Dual targets of a transcriptional activator that tracks on DNA

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The sliding clamp of the bacteriophage T4 DNA polymerase, gp45, is also the proximal effector for activation of transcription of T4 late genes. We have identified the phage T4-encoded σ factor gp55 and the co-activator gp33 as targets of gp45 in promoter complexes, and have shown that a conserved carboxy-terminal amino acid sequence of gp55 and gp33 is required for interaction with gp45. The respective contribution of each target–gp45 interaction to activation of transcription has been assessed by measuring promoter opening rates. The opening rate supported by interaction with both targets is far greater than the arithmetical sum of the separate contributions of each target, implying a synergistic activation of transcription through at least two separate interactions of the trimeric gp45.

Keywords: bacteriophage T4 late genes/DNA-tracking proteins/T4 gp33/T4 gp55/transcriptional regulation

Introduction

The late genes of bacteriophage T4 are transcribed from extremely simple promoters, consisting of TATAAATA, centered at –10 relative to the transcription start. Transcription of late genes is carried out by *Escherichia coli* RNA polymerase core, E, in association with the phage T4 gene 55-encoded σ factor, gp55. Recognition of T4 late promoters and initiation of transcription on supercoiled DNA requires only this form of RNA polymerase *in vitro* (Kassavetis *et al.*, 1983; Elliott and Geiduschek, 1984), while transcription of late genes *in vivo* requires concurrent DNA replication (Riva *et al.*, 1970). Late gene expression is coupled to DNA replication through the sliding clamp of the T4 DNA polymerase, which serves as a transcriptional activator.

An *in vitro* system in which late transcription is activated by DNA replication proteins requires the previously mentioned E.gp55 supplemented by the small RNA polymerase-binding protein gp33. Gp33 is a prototypical transcriptional co-activator; it is required for activation of transcription by the DNA polymerase accessory proteins, but represses basal transcription in their absence (Herendeen *et al.*, 1990). Activation is accomplished by the DNA polymerase accessory proteins, in the presence

of ATP or dATP. The DNA polymerase accessory proteins serve as enhancer-binding proteins, in the sense that they activate transcription by interacting initially with nicks, gaps or primer–template junctions that are located at a distance from the promoter (Herendeen *et al.*, 1989). Activation from a distance is, however, accomplished by a ‘tracking’ mechanism, in which the transcriptional activator is loaded at the enhancer, subsequently diffuses in the one-dimensional space afforded by DNA, and ends up bound to the T4 late RNA polymerase holoenzyme at the upstream end of the open T4 late initiation complex; hence a clear and unobstructed pathway between enhancer and promoter is required (Herendeen *et al.*, 1992; Tinker *et al.*, 1994a; Sanders *et al.*, 1995; for reviews, see Brody *et al.*, 1995; Geiduschek *et al.*, 1997).

The roles of gp45 and of the gp44/62 complex in DNA replication and in activation of transcription have been strikingly illuminated with the recent solution of the crystal structures of proliferating cell nuclear antigen (PCNA) and the β subunit of *E.coli* DNA polymerase III, which are the eukaryotic and bacterial counterparts of gp45. These proteins are highly symmetric tori with a central hole large enough to accommodate double-stranded DNA (Kong *et al.*, 1992; Krishna *et al.*, 1994), and they become topologically confined to DNA in an ATP-dependent process by the action of a partner (RF-C in eukaryotes, the γ complex in eubacteria and the gp44/62 complex in the T4 system) acting at a nick, gap or primer–template junction. Once confined to DNA, these toroidal ‘sliding clamps’ interact with various ligands, thereby confining them to the one-dimensional space of the DNA ‘track’. This view relegates gp44/62, RF-C and the γ complex to the role of molecular chaperones that use ATP to load their cognate tracking effectors onto DNA (for review, see Kelman and O’Donnell, 1995). Indeed, under conditions of molecular crowding, gp45 can effect both activation of transcription and processive DNA synthesis in the absence of its cognate loading enzyme and in the absence of ATP (Reddy *et al.*, 1993; Sanders *et al.*, 1994).

Identification of gp45 as the direct activator of T4 late transcription has left events at the promoter largely unexplained. In particular, the components of phage-modified *E.coli* RNA polymerase that interact with gp45 and mediate its effects remain unknown. While many transcriptional activators exert their effects on transcription by binding to the carboxy-terminus of the α subunit of RNA polymerase (α CTD), previous work has shown that the α CTD is not a target of the activator gp45 (Tinker *et al.*, 1995). In fact, inactivation of interactions with the α CTD by ADP ribosylation at Arg265 seems to be one of the tricks by which T4 shuts down host transcription (K.Severinov, H.Tang, L.Snyder, R.L.Gourse, R.H.Ebright and A.Goldfarb, personal communication).

