the absence of middle transcription [Fig. 2B, filled circles]. Expression of recombinant gp2 during N4am98 phage infection results in a DNA synthesis pattern resembling that of wild-type infection, indicating that recombinant gp2 is functional [Fig. 2B, squares].

Hexahistidine-tagging of gp2 resulted in inactive protein, therefore, standard procedures were used to purify the native protein. Gp2 produced in strain BL21(DE3)/pLysS/pSH2 fractionated with the membrane and chromosomal DNA of lysed cells. Sonication, high salt, and/or detergent treatment did not efficiently release soluble active gp2 from the DNA/membrane complex (data not shown); successful release was achieved by DNase I treatment. Solubilized gp2 was purified, and samples

![Figure 1](http://genesdev.cshlp.org/files/2009/12/genesdev_cshlp_2009_10_10_15207_F1.jpg)

**Figure 1.** Sequence of the p17-coding region reveals a 128-amino acid ORF. The N4am98 mutation, a CAG (Gln, amino acid 58)-to-TAG (amber) transition, is bold. The tryptophan (amino acid 30) is boxed.
from the various purification steps were analyzed by SDS-PAGE (Fig. 2C). A single 15-kD protein was purified to homogeneity.

**Gp2 is a single-stranded DNA-binding protein**

The association of gp2 with the DNA/membrane complex and its specific release by DNase I treatment suggested that gp2 is a DNA-binding protein. Purified gp2 did not form complexes with promoter-containing dsDNA in gel mobility-shift assays (data not shown). However, gp2 formed complexes in reactions with either the template or complementary strands of the DNA fragment tested (Fig. 3A). Because of the small amount of labeled ssDNA probe used (<1 nM), the gp2 concentration required for half-maximal binding provides an estimate of the binding constant (Carey 1991). The estimated $K_d$ for ssDNA is $\sim$30–60 nM. It is worth noting that increasing concentrations of gp2 lead to further retardation of the ssDNA/gp2 complexes.

The affinity of gp2 for ssDNA, dsDNA, and RNA was investigated by equilibrium competition experiments (Fig. 3B). ssDNA is a $\sim$3000-fold more efficient competitor than RNA or dsDNA (Fig. 3B, cf. lanes 8, 14, 15). Circular M13 viral ssDNA was an efficient competitor of gp2 binding; therefore, binding to the ends of linear DNA is not gp2’s predominant binding mode (Fig. 3B, lanes 15–20). The inability of supercoiled plasmid DNA to compete for gp2 binding (data not shown) suggests that torsional stress cannot unwind dsDNA to a sufficient extent for gp2 invasion/interaction. The binding of gp2 to both strands of the fragment tested and the ability of non-N4 ssDNA to effectively compete binding indicate that gp2 is not a sequence-specific ssDNA-binding protein.

To characterize the interaction of gp2 with ssDNA, the quenching of gp2’s intrinsic fluorescence upon ssDNA binding was measured (Lohman and Mascotti 1992). The fluorescence emission spectrum of gp2 shows a maximum at 340 nm (Fig. 4A), suggesting that a single tryptophan residue (amino acid 30, Fig. 1) is partially solvent exposed (Lakowicz 1983). The fluorescence increased linearly with increasing gp2 concentrations (data not shown).

Single-stranded DNA-binding proteins bind DNA in a sequence-independent manner, although they display preferences for certain nucleotide bases (Chase and Williams 1986). To compare the relative affinities of gp2 for oligonucleotide homopolymers of different base composition, fluorescence was measured while 30-
base oligonucleotides, [dT]30, [dC]30, or [dA]30, were titrated into a solution containing 0.5 µM gp2. (●) [dT]30; (▲) [dC]30; (■) [dA]30. (C) Dependence of gp2 binding on oligonucleotide length. Poly-[dT] oligonucleotides were titrated into a solution containing 0.25 µM gp2 and 300 mM NaCl. (●) 20 mer; (▲) 30 mer; (■) 40 mer; (▼) 50 mer.

