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Rules governing the efficiency and polarity of loading a tracking clamp protein onto DNA: determinants of enhancement in bacteriophage T4 late transcription

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The bacteriophage T4 DNA polymerase accessory proteins confer processivity and high speed on replicative DNA chain elongation: the gene 45 protein, gp45, tracks along DNA and serves as the sliding clamp of the viral DNA polymerase; the gene 44/62 protein complex, gp44/62, is an ATP-dependent loading enzyme that mounts gp45 on DNA. Gp45 also activates T4 late transcription. Transcriptional enhancement by gp45 requires a particular orientation that is imposed by gp44/62 at the DNA loading site. Loading and orienting gp45 on DNA, tracking along DNA and interaction with RNA polymerase have been analyzed by measuring transcriptional activation. The efficiency of loading gp45 at different DNA structures and the resulting transcriptional activation have been compared, and sources of interference with transcriptional activation have been examined. All observations are compatible with a mechanism in which the loading enzyme recognizes the polarity of single-stranded DNA and imposes a corresponding polarity of DNA entry on gp45. Primer–template junctions are the most efficient DNA loading sites for gp45 and can generate very rapid opening at promoters that are located at a distance of >1 kbp. In contrast, gp45 does not track efficiently across single-stranded DNA.

Keywords: DNA tracking proteins/enhancers/gene regulation/processivity factor/T4 late genes

Introduction

The primary replicative DNA polymerases of eukaryotes, prokaryotes and certain viruses share common elements of structure and function: they are composed of a core enzyme, capable of relatively non-processive DNA synthesis and exonucleolytic degradation, and a set of accessory proteins that confer processivity on the core enzyme (Kornberg and Baker, 1992; Kuriyan and O'Donnell, 1993; Nossal, 1994). The accessory proteins comprise two components: a processivity factor proper, the 'sliding clamp', and an ATP-dependent assembly factor, the 'clamp loader'. The structures of two sliding clamps, the β subunit of *Escherichia coli* DNA polymerase III holoenzyme and yeast proliferating cell nuclear antigen (PCNA), have been determined: they are toroidal multimers, capable of encircling DNA (Kong *et al.*, 1992; Krishna *et al.*, 1994). Indeed, eukaryotic (PCNA), bacterial (β) and viral [bacteriophage T4 gene 45 protein (gp45)] clamps have been shown to 'track', that is to diffuse freely on DNA although confined topologically, once they have been loaded onto DNA at an appropriate site by the cognate 'clamp loader' (Stukenberg *et al.*, 1991; Burgers and Yoder, 1993; Tinker *et al.*, 1994b; Podust *et al.*, 1995). How the clamp loaders, clamps and DNA loading sites interact can also be understood through analysis of DNA replication and through measurements of the ATPase activities of these clamp loaders (reviewed by Young *et al.*, 1992, 1994; Nossal, 1994).

A role for one set of DNA polymerase accessory proteins in transcriptional activation has been identified recently, permitting examination of accessory protein loading and tracking in the context of transcription. The late genes of bacteriophage T4 are transcribed from extremely simple promoters, consisting of TATAAATA centered ~10 bp upstream of a transcriptional start site. The ability to recognize these promoters is conferred on *E.coli* RNA polymerase core by a small σ-family protein encoded by T4 gene 55 (gp55), but transcription is weak, particularly in relaxed or linear DNA, and further suppressed by the small RNA polymerase-bound T4 gene 33 protein. Gp33 is a transcriptional co-activator; it confers the ability to support activation of transcription by gp45, the 'sliding clamp' of the T4 DNA polymerase holoenzyme (Herendeen *et al.*, 1990). Although gp45 alone can activate gp55-directed transcription in the presence of gp33 under conditions of macromolecular crowding (Sanders *et al.*, 1994), under conventional reaction conditions it additionally requires the 'clamp loader' encoded by T4 genes 44 and 62 (the gp44/62 complex). The latter loads gp45 onto DNA in an ATP hydrolysis-dependent process at enhancer-like entry sites that can be located at a considerable distance from the promoter (reviewed by Brody *et al.*, 1995).

Once gp45 has been loaded, it tracks along DNA, encounters RNA polymerase, and eventually becomes stably associated with the upstream end of the activated open complex (Tinker *et al.*, 1994a). Tracking along DNA is an essential part of this transcriptional activation mechanism: a continuous and open path along DNA connecting an enhancer and its promoter is required for activation by the T4 DNA polymerase accessory proteins (Herendeen *et al.*, 1992).