Fig. 1. Proteins generated for use in the study. (A) The carboxy-termini of gp43 (T4 DNA polymerase), gp55 and gp33, and C-truncated proteins constructed for this work. The conserved carboxy-termini are shaded. (B) An SDS-Tricine gel (Schagger and von Jagow, 1987) with ~250 ng of each of the indicated proteins was run and stained with Coomassie blue. A slight distortion of the bands in lanes 1–4 (gel-purified gp55 and derivatives) is due to the presence of guanidine hydrochloride in the storage buffer.

The elimination of a role for the α CTD in activation of T4 late transcription leaves several candidate targets for the activator gp45. A suppressor genetic analysis by Coppo and co-workers (Coppo *et al.*, 1975) has suggested gp55 as a target of gp45, the essential role of gp33 in late transcription strongly suggests it as a direct target (Bolle *et al.*, 1968; Herendeen *et al.*, 1990), and the amino-terminal domain of α as well as β or β' would be candidates on general grounds. Multiple targets are conceivable: prokaryotic and eukaryotic transcriptional activators are commonly multivalent and can act synergistically through multiple interactions to generate massive accelerations of assembly of transcription complexes (Busby *et al.*, 1994; Sauer *et al.*, 1995).

In the experiments that are described below, we identify gp55 and gp33 as targets of the transcriptional activator gp45. We demonstrate that gp45 binds directly to gp33 and gp55, and that each interaction contributes to enhancement of the rate of promoter opening. A synergistic effect of the two interactions is noted, and implications for the activation mechanism are discussed.

Fig. 2. Removal of the carboxy-terminus of gp55 greatly diminishes, but does not abolish, enhancement of transcription. Single-round transcription in the presence of the indicated components ('acc. prot.' indicating the DNA polymerase accessory proteins, gp45, 44 and 62) was compared as specified in Materials and methods. The 420 nucleotide T4 late transcript and a recovery marker are indicated at the side.

Results

The five carboxy-terminal amino acids of gp33 are essential for activation of T4 late transcription by gp45, but non-essential for two other functions of the protein: RNA polymerase binding and repression of unenhanced transcription (Winkelman *et al.*, 1994). A preliminary analysis of gp55 implicated the carboxy-terminus as necessary for enhancement of transcription but not for unenhanced transcription (J.Winkelman, unpublished). The carboxy-terminal three amino acids of the T4 DNA polymerase, gp43, have been identified as essential for response to the DNA polymerase accessory proteins, but not essential for dispersive DNA chain elongation or exonuclease activity (W.Konigsberg, personal communication; Berdis *et al.*, 1996). The common theme of requirement for response to the sliding clamp and for no other function suggests that these carboxy-termini might be determinants of binding to the gp45 sliding clamp. A similarity of amino acid sequence at the carboxy-termini of gp43, 55 and 33 (also noted by Berdis *et al.*, 1996) reinforces their candidacy as gp45-binding determinants (Figure 1).

Carboxy-terminally truncated and histidine-tagged variants of gp55 and gp33 were generated in order to pursue this line of reasoning. Transcription assays were performed to reveal the ability of truncated proteins to support enhanced assembly of T4 late transcription complexes. Histidine-tagged proteins were also immobilized for tests of direct interaction with gp45. Figure 1A shows a diagram of the gp33 and gp55 derivatives that were generated, and Figure 1B shows an SDS gel of the purified proteins.

Truncations of the carboxy-termini of gp55 and gp33 diminish enhancement of transcription

The abilities of three carboxy-terminal truncations of gp55 to support activation of T4 late transcription by gp45 in the presence of gp33 or the carboxy-terminally truncated gp33CΔ5 are analyzed in Figure 2. The template for transcription was pDH310 DNA nicked at a unique site in the non-transcribed strand of a T4 late transcription unit (defined by a consensus T4 late promoter and a terminator, yielding a 420 nucleotide transcript). The nick

serves as the loading site of the gp45 sliding clamp transcriptional activator by the gp44/62 complex clamp loader (Herendeen *et al.*, 1989, 1992; Tinker *et al.*, 1994a; Sanders *et al.*, 1995). The odd-numbered lanes show gp33-repressed basal transcription on a nicked, circular DNA template. Gp55 mutants CΔ8 and CΔ25 displayed essentially the same level of repressed transcription as did full-length gp55. The ability of gp55 to support enhanced transcription (compare lanes 2 and 1) was diminished but not entirely abolished by truncation of the carboxy-terminus of gp55 (compare lanes 6 and 10 with lanes 5 and 9, respectively). The transcriptional properties of gp55CΔ8 and gp55CΔ25 under unenhanced, repressed and enhanced conditions were indistinguishable (Figure 2 and data not shown).