The dependence of complex formation on oligonucleotide length was determined by measuring gp2 fluorescence, while [dT]n oligonucleotides of different lengths were titrated into a solution containing gp2. The data were fitted to a plot of fluorescence versus concentration of oligonucleotide added [Fig. 4C]. The curve fit of the data indicated that the relative affinities of gp2 for the oligonucleotides are [dT]50 > [dT]40 > [dT]30 > [dT]20. A binding site size between 3 and 4 nucleotides per monomer was calculated from the fluorescence titrations with [dT]40 and [dT]50 [Lohman and Maschotti 1992]. The [dT]30 oligonucleotide does not produce the same degree of quenching as [dT]40 and [dT]50. This is reminiscent of Eco SSB’s behavior, in which complexes with a binding stoichiometry of 65 bases per tetramer result in 89% quenching, whereas complexes with 35 bases per tetramer binding mode result in only 53% quenching [Lohman and Overman 1985].

To confirm that the observed fluorescence quenching reflects gp2/ssDNA complex formation, binding reactions containing end-labeled oligonucleotides and increasing amounts of gp2 were analyzed by electrophoretic mobility gel shift assays [EMSA; Fig. 5A]. From the fluorescence data, we calculated that 50 nM [dT]40 or [dT]50 oligonucleotides should be >90% bound by 0.25 µM gp2 [Fig. 4C], however, a lower degree of complex formation was observed in EMSA [Fig. 5A], probably reflecting complex dissociation during electrophoresis. In agreement with the fluorescence data, gp2 did not form a stable complex with the [dT]20 oligonucleotide [Fig. 5A]. Gp2/DNA complexes were more stable with longer oligonucleotides [Fig. 5A]. Surprisingly, the complex with [dT]30 has lower mobility than the complex with [dT]40 (see Discussion).

The effect of increasing NaCl concentration on gp2 binding could not be determined by fluorescence quenching. Although the fluorescence maximum at 340 nm did not change appreciably during the titration with NaCl, the fluorescence intensity increased reaching a maximum at 0.8 M NaCl [data not shown], suggesting
either a change in protein conformation or aggregation state. Therefore, gp2 was incubated with increasing NaCl concentrations prior to addition of poly[dT]40, and the complexes were analyzed by EMSA [Fig. 5B]. Gp2 binding to DNA increased as the NaCl concentration increased from 0 to 0.75 M. At concentrations above 1 M NaCl, DNA binding decreases.

Recombinant gp2 activates N4 RNAPII transcription on single-stranded DNAs by RNAP recruitment to single-stranded templates

Transcription reactions were performed to investigate the effect of gp2 on N4 RNAPII activity. Gp2 did not activate transcription of double-stranded linear or supercoiled, promoter-containing templates [data not shown]. Therefore, we tested the effect of gp2 on N4 RNAPII transcription on both the template and complementary strands of a middle promoter-containing N4 DNA fragment, from which a 107-nucleotide run-off transcript was expected (Abravaya and Rothman-Denes 1989b, Fig. 6A). However, gp2-stimulated transcription initiated at a number of sites under these conditions on both strands. Although the expected 107-nucleotide transcript was synthesized when the template strand was used, the major transcript [147 nucleotides] produced on this strand did not coincide with the previously identified in vivo start site on this fragment.

The 5′ ends of RNAs transcribed from the complementary strand in the absence and presence of gp2 were compared using S1 nuclease protection and eightfold less RNA when synthesized in the presence of gp2 [Fig. 6B]. Clusters of three major fragments, probably resulting from S1 nuclease invasion of the rU:dA base pairs at the end of the hybrid, were detected whether the RNAs were synthesized in the absence or presence of gp2. A similar result [data not shown] was obtained when the major transcript originating from the template strand was analyzed [data not shown]. Therefore, gp2 did not affect RNAPII start-site selection on single-stranded templates.

Run-off transcription reactions were carried out at increasing concentrations of N4 RNAPII [Fig. 6C]. In the absence of gp2, RNA synthesis increased with increasing RNAP concentrations, and longer products were detected. Gp2 addition inhibited the synthesis of longer products, although it promoted 3′ nontemplated addition. Moreover, Gp2 addition did not activate at the highest RNAP concentration, suggesting that gp2 activates transcription on single-stranded templates through N4 RNAPII recruitment.