The most studied enhancers of T4 late transcription have been nicks in DNA, which can activate transcription from upstream or downstream of a target promoter. A characteristic polarity distinguishes these *cis*-acting sites from conventional enhancers: the nick must be in the non-transcribed strand of its target transcription unit (Herendeen *et al.*, 1989). It has been suggested that polarity of transcriptional activation is due to assembly of an asymmetric gp44/62–gp45 complex at the nick-as-enhancer, imposing a particular orientation on gp45 as it tracks along DNA and thereby determining the orientation of RNA polymerase that is compatible with formation of

an activated promoter complex (Herendeen *et al.*, 1992; Tinker *et al.*, 1994a).

The experiments that are presented below explore the process of gp45 loading, orientation, tracking along DNA and interaction with RNA polymerase through the lens of transcriptional activation. We compare nicks and DNA duplex–single strand junctions as entry sites for gp45, compare the transcriptional enhancement efficiencies generated at these sites, analyze sources of interference with transcriptional activation, enumerate the restrictions on polarity of transcriptional activation and account for the entire body of these observations with a single molecular model.

Results

Single-stranded extensions activate transcription in the presence of the DNA polymerase accessory proteins

The first experiment examines the ability of different DNA structures to serve as enhancers of T4 late transcription (Figure 1a). The plasmid used to generate these templates, pDH82, has two T4 late transcription units in opposite orientation; cleavage by gpII endonuclease introduces a nick into the non-transcribed strand of the larger (420 nt) transcription unit and the transcribed strand of the smaller (314 nt) transcription unit. Transcription of this nicked circular DNA responds to the DNA polymerase accessory proteins in a strand-specific manner, as demonstrated previously (Herendeen *et al.*, 1989), with synthesis of 420 nt RNA selectively enhanced (Figure 1b, lanes 1 and 2). Transcription of linear DNA with blunt ends (lanes 3 and 4) or short (4 nt) overhangs (lanes 5 and 6) is not activated significantly by the DNA polymerase accessory proteins. Linear templates bearing ~100–200 nt long single-stranded tails allow the DNA polymerase accessory proteins to activate transcription at both promoters (lanes 7–10); this occurs whether the template bears 3′-recessed (primer–template junctions, lanes 7 and 8) or 5′-recessed (3′ tails, lanes 9 and 10) termini. Templates bearing a primer–template junction at one end and a 3′ tail at the other only activate the promoter that lies upstream of a primer–template junction (lanes 11–14). The mechanisms governing this preferential activation are analyzed in the experiments that follow.

The prominent background of transcripts shorter than ~420 nt in lane 8 and transcripts longer than ~420 nt in lane 10 clearly and reproducibly depends on the DNA polymerase accessory proteins. The size range of these 'background' transcripts in lane 8 is consistent with transcription initiating at a variant T4 late promoter (TCTAAATA; marked in Figure 1a) and terminating in or near the set of single-stranded ends that are generated by the exonuclease (exo) III digestion. The sizes of the transcripts in lane 10 are consistent with initiation at the same variant promoter and termination at the heterogeneous single strand–duplex junctions generated by digestion with T7 gene 6 protein. 3′-tailed templates with a single strand–duplex junction at the *BglI* site yield a single ~730 nt RNA, consistent with run-off transcription initiating at the same variant promoter (data not shown). The method used to prepare top-long and top-short DNA (lanes 11–14) removes the DNA segment containing this variant late promoter. We attribute similar 'background' transcripts in experiments that are presented below to similarly active variant late promoters.