Reasoning that an interaction of gp45 with gp33 alone might be sufficient to generate a response to the accessory proteins, we sought to eliminate this 'background' activation by using the carboxy-terminally truncated gp33CΔ5 which is defective in the ability to generate enhanced transcription (Winkelman *et al.*, 1994). The five carboxy-terminal amino acids of gp33 were required for any detectable response to the DNA polymerase accessory proteins under these conditions (compare lanes 4, 8 and 12 with lanes 2, 6 and 10).

Gp55 tracks along DNA with gp45; gp55CΔ8 does not

The effect of removing the carboxy-terminus of gp55 on interaction with gp45 was examined in a 'co-tracking' assay (Tinker-Kulberg *et al.*, 1996). It has been shown that gp55 can be cross-linked photochemically to DNA through its interaction with tracking gp45. The probe for such an assay is a single-stranded, circular M13 DNA with a long duplex region, the single-strand–duplex junctions of which serve as loading sites for gp45 by the gp44/62 complex. A single residue of the photoactive nucleotide analog 5[N'-(p-azidobenzoyl)-3-aminoallyl]dUMP (N₃Rd-UMP) and an immediately adjacent radioactively labeled nucleotide are incorporated into the interior of the duplex DNA segment. Upon UV irradiation, proteins in the vicinity of the aryl azide can be cross-linked to DNA. Nuclease digestion leaves the cross-linked nucleotide and, in a fraction of molecules, the adjacent labeled nucleotide as well, attached to protein. Since the mobilities of proteins thus cross-linked and nuclease-treated are barely changed by the attached oligonucleotide, they are detected and identified as radioactive bands nearly co-migrating with silver-stained, uncross-linked proteins. In these experiments, photochemical cross-linking of gp45 requires its loading enzyme and ATP or dATP. Cross-linking of gp45 is prevented by ATP-γ-S, or by blocking the double-stranded DNA track between the gp45-loading site and the cross-linking nucleotide. Cross-linking of gp55 in this assay is entirely dependent on the presence of tracking gp45 (Tinker-Kulberg *et al.*, 1996).

Figure 3 shows the result of an analysis of gp55–gp45 co-tracking. Cross-linking of the single-stranded DNA-binding protein gp32 can be seen in all lanes. Gp45 was cross-linked in the presence of dATP (Figure 3A, lane 1). Addition of gp55 (lane 2) produced a strong radioactive tagging of an ~22 kDa protein, previously identified as gp55 co-tracking with gp45 (Tinker-Kulberg *et al.*, 1996).

Fig. 3. Gp55 co-tracks with gp45, while gp55CΔ8 does not. The indicated proteins were combined with 4 fmol of DNA photoprobe, incubated for 15 min, then UV irradiated for 4 min. Reaction products were processed and analyzed as specified in Materials and methods. (A) Co-tracking requires the carboxy-terminus of gp55 and ATP (or dATP) hydrolysis. Participating components are specified at the top of the figure. The locations of cross-linked proteins in the gel are shown at the right; brackets indicate that cross-linking was not detected. (B) Gp55CΔ8 does not displace gp55 from its interaction with gp45. Increasing quantities of gp55CΔ8 were added to a co-tracking assay containing 200 nM gp55.

No co-tracking signal was observed when gp55CΔ8 was substituted for gp55 (lane 3), although gp45 was still cross-linked efficiently. Co-tracking was abolished by substitution of ATP-γ-S for dATP (lanes 5 and 6), and was strictly dependent on the presence of gp45 and of the gp44/62 complex (lanes 7 and 8). Addition of gp55CΔ8 up to a concentration of 1 μM generated no cross-linking signal (data not shown). The faster-running material labeled 'non-specific' was independent of the presence of DNA polymerase accessory proteins or gp55 (lanes 1, 5 and 6) and was not analyzed further.