To further analyze the interaction of N4 RNAPII and gp2, the ability of gp2 and RNAP to form complexes on single-stranded templates was tested by EMSA [Fig. 7A]. In the absence of gp2, RNAP formed complexes inefficiently, and only at the highest concentration tested [Fig. 7A, lane 5, denoted by *]. The formation of gp2/DNA complexes was dependent on gp2 concentration [Fig. 7A, cf. lanes 6 and 11]. At both gp2 concentrations tested, an increase in complex formation was observed with in-
creasing RNAP concentrations, although the mobility of the resulting complex did not change appreciably. In both cases, the extent of complex formation was higher than that observed with the individual proteins (Fig. 7A, cf. lanes 5, 6 and 10, lanes 5, 11 and 15). This effect could arise from cooperative binding through gp2–N4 RNAPII interactions, or if the binding of gp2 elicited a DNA conformational change that facilitates the binding of N4 RNAPII. This latter scenario does not necessarily imply protein–protein interactions or that gp2 must be present in the final complex. To analyze the composition of the complex, reactions containing hexahistidine-tagged N4 RNAPII and gp2 with or without DNA were subjected to gel electrophoresis. The native gel was electrobblotted to nitrocellulose and probed with antibodies against gp2 or recombinant RNAPII (Fig. 7B). In the absence of ssDNA, a band migrating with lower mobility than free gp2 was detected using anti-gp2 antibodies when RNAPII is present (Fig. 7B, left panel, denoted by *). When antibodies against RNAPII were used, a band with slightly lower mobility than free RNAPII and comigrating with the band reacting with anti-gp2 antibodies was evident (Fig. 7B, right panel, denoted by *). These results indicate that gp2 comigrates with N4 RNAPII in the absence of ssDNA. In the presence of ssDNA, three species that react with both anti-gp2 and anti-RNAPII antibodies were detected. The reason for the difference in migration of these three species is unknown.

To conclusively determine whether gp2 interacts with N4 RNAPII, purified gp2 and recombinant RNAPII (hexahistidine-tagged at the N terminus of the p7 subunit) were applied to a metal-affinity column (Fig. 7C). All input N4 RNAPII was retained on the column, although some gp2 was not. The column was washed with 1 M NaCl buffer; no p4 was detected in the wash, indicating that the interaction between the two RNAPII subunits is resistant to high-salt concentrations. P7, p4, and gp2 were detected in equimolar amounts in the 100-mM imidazole eluate. Similar results were obtained when the column was preloaded with RNAPII and excess gp2 was applied. In contrast, Eco SSB did not interact with N4 RNAPII and, conversely, gp2 did not interact with N4 vRNAP (data not shown). Therefore, gp2 binds specifically to RNAPII in the absence of ssDNA through an interaction that is resistant to 1 M NaCl concentration. Because gp2 binds to ssDNA as an oligomer, the stoichiometry of interaction suggests that the gp2 oligomer might dissociate upon interaction with N4 RNAPII. These results support our hypothesis that gp2 recruits N4 RNAPII to single-stranded templates through specific protein–protein interactions. The site(s) of gp2 interaction on N4 RNAPII remains to be identified.

Discussion

The activator of N4 RNAPII is a single-stranded DNA-binding protein

The involvement of gp2 in in vivo N4 middle RNA synthesis, its tight association with the DNA/inner membrane of infected cells (Zehring et al. 1983), and its ability to direct RNAPII-specific transcription in a semipurified system (Abravaya and Rothman-Denes 1989a) suggested that gp2 might specifically recognize N4 middle promoters either by itself or after interaction with RNAPII. Yet, characterization of the purified recombinant protein revealed that gp2 is a nonspecific single-stranded DNA-binding protein.