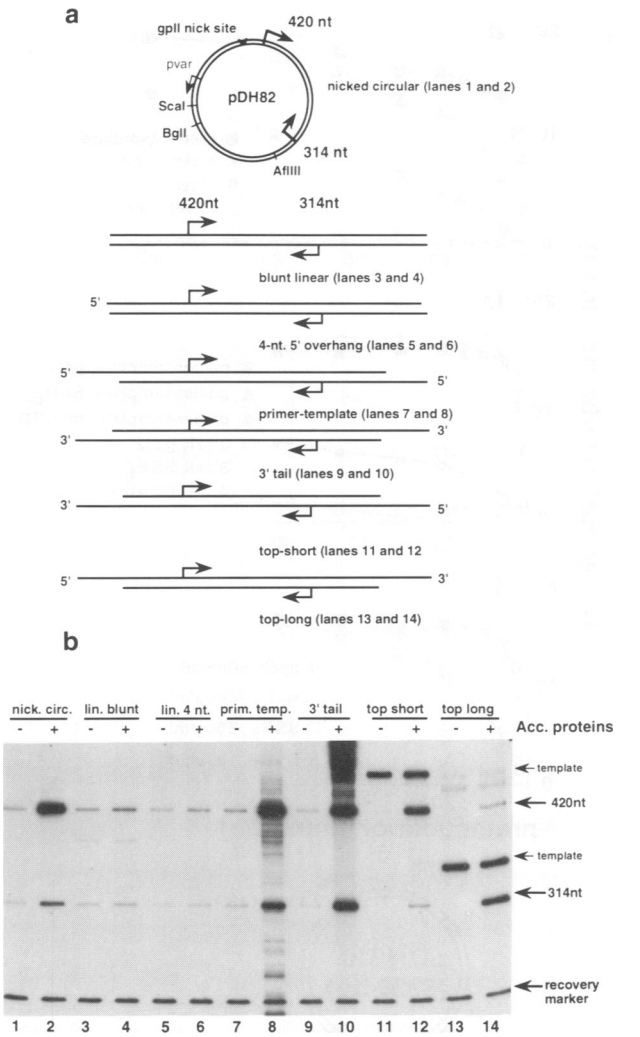

Fig. 1. DNA structures that serve as enhancers of T4 late transcription. (**a**) DNA templates are derived from pDH82 (3882 bp), whose opposed T4 late transcription units, sites of nicking by gpII endonuclease, and of cutting by *ScaI*, *BglI* and *AflIII*, are shown at the top of the figure, approximately to scale. The location of a variant T4 late promoter is also indicated. (**b**) Single-round transcription assays in the absence or presence of the DNA polymerase accessory proteins gp44, 62 and 45 were performed on the DNA shown in (a), as described in Materials and methods. The 420 and 314 nt transcripts are indicated at the right. Additional transcripts in lanes 8 and 10 are referred to in the text. The additional bands in lanes 11–14, also marked at the right, are labeled DNA that is generated during template preparation.

A primer–template junction is the preferred site of entry of the transcriptional activator

The relative efficiencies of different DNA loading sites can be compared by measuring the relative rates of promoter opening in a single-round transcription assay. For the experiments of Figure 2, transcription proteins were mixed at 0°C and added to DNA, single-stranded DNA binding protein (SSB) and dATP pre-equilibrated at 25°C, incubated at that temperature for the indicated time, then challenged to initiate a single round of transcription by adding a mixture of ribonucleoside triphosphates and

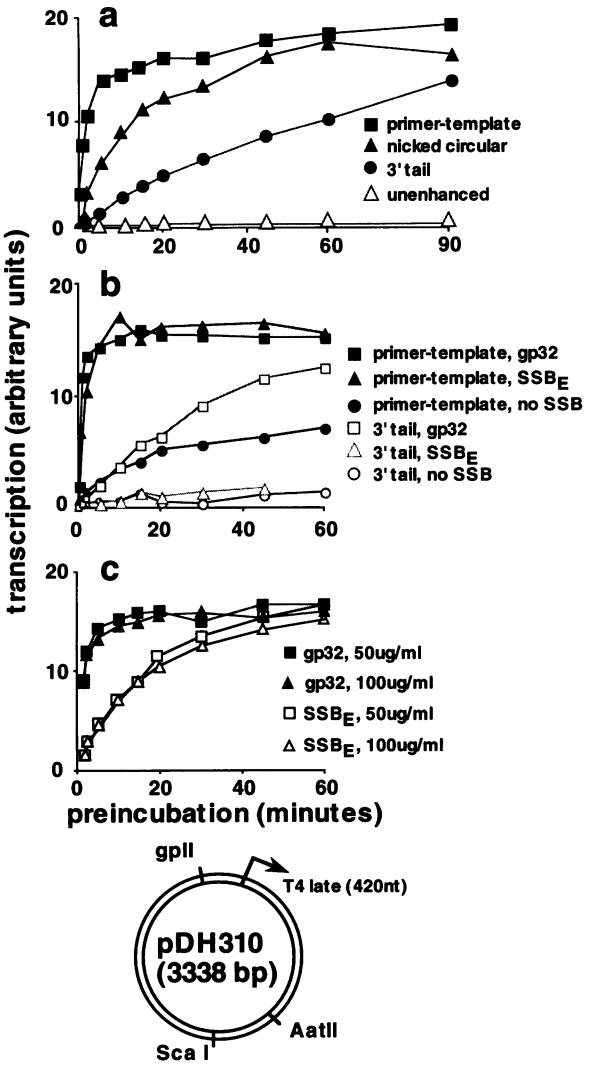

Fig. 2. Efficiencies of transcriptional activation generated by different gp45 loading sites. A mixture of T4-modified RNA polymerase, gp55, gp33 and the DNA polymerase accessory proteins (gp44/62 complex and gp45) was added to pDH310 (shown at the bottom of the figure) DNA, gp32 and dATP pre-equilibrated at 25°C. Aliquots were withdrawn at the times noted on the abscissa and added to a mixture of unlabeled and radioactive ribonucleoside triphosphates and rifampicin pre-equilibrated at 25°C for a single round of transcription. The yield of 420 nt RNA is reported on the ordinate (in arbitrary units). Further experimental details are provided in Materials and methods. (**a**) Comparison of primer–template and 3′-tailed junctions with