The failure to detect a protein in a cross-linking assay does not constitute proof of its absence. Chemical specificities of photochemical cross-linking intermediates are not well characterized, and removal of eight carboxy-terminal amino acids of gp55 may well have removed a unique cross-linking target or placed it out of reach of the cross-linker. Reasoning that a protein capable of binding gp45, but incapable of cross-linking to DNA would displace gp55 and thus inhibit its cross-linking, a mixing experiment was performed. Gp55CΔ8 was added to samples containing the DNA polymerase accessory proteins, dATP and gp55

Fig. 4. Gp45 interacts directly and specifically with gp55 and with gp33. **(A)** Affinity chromatography of gp45 on histidine-tagged gp55 (lanes 2–4) and on histidine-tagged gp55CΔ8 (lanes 5–8) immobilized on nickel-NTA-agarose. **(B)** Affinity chromatography of gp45 on histidine-tagged gp33 (lanes 2–4) and on histidine-tagged gp33CΔ5. Lane 1 of each panel shows one-fifth of the amount of gp45 loaded onto the column. Lanes 2 and 5 of each panel show one-fifth of the pooled flow-through and the first two column volumes of wash; lanes 3 and 6 of each panel show one-fifth of the next three column volumes of wash, and lanes 4 and 7 of each panel show one-fifth of the SDS/EDTA eluate of each column. Experimental details are specified in Materials and methods.

(Figure 3B). Addition of gp55CΔ8 up to 1 μ M (a 5-fold molar excess) did not diminish gp55 cross-linking. We conclude that gp55CΔ8 does not bind to gp45 and track along DNA with it; nor is there a gratuitous inhibitor of gp55 cross-linking present in the gp55CΔ8 preparation. Gp55 binds to and co-tracks with gp45; co-tracking is abolished by removal of the eight carboxy-terminal amino acids of gp55.

Gp45 interacts directly and specifically with gp33 and gp55

Interaction of gp45 with candidate targets was examined by affinity chromatography. Hexahistidine derivatives of gp55 and gp33 were assayed for the ability to support unenhanced transcription, and His₆-tagged full-length proteins additionally were assayed for their ability to support enhanced transcription, using promoter opening rate measurements of the type shown below in Figure 5. All were found to be equivalent in activity to the untagged proteins (Sanders, 1996).

His₆-gp55 and -gp33 were immobilized on Ni-NTA agarose, generating concentrations of 2 mg of immobilized protein per ml of settled resin (~90 μ M gp55 and ~150 μ M gp33). The binding of each protein to gp45 was seen to depend on carboxy-terminal amino acids. Gp45 was adsorbed quantitatively to immobilized full-length gp55; no gp45 appeared in the flow-through or wash fractions (Figure 4A, lanes 2 and 3), but it was quantitatively recovered in the SDS/EDTA eluate, along with gp55 (lane 4). In contrast, nearly all the gp45 flowed through the gp55CΔ8 column (lane 5), and essentially none appeared in the wash or eluate fractions (lanes 6 and 7). Likewise, gp45 was largely retained on a column of immobilized

full-length gp33 (lane 4) but flowed through a column of gp33CΔ5 (lane 5). It should be also noted that small amounts of gp45 reproducibly appeared in the flow-through and wash fractions (visible in the primary data and in the original photographs; lanes 2 and 3) from columns of immobilized gp33. Bearing in mind that the molar concentration of immobilized gp33 was higher than the molar concentration of immobilized gp55, we interpret this leaching as a reflection of a lower affinity of gp45 for gp33 than for gp55. Consistent with this view, retention of gp45 on a column of immobilized gp55 was found to be stable to washing with high salt (0.7 M NaCl) while retention on a column of gp33 was not (data not shown). Thus, gp45 binds directly and specifically to gp55 and to gp33, and it appears that gp45 has a higher affinity for gp55 than for gp33.

Synergistic activation of promoter opening mediated through the carboxy-termini of gp33 and gp55

Rates of promoter opening were measured to assess the separate and combined contributions of the carboxy-termini of gp33 and gp55 to enhancement of transcription. In these assays, protein components were combined on ice, then equilibrated to 25°C. Promoter opening was initiated by adding DNA (also at 25°C). Aliquots were withdrawn at the indicated times and added to a mixture of ribonucleoside triphosphates and rifampicin to generate a single round of transcription from already formed open promoter complexes. The transcription products were then processed and analyzed as described in Materials and methods.