Gp2 displays the benchmark properties of single-stranded DNA-binding proteins (Chase and Williams
Carter et al. 1986. Although it is difficult to assign gp2 to a particular class of single-stranded DNA-binding proteins due to the lack of sequence homology or structural information, gp2 shares several properties with other single-stranded DNA-binding proteins. The base preference of gp2 binding for pyrimidines (dT > dC > dA) has also been observed for T4 gp32 [Newport et al. 1981], Eco SSB [Overman et al. 1988], and the heterotrimeric human RPA [Kim et al. 1992]. The gp2-binding site size was estimated to be 3–4 nucleotides per monomer. The F gene V family of SSBs displays similar binding stoichiometries [Kansy et al. 1986; Bulsink et al. 1988]. M13 gene V protein binds as a dimer to form stable complexes on short oligonucleotides [Folkers et al. 1994]. In contrast, gp2 requires at least 30 nucleotides of DNA for association. Gp2 elutes as a 150-kD complex upon gel-filtration chromatography in 0.15 and 1 M NaCl, indicating oligomerization [R.H. Carter, unpubl.]. Gp2/30-mer complexes migrate more slowly and are less stable than gp2/40-mer complexes, whereas complexes with longer oligonucleotides migrate with progressively lower mobility as expected from the increase in length of the DNA. An increase in mobility upon binding of Eco SSB and replication protein A [RPA] to longer oligonucleotides has been observed [Blackwell and Borowiec 1994, Mitra et al. 1997]. RPA binds to 8–10 nucleotides through two ssDNA-binding domains [DBD-A and DBD-B] of the RPA70 subunit [Pfuetzner et al. 1997; Walther et al. 1999], to form an unstable complex [Blackwell and Borowiec 1994]. In a second mode, in which 30 nucleotides are occluded, a stable complex is formed through additional interactions of a third ssDNA-binding domain of RPA70 [DBD-C] and the RPA32 DNA-binding domain with ssDNA [Kim et al. 1992; Bochkareva et al. 2001, 2002]. We surmise that, in spite of a 3–4-nucleotide binding site size, the instability of gp2/20-mer complexes and the relative slow migration of gp2/30-mer complexes must reflect different modes of interaction between monomers in the oligomer and ssDNA. The oligomerization state of gp2 as well as its implications to single-stranded DNA binding is under investigation.

**A model for N4 RNAPII promoter recognition**

Phylogenetic analysis of T7-like RNA polymerases indicates the existence of three subfamilies, the phage (T7, T3, SP6, K11) polymerases, the nuclear-encoded mitochondrial and chloroplast polymerases, and the mitochondrial plasmid-encoded enzymes [Cermakian et al. 1997]. Surprisingly, both bacteriophage N4-coded enzymes [vRNAP and N4 RNAPII] cluster with the plasmid-encoded polymerases [Kazmierczak et al. 2002]. T7-like RNA polymerases contain four functionally important motifs, DxxGR, A, B, and C [Delarue et al. 1990]. The T7 RNAP structure resembles a cupped hand with thumb and fingers subdomains rising on each side of a palm subdomain, where Motifs DxxGR, A, and C are located, whereas Motif B lies in the fingers subdomain [Jeruzalmi and Steitz 1998]. Comparison of the sequences of N4 RNAPII subunits (p7/p4) with the T7 RNAP sequence indicates the presence within N4 RNAPII of the four motifs as well as other blocks of sequence similarity lying within the fingers, palm, and thumb subdomains [Willis et al. 2002]. The DxxGR motif lies near the C terminus of the p7 subunit, whereas motifs A, B, and C are present in the p4 subunit. Promoter recognition by T7 RNA polymerase is achieved by insertion of the “specificity loop” [amino acids 739–770] into the DNA major groove (−8 to −12 bp) and of a flexible surface loop [amino acids 93–101] into the minor groove of an A + T rich sequence (−13 to −17 bp; Raskin et al. 1993; Rong et al. 1998; Cheetham and Steitz 2000]. The N4 RNAPII p4 polypeptide contains a segment of unknown function whose position roughly corresponds to that of the T7 “specificity loop,” whereas the N4 RNAPII N-terminal domain is truncated by 156 amino acids relative to T7 RNAP, suggesting that the flexible surface loop is absent [Willis et al. 2002]. N4 RNAPII is inactive on double-stranded templates containing in vivo sites of transcription initiation [Zehring and Rothman-Denes 1983]. Although gp2 is essential in vivo for N4 middle transcription, it does not bind to promoter-containing, double-stranded templates or endows N4 RNAPII with the ability to initiate transcription at middle promoters. We have shown that (1) gp2 is a nonspecific single-stranded DNA-binding protein that stimulates N4 RNAPII transcription on single-stranded DNA, (2) gp2 and N4 RNAPII bind cooperatively to ssDNA, and (3) gp2 interacts with RNAPII to form a complex in which RNAPII and gp2 are present in equimolar amounts. We conclude that gp2 activates RNAPII transcription by recruiting the polymerase to single-stranded templates.

What is the role of N4 gp2 in promoter recognition by N4 RNAPII? N4 middle promoter-containing fragments were identified on the basis of their ability to direct transcription of downstream plasmid sequences after phage infection [Abravaya and Rothman-Denes 1989b]. In vitro transcription from middle promoters present in supercoiled plasmids was observed upon addition of extracts from N4-infected cells, implying that plasmid-borne promoters are suitable templates for specific transcription [Abravaya and Rothman-Denes 1989a]. However, in vivo expression of recombinant N4 RNAPII and gp2 did not support transcription from plasmid-borne middle promoters [R.H. Carter, unpubl.], indicating that an additional phage-coded factor is necessary for N4 middle transcription. On the basis of the properties of gp2, N4 RNAPII, and N4 middle promoters, we propose that a yet-unidentified N4-coded protein is responsible for site-specific DNA binding through interaction with the upstream conserved sequences of the promoter. We further propose that binding of this protein induces unwinding of a region downstream to its binding site. Such an activity is a hallmark of proteins involved in initiation of E. coli [dna A protein] and bacteriophage λ [λ O protein] DNA replication [for review, see Kornberg and Baker 1992]. Unwinding would allow the site-specific recruitment of gp2 and concomitant or subsequent recruitment of RNAPII through interactions with gp2. Moreover,
single strandedness between the conserved promoter elements that are present at a variable distance (12–20 bp) would provide flexibility to allow recognition of the two conserved sequences [Tomonaga et al. 1998; Hammer et al. 2001].

Promoter recognition by T7-like RNA polymerases

Except for T7 RNA polymerase and the closely related phage T3, SP6, and K11 enzymes, all other members of the T7 RNAP-like family that have been characterized thus far require additional factors for transcription initiation. The catalytic cores of the yeast and human mitochondrial RNA polymerases are inactive on promoter-containing templates [Fisher and Clayton 1985; Kelly and Lehman 1986; Schinkel et al. 1987]. Specific initiation by yeast mitochondrial RNAP requires a 39.5-kD transcription factor, Mtf1 [Winkley et al. 1985, Schinkel et al. 1987]. Mtf1 cannot recognize promoter sequences on its own [Schinkel et al. 1988]; however, it associates with the catalytic core [RPO41] in solution, forming a holoenzyme complex that is competent for promoter recognition [Mangus et al. 1994]. Human mitochondrial RNA polymerase, POLRMT [Tiranti et al. 1997], accurately transcribes templates containing the human mitochondrial promoters only when supplemented with the 24.4-kD high-mobility box protein TFAM [Fisher and Clayton 1985; Parisi and Clayton 1991] and either TFB1M or TFB2M transcription factors [Falkenberg et al. 2002]. It has been suggested previously that yeast Mtf1 has similarities to bacterial σ factors [Jang and Jaehning 1991], surprisingly, the crystal structure reveals that it is structurally homologous to rRNA dimethyltransferase 16S/15′ (Schubot et al. 2001). The human factors, TFB1M and TFB2M, have sequence similarity to bacterial 16S rRNA dimethyltransferases, and each factor independently interacts stochiometrically with POLRMT to form a heterodimer [Falkenberg et al. 2002]. It has been suggested that the homology of mitochondrial transcription factors to bacterial 16S rRNA methyltransferases might reflect their recruitment to the mitochondrial transcription apparatus through evolution [Schubot et al. 2001; Falkenberg et al. 2002]. On the other hand, there is no evidence supporting a direct interaction between yeast Mtf1, human TFB1M, or TFB2M and promoter sequences. The existence of a region homologous to the T7 RNAP “specificity loop” in RPO41 led to the suggestion that determinants of promoter recognition reside in the catalytic core [Schadel and Clayton 1995]. In this context, the transcription factors would bind to the catalytic core and induce a conformational change leading to interaction of the catalytic core with the promoter (Schubot et al. 2001).

In contrast to the mitochondrial enzymes, the bacteriophage N4-encoded RNA polymerases use single-stranded DNA-binding proteins for promoter recognition. Bacteriophage N4 virion RNAP, the most distantly related member of the T7-like RNAP family [Kazmierczak et al. 2002], is inactive on linear double-stranded templates, but transcribes denatured genomic N4 DNA or promoter-containing single-stranded DNAs with in vivo specificity [Falco et al. 1978; Haynes and Rothman-Denes 1985]. E. coli single-stranded DNA-binding protein [Eco SSB] specifically activates N4 vRNAP transcription at N4 early promoters on supercoiled templates by providing the 5–7-bp stem, 3-nucleotide loop DNA hairpin structure required for promoter recognition [Haynes and Rothman-Denes 1985; Glucksman et al. 1992, Markiewicz et al. 1992]. In addition, Eco SSB binds to the RNA transcript as it exits from the enzyme, preventing formation of a persistent RNA:DNA hybrid, and therefore, allowing template recycling [Davydova and Rothman-Denes 2003]. We expect that further analysis of transcription initiation by N4 RNAPII will provide insights into the mechanism of promoter recognition by mitochondrial RNA polymerases and the role of single-stranded DNA-binding proteins in transcription activation [Rothman-Denes et al. 1998].