Unenhanced promoter opening rates of RNA polymerase supplemented with gp55 and gp55CΔ8, respectively, are compared in Figure 5A. Unenhanced promoter opening rates supported by full-length and truncated gp55 were nearly identical and similarly (~4-fold) higher with supercoiled DNA than with linear DNA (compare lanes 1–14 with lanes 15–28). Promoter opening rates on linear DNA supported by the two σ factors in the presence of gp33, or the truncated gp33CΔ5 are compared in Figure 5B. In the presence of either gp33 or gp33CΔ5, repressed unenhanced transcription supported by either of the two gp55s were equivalent. Thus the carboxy-termini of gp33 and gp55 play no significant role in determining unenhanced and repressed rates of promoter opening.

In contrast, enhancement by the DNA polymerase accessory proteins uncovered clear differences between gp33, gp55 and their truncated counterparts. In the presence of full-length gp55 and full-length gp33, enhanced promoter opening rates were high, with nearly complete open promoter complex formation generated within 1 min (cf. Sanders *et al.*, 1995). In the presence of gp55CΔ8 and full-length gp33, promoter opening rates were found to be ~10-fold lower, with ~5 min required for 50% of maximal opening (Figure 5C). The effect of substitution of gp33CΔ5 for gp33 was nearly absolute, with rates of promoter opening dropping nearly to repressed levels, yet a further drop in promoter opening rate upon deletion of the carboxy-terminus of gp55 was still seen, consistent with full-length gp55 contributing to activation of transcription, even in the absence of the gp33–gp45 interaction (cf. Sanders *et al.*, 1994). The contributions of the inter-

Fig. 5. Enhanced, but not unenhanced or repressed, promoter opening rates are affected by deletion of the carboxy-termini of gp55 and gp33. Promoter opening rates supported by gp55 and gp33 variants were compared by combining transcription components under conditions similar to those of Figure 2, and withdrawing aliquots at the indicated times for a single round of transcription. Experimental details are specified in Materials and methods. (A) Unenhanced promoter opening rates of E.gp55 and E.gp55CΔ8 on supercoiled DNA (lanes 1–7, ▲; and 8–14, □, respectively) and on linear DNA (lanes 15–21, ■; and 22–28, ○). (B) Repressed promoter opening rates of E.gp55 in the presence of gp33 or gp33CΔ5 (lanes 1–7, □; and lanes 8–14, ■, respectively) and E.gp55CΔ8 in the presence of gp33 or gp33CΔ5 (lanes 15–21, ▲; and lanes 22–28, ○; respectively). (C) Enhanced promoter opening rates of E.gp55 and E.gp55CΔ8 in the presence of gp33 (lanes 1–7, □; and lanes 8–14, ▲, respectively) and gp33CΔ5 (lanes 15–21, ■; and lanes 22–28, △, respectively).

actions of the two targets of gp45 is apparently synergistic, in that both targets together generate a response to gp45 that is much greater than the sum of the individual contributions [an ~10-fold loss in promoter opening rate

accompanied loss of the gp55–gp45 interaction, an ~50-fold loss accompanied loss of the gp33–gp45 interaction and a (very) ~450-fold loss accompanied loss of both gp45–gp33 and gp45–gp55 interactions].

Discussion

Interaction of gp45 with the co-activator gp33 and with the late promoter-recognition protein gp55

Binding of gp45 to its two known targets, the T4 late σ factor, gp55, and the co-activator, gp33, requires a carboxy-terminal determinant that is conserved between gp33, gp55 and the DNA polymerase, gp43. The likelihood that the shared sequence (S/T)LDLF(F/Y/L) at or very near the carboxy-termini of gp33, 43 and 55 binds specifically to a common target on gp45 is consistent with its absence from the databases of prokaryotic proteins and its general rarity at the carboxy-termini of proteins (R.L.Doolittle, personal communication). A number of other prokaryotic transcriptional activators also mediate their effects through their carboxy-termini (Li *et al.*, 1994; Choi *et al.*, 1995; Monsalve *et al.*, 1996). These appear to be examples of a common modularity of construction that is already well recognized in the eukaryotic transcription activators (reviewed by Tjian and Maniatis, 1994; Struhl, 1995; Triezenberg, 1995).