Materials and methods

Bacterial strains and plasmids

E. coli strain W3350(DE3)/pLysS was used for phage infections [Choi et al. 1995]. E. coli strain BL21(DE3)/pLysS was used for gp2 overproduction. ORF2 was PCR-amplified from N4 genomic DNA using primers [5′-GCCGAATTCATATGGCTATGCACTACTTTTGC-3′ and 5′-GGGGGATCCTAGAAAGTCAGTTTGG-CAGACC-3′] that introduce NdeI and BamHI restriction enzyme sites. These were used to clone the ampiclon into plasmid pET11a (Novagen) to create expression plasmid pSH2. The resulting recombinant protein does not contain any vector-encoded sequences.

DNA templates

The N4 Mc fragment was isolated from pBR(Mc) [Malone et al. 1988] and cloned into the BamHI site of M13mp7. BamHI digestion of the single-stranded viral M13 DNA containing the fragment inserted in either orientation released the 171 base-long template or complementary strands of the Mc fragment, which were used as templates in transcription reactions. Template MCC101–165 (65 mer), which was used for S1 mapping and gel-retardation assays, corresponds to the sequence of the complementary strand of the Mc fragment from positions 101 to 165. Deoxyoligonucleotides were purchased from Integrated DNA Technologies, gel purified, and quantitated by UV absorbance.

Measurement of in vivo N4 DNA synthesis and labeling of proteins after induction or phage infection

E. coli strain W3350(DE3)/pLysS/pSH2 was infected with N4 phage and DNA synthesis was measured [Guinta et al. 1986]. To induce ORF2 expression, cells were treated with 2 mM isopropyl-β-D-galactoside (IPTG) 15 min prior to phage addition. Proteins were labeled as described [Willis et al. 2002].

Purification of gp2 from the T7-based expression system

Gp2 activity was measured by supplementation of fractions with RNAPII [Abravaya and Rothman-Denes 1989a].
BL21(DE3)/pLYS/pSH2 cells were grown in M9 medium [Miller 1972] containing ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL). When the culture reached OD₆₀₀ = 0.05, IPTG was added to 400 µM, and 3 h later, cells were harvested by centrifugation. Lysis and preparation of the DNA/membrane complex was performed as described [Zehring and Rothman-Denes 1983] with the following modifications. The DNA/membrane pellet was resuspended in 150 mM NaCl A-10 Buffer (10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 10 mM Tris-HCl at pH 7.9 [4°C], 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride) by repeated passage through 18- and 23-gauge needles. The sheared DNA/membrane complex was collected by centrifugation and resuspended in 150 mM NaCl A-10 buffer. DNasel was added to 5 U/mL final concentration, and the mixture was incubated for 20 min at 37°C. After centrifugation, gp2 was recovered in the supernatant. Proteins precipitating between 25% and 50% ammonium sulfate were resuspended in 150 mM NaCl-BTP buffer (20 mM bis-Tris-propane-HCl at pH 7.0, 10% glycerol, 1 mM DTT) and applied onto a Sephacryl S-300 HR [26/60] (Pharmacia LKB) column equilibrated with the same buffer. Column fractions were analyzed by SDS-PAGE and assayed for nuclease activity. Gp2-containing fractions were pooled and applied onto a Macro-Prep Ceramic Hydroxypatite (Bio-Rad) column equilibrated with 150 mM NaCl-BTP buffer. After washing with the same buffer, gp2 was step eluted with PS Buffer [100 mM NaPO₄ at pH 7.0, 10% glycerol, 1 mM DTT] in BTP. Gp2 eluted between 15 and 35 mM NaPO₄ with trace amounts of contaminating nucleases. The eluate was loaded onto a Q-Sepharose [Pharmacia LKB] column equilibrated with 100 mM NaCl-BTP buffer. Gp2 was eluted with a linear gradient of 100–500 mM NaCl in BTP and was nuclease free. Pooled fractions were concentrated with Centricon-30 [Amicon] and dialyzed against 20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM DTT, 50% glycerol. Aliquots were stored at −80°C.

Column fractions were assayed for nuclease activity by following the conversion of supercoiled pUC19 plasmid to the nicked form. Reaction mixtures [5 µL] containing 2 µL of DNasel reaction mix [0.1 µg pUC19 supercoiled plasmid, 125 mM Tris-HCl at pH 7.5, 25 mM MgCl₂, 0.25 mM EDTA, 75 µg/mL BSA, 1.25 mM DTT] and 3 µL protein fraction were incubated for 1.5 h at 37°C, quenched by addition of 1 µL load buffer [12% Ficoll, 60 mM EDTA, 1% SDS] and analyzed on a 1% agarose gel.

Cloning and purification of N4 RNAPII containing an N-terminal hexahistidine tag in the p7 subunit

A DNA fragment encompassing ORF15 (p7) and ORF16 (p4, Willis et al. 2002) was generated by PCR amplification using N4 genomic DNA as template and Phu DNA polymerase (Stratagene) as per manufacturer's recommendations. Primers: 5'–GCTTCTGGATCTATATGACATCAGA-3'; 5'–GCAC TGAGTTAAGATAGCGTATTCGGT-3'. The PCR primers introduced Xhol and PstI restriction sites at the 5' end of ORF15 and the 3' end of ORF16, respectively. The PCR product was cut with the appropriate enzymes and cloned into the expression plasmid pBAD/HisB [Invitrogen], digested previously with PstI and Xhol, to generate pAD1. The recombinant p7 subunit contains a 48 amino acid vector-encoded terminal leader sequence (MGGSHHHHHHGMASMTGGQQMGRDLY DDDDRDPSSRSAATGWFEAW), which includes the hexahistidine tag and the Xpress epitope [underlined], and lacks the N-terminal methionine of p7. Induction of recombinant RNAPII expression complemented N4 phase am15/am23, which contains mutations in ORFs 15 and 16 (Willis et al. 2002).

E. coli BL21 cells bearing pAD1 were grown at 37°C to OD₆₀₀ = 0.5 in Lenox L Broth (LB) medium containing 100 µg/mL ampicillin. After centrifugation, cell pellets were resuspended in LB medium containing 0.2% arabinose and grown 1 h at 37°C. After low-speed centrifugation, pelleted cells were resuspended in sonication buffer [20 mM Tris-HCl at pH 8.0, 20 mM NaCl, 1× Complete protease inhibitor, EDTA-free [Roche]] and sonicated in pulses on ice. After low-speed centrifugation, the cleared lysate was applied to a Talon Co₃⁺-IMAC resin column [Clontech] equilibrated in sonication buffer. The column was washed with 20 mM Tris-HCl [pH 8.0], 1 M NaCl, followed by cold sonication buffer. Protein was eluted with 20 mM Tris-HCl [pH 8.0], 20 mM NaCl, 100 mM imidazole and concentrated on HiTrap Q [Amersham-Pharmacia]. Protein fractions were pooled and applied to a Macro-Prep Ceramic Hydroxypatite (Bio-Rad) column equilibrated with 20 mM sodium phosphate (pH 7.3) buffer, and eluted with a 20–800 mM sodium phosphate [pH 7.3] linear gradient. Eluted protein was diluted 1:1 (v/v) in glycerol and stored at −20°C. The purified recombinant enzyme was as active as endogenous N4 RNAPII [Zehring and Rothman-Denes 1983] and was activated by gp2.

EMSA

ssDNAs were end-labeled with T4 polynucleotide kinase [New England Biolabs] and [γ³²P]ATP [3000 Ci/m mole, Amersham]. Conditions for assays are specified in the figure legends. Samples [10 µL] contained 300 µg/mL BSA and 10%–15% glycerol, and were analyzed on 5%–9% polyacrylamide (80:1, acrylamide to bisacrylamide), 2.5% glycerol, and were analyzed on 5%–9% polyacrylamide (80:1, acrylamide to bisacrylamide), 2.5% glycerol, 1× gel shift running buffer (380 mM glycine, 2 mM EDTA, 50 mM Tris-HCl at pH 8.5). Cesium chloride-purified ppB[Mc] was used as the dsDNA competitor. Cesium chloride-pelleted RNA isolated from rifampicin-pretreated, W3350 cells infected with wild-type N4 phage was used as the RNA competitor and was denatured by boiling immediately prior to use. M13mp18 viral DNA was used as the ssDNA competitor.