The significance of gp55 co-tracking with gp45

Evidence for this co-tracking has been presented recently (Tinker *et al.*, 1996); co-tracking assays are used here simply to provide evidence for a defect in gp45–gp55 and gp45–gp33 interaction. What are the implications of the gp45–gp55 interaction for the mechanism of transcriptional activation by gp45? Recruitment of RNA polymerase core to a co-tracking or promoter-bound gp45–gp55 complex has been proposed as a means by which activation of transcription might be mediated (Tinker *et al.*, 1996), with gp45 functioning (much as it does for DNA replication) as a sliding clamp that increases the duration of one-dimensional promoter scanning by RNA polymerase. Whether that could be the rate-determining step remains to be seen. It also seems plausible that recruitment of RNA polymerase core might be rate limiting for initiation of transcription in the infected cell. Much of the RNA polymerase in an *E.coli* cell is likely to be in the form of transcribing or recently released core, rather than holoenzyme (McClure, 1985). RNA polymerase core binds non-specifically to DNA. Since co-tracking of gp45 with gp55 places the latter in close proximity to DNA, it might facilitate recruitment of DNA-bound RNA polymerase core by gp55.

Interaction of gp45 with gp55 and gp33 produces synergistic activation of transcription

Many examples of, and possibilities for, synergistic activation of transcription in the eukaryotes (Ptashne and Gann, 1990; Lieberman and Berk, 1994; Chi *et al.*, 1995; Kobayashi *et al.*, 1995; Sauer *et al.*, 1995; Tanaka, 1996) and eubacteria (Busby *et al.*, 1994; Joung *et al.*, 1994) have been described. Synergy can result when multiple interactions impinge on a single rate-limiting step, or on successive steps in a coupled reaction sequence (reviewed by Herschlag and Johnson, 1993).

In eukaryotic transcription, the preponderance of current advocacy is for a dominant role of transcriptional activators in recruitment of transcription initiation factors or RNA polymerase II holoenzyme (Barberis *et al.*, 1995; Farrell *et al.*, 1996; reviewed by Hori and Carey, 1994; Maldonado

and Reinberg, 1995; Struhl, 1995, 1996). Since transcriptional activators also play roles in reconfiguring chromatin (Kingston *et al.*, 1996; Krude and Elgin, 1996; Sväen and Hörz, 1996; Wilson *et al.*, 1996; Wolffe and Pruss, 1996) and in steps of transcriptional initiation, including promoter clearance, that follow polymerase recruitment (Xiao *et al.*, 1994; Rasmussen and Lis, 1995; Edwards and Kane, 1996; Stargell and Struhl, 1996a,b), one anticipates that synergy in eukaryotic transcriptional activation can be generated through action on multiple stages of transcription.

In prokaryotic (eubacterial) transcription, individual activators generate their effects by increasing recruitment to the promoter, the rate of promoter opening (reviewed by McClure, 1985; Record *et al.*, 1996) or, conceivably, promoter clearance (Monsalve *et al.*, 1996; Ring *et al.*, 1996). The synthetic constructs that exhibit synergistic activation in *E.coli* have not yet been analyzed in the quantitative detail that would allow a precise specification of mechanism. For the case of synergistic activation of the weakly binding and slowly opening λP_{RM} promoter by cAMP receptor protein and λcI protein from DNA-binding sites located respectively nine and four helical turns upstream of the transcriptional start (Joung *et al.*, 1994), the two activators are expected primarily to stimulate polymerase recruitment and promoter opening, respectively (based on their modes of action at comparably placed but separate sites; Hawley and McClure, 1982; Shih and Gussin, 1983; Malan *et al.*, 1984; Li *et al.*, 1997).

Considering the synergy of activation of T4 late transcription, it is highly plausible that gp55 and gp33 are able to bind concurrently to the trimeric gp45. Thus, these two RNA polymerase-bound proteins might, in principle, make reinforcing contributions to polymerase recruitment. That this is probably not their primary mode of action is suggested by the fact that the greater effect on transcriptional activation is exerted by the more weakly binding gp33 (Figure 5C). In exploratory experiments, Malik and Goldfarb (1988) also provided indications that unenhanced T4 late transcription is more limited in promoter opening than polymerase binding.

Materials and methods

Materials

Labeled and unlabeled nucleoside triphosphates, restriction enzymes, exonuclease III, proteinase K, DNase I, S1 nuclease, rifampicin, bovine serum albumin (BSA) and insulin were purchased. Unless noted otherwise, all other proteins were overproduced in *E.coli* BL21 (DE3) bearing the appropriate overexpression plasmid, grown at 37°C, and induced at $A_{600} = 0.6$ by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to 0.5 mM final concentration. Induction was continued for 2 h, and cells were harvested and stored at –70°C. Gp55 and its truncated derivatives were prepared from washed inclusion bodies as described for σ^{70} (Igarashi and Ishihama, 1991), then loaded onto preparative SDS gels. The appropriate bands were excised, proteins eluted, precipitated with acetone, then dissolved in guanidine storage buffer [6 M guanidine-HCl, 5% (v/v) glycerol, 200 mM NaCl, 20 mM Na HEPES, pH 7.8] essentially as described by Hager and Burgess (1980), with the exception that insulin, rather than BSA, was used as carrier in the precipitation step. Protein concentrations were estimated roughly from the intensity of Coomassie staining of the purified proteins fractionated on SDS–polyacrylamide gels, compared with gp55 of known concentration. The denatured proteins were diluted ~20-fold in transcription buffer immediately before addition to other reaction components.

Histidine-tagged gp55s were prepared from washed inclusion bodies, prepared as above, suspended in 6 M urea, 40 mM Na HEPES, pH 7.8,

250 mM NaCl, 5 mM β -mercaptoethanol, 10 mM imidazole. The crude gp55-containing solutions were incubated with Ni-NTA agarose (5 mg protein per ml resin) at 4°C for 2 h on a rotating wheel, then the slurry was loaded into a column, and washed with 5 volumes of the above buffer. Bound proteins were eluted with the above buffer supplemented with 500 mM imidazole. Protein solutions were then concentrated to 200–300 μ M with a centrifugal ultrafiltration device, and stored at 4°C. Protein concentrations were estimated by the method of Bradford (1976), using a sample of gp55 of known concentration as a standard.

Histidine-tagged gp33 and histidine-tagged gp33C Δ 5 were purified as follows: induced cells were suspended in lysis buffer [40 mM Na HEPES, pH 7.8, 200 mM NaCl, 10 mM MgCl₂, 5% (v/v) glycerol, 5 mM β -mercaptoethanol, 10 mM imidazole, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]] and lysed by sonication. Lysates were centrifuged at 10 000 g, Ni-NTA agarose (1 ml resin per 40 mg protein) was added to the supernatant, incubated at 4°C for 2 h on a rotating wheel, then the slurry was loaded into a column. The column was washed with 5 volumes of lysis buffer, and bound proteins were eluted with three column volumes of a buffer consisting of 40 mM Na HEPES, pH 7.8, 100 mM NaCl, 5% (v/v) glycerol, 0.05% (w/v) Brij 58, and 500 mM imidazole. Eluted proteins were dialyzed into 20 mM K HEPES, pH 7.8, 100 mM potassium acetate, 50% (v/v) glycerol, 0.05% (w/v) Brij 58, 1 mM β -mercaptoethanol, and stored at –20°C. Proteins were estimated to be >90% pure, based on examination of SDS–polyacrylamide gels. Protein concentrations were determined by the method of Bradford (1976), with a sample of gp33 of known concentration as standard.

The plasmid pDH310, used for construction of templates for transcription, has been described (Herendeen *et al.*, 1989). Preparation of nicked and *exo*III-treated DNA has been described (Herendeen *et al.*, 1989; Sanders *et al.*, 1995). The genes for overproduction of variants of gp55 and gp33 were made by subcloning PCR products generated with mutagenic oligonucleotide primers and *Pfu* and/or *Taq* DNA polymerase into the appropriate expression vectors. The PCR templates were the previously described gp55 and gp33 overproduction plasmids, pPLHG55 (Williams *et al.*, 1987) and pKW7 (Williams *et al.*, 1989), respectively. PCR products for generating gp55C Δ 8, gp55C Δ 25, gp55C Δ 45 and gp33C Δ 5, made from DNA templates constructed by J.Winkelman, were subcloned into the *Eco*RI–*Bam*HI sites of pGEM1 (Promega). PCR products for generating the histidine-tagged versions of gp55, gp55C Δ 8, gp33 and gp33C Δ 5 were subcloned into the *Nco*I–*Bam*HI sites of a derivative of pET21b (Studier and Moffatt, 1986), in which a double-stranded oligonucleotide encoding six histidines and bearing a unique *Nco*I site was inserted into the unique *Nde*I site. This plasmid was the kind gift of A.Kumar.