Gp2 fluorescence spectra and titrations

Fluorescence measurements were made in an Alphascan [Photon Technologies Inc.] fluorimeter at 22°C. Experiments were performed in 20 mM Tris-HCl [pH 8.0], 1 mM EDTA, and NaCl as indicated in the figure legends. Fluorescence excitation was performed at 292 nm. Fluorescence intensity was detected at a right angle from the incident excitation from 300 to 400 nm in 0.5-nm increments with 0.25-sec integration time. Each data point in titration experiments corresponds to the fluorescence intensity at 340 nm, the maximal value for gp2. Nucleic acids were added incrementally, and the reaction mixtures were allowed to equilibrate with stirring for 10 min at 22°C. Values were obtained by averaging three independent scans and subtracting the average of three blank scans. The 340-nm fluorescence [F] at each point in titration experiments was plotted as quenching [1-F/F₀], in which F₀ is the value for the initial fluorescence of the gp2 solution. The quenching data were fitted to the equation y = [m₁ * m₀/(m₂ + m₀)] + m₃, in which m₀ = quenching, m₁ = maximum quenching, m₂ = Kd, and m₃ = minimum quenching.

In vitro transcription with purified N4 RNAPII and gp2

Transcription reactions [5–15 µL] contained 10 mM MgCl₂, 0.1 mM EDTA, 1 mM each ATP, CTP, GTP, 0.1 mM UTP [α-³²P]UTP [25 Ci/m mole, Amersham], 100 µg/mL BSA, 20 mM Tris-HCl [pH 8.0] and template, RNAPII and gp2 concentrations.
as indicated in figure legends. Reactions were preincubated at 37°C for 5 min and initiated upon the addition of the four rNTPs. After 2 min of incubation at 37°C, the reactions were terminated by adding 1.5 volumes stop buffer (95% formamide, 1 mM EDTA) and analyzed on 8% or 10% acrylamide/8 M urea gels. RNAPII was purified as described previously (Willis et al. 2002).

**S1 nuclease protection**

An end-labeled oligonucleotide spanning positions 101–165 of the complementary strand, from which all observed full-length run-off transcripts (all shorter than 140 nt) originate, was used as a probe. S1 nuclease protection and analysis was described as (Ausubel et al. 1999).

**Detection of gp2–N4 RNAPII interactions on native gels**

Reaction mixtures (15 µL) containing the specified combinations of 8 pmole gp2, 20 pmole hexahistidine-tagged N4 RNAPII, 20 pmole MCC101–165 ssDNA in 10 mM MgCl2, 0.1 mM EDTA, 20 mM Tris-HCl (pH 8.0) were incubated at 25°C for 25 min before loading onto a 6% polyacrylamide gel (40:0.6 acrylamide to bis-acrylamide). Electrophoresis was performed at 4°C in 50 mM Tris-acetate (pH 8), 2 mM EDTA.

Electrotransfer of proteins to nitrocellulose membranes (NitroPure, Micron Separations, Inc) was performed using 1× transfer buffer (25 mM Tris, 192 mM Glycine at pH 8.3). Anti-gp2 rabbit antibodies were affinity purified from serum by overnight adsorption to and subsequent elution from nitrocellulose containing gp2 protein. Western analysis of gp2 was performed as described [Invitrogen] according to the supplier’s specifications.

**Detection of gp2–N4 RNAPII interactions on metal-chelating column**

Hexahistidine-tagged RNAPI [100 pmole] and gp2 [300 pmole] in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl (150 µL total volume) were loaded onto a 50-µL Talon Co2+-IMAC resin column. The column was washed with 1.5 mL of the same buffer, followed by 100 µL of 20 mM Tris-HCl (pH 8.0), 1 M NaCl. Bound proteins were eluted with 150 µL of 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 100 mM imidazole. Gp2 was not retained by the column in the absence of N4 RNAPII. Proteins were analyzed on 15% SDS-PAGE followed by silver staining.

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Phage N4 RNA polymerase II recruitment to DNA by a single-stranded DNA-binding protein

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