Transcription reactions

For the experiment shown in Figure 2, 100 fmol of nicked pDH310 DNA, 1 pmol of RNA polymerase core from uninfected *E.coli*, 5 pmol of gp55 or the specified variant protein, 4 pmol of gp33 or gp33C Δ 5, 3.6 pmol of gp45 trimer and 3.3 pmol gp44/62 complex was incubated at 25°C for 30 min in 20 μ l of transcription buffer [33 mM Tris acetate, pH 7.8, 240 mM potassium acetate, 10 mM magnesium acetate, 1 mM dATP, 1 mM dithiothreitol (DTT) and 150 μ g/ml BSA]. A single round of transcription was initiated by adding 5 μ l of 5 mM GTP, 5 mM ATP, 0.5 mM CTP, 0.5 mM [α -³²P]UTP (4000 c.p.m./pmol) and 125 μ g/ml rifampicin in transcription buffer. Incubation was continued for 10 min, and the reactions were stopped by the addition of 7 volumes of 20 mM Na₃ EDTA, 0.4% (w/v) SDS, 40 mM Tris–HCl, pH 8.0, 250 mM NaCl, 250 μ g/ml yeast tRNA, 50 μ g/ml proteinase K and 1000 c.p.m. of a labeled DNA fragment that served as a recovery marker. Samples were incubated for 30 min at 37°C, extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol and suspended in buffered 80% formamide. Nucleic acids were fractionated on 5% polyacrylamide–7 M urea gels, quantified by radioanalytic phosphorimaging and visualized by autoradiography.

Promoter opening rate assays shown in Figure 5 were performed as described (Sanders *et al.*, 1995), with the exception that the reaction medium contained 33 mM K HEPES, pH 7.8, 10 mM magnesium acetate, 240 mM potassium acetate, 0.05% (w/v) Brij 58, 150 μ g/ml BSA and 1 mM DTT.

Analytical affinity chromatography

Two mg of histidine-tagged proteins were immobilized per ml of settled Ni-NTA–agarose. The resulting materials were washed extensively with a buffer consisting of 20 mM K HEPES, pH 7.8, 160 mM potassium acetate, 10 mM magnesium acetate, 0.05% (w/v) Brij 58, 5% (v/v) glycerol and 1 mM β -mercaptoethanol. These resins were stored at 4°C

and used for all subsequent experiments. Columns (20 μ l) of each resin were constructed immediately prior to experiments. Eighty pmol of gp45 trimer in one column volume (20 μ l) of the above buffer were loaded onto each column, then washed with 5 volumes (100 μ l) of buffer. Proteins were eluted with three column volumes of 100 mM EDTA and 0.1% (w/v) SDS. The flow-through and first two column volumes of wash (60 μ l) were pooled and designated ‘flow-through’. The next three column volumes were pooled and designated ‘wash’ and the final three column volumes were designated ‘eluate’. SDS sample buffer was added to each fraction, the samples were heated to 100°C for 3 min, then 1/5 of each sample was fractionated on an SDS–polyacrylamide gel. Proteins were visualized by Coomassie staining.

Photochemical cross-linking assays

The construction of photochemically active DNA probes has been described (Tinker *et al.*, 1994a). Ligation was ~80% efficient. The photoprobe used in these assays was the –39 probe described in the above reference, and tracking was assayed essentially as described (Tinker *et al.*, 1994b). Briefly, proteins (2.7 pmol of gp45 trimer, 3.3 pmol of gp44/62 complex, 200 pmol of gp32 and 3 pmol of gp55, unless indicated otherwise) were combined with 4 fmol of photoprobe in 15 μ l of 33 mM K HEPES, pH 7.8, 160 mM potassium acetate, 10 mM magnesium acetate, 0.05% (w/v) Brij 58, 5% (v/v) glycerol, 1 mM β -mercaptoethanol and 0.25 mM dATP or 0.25 mM ATP- γ -S. Samples were incubated at 25°C for 15 min, irradiated for 4 min with a germicidal lamp (254 nm) from a distance of 60 cm, treated with DNase I and S1 nuclease, resolved on 13% SDS–polyacrylamide gels, silver-stained, dried, analyzed by radioanalytic phosphorimaging and autoradiographed, as described (Bartholomew *et al.*, 1995).

Acknowledgements

We are grateful to J.Winkelman for early experiments on this topic and for numerous helpful discussions and suggestions, to R.L.Doolittle for advice, to A.Kumar and K.Severinov for materials, and to W.Konigsberg, M.M.Susskind and L.Rothman–Denes for sharing information in advance of publication. Our research has been supported by a grant from the NIGMS.

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Received on December 24, 1996; revised on February 26, 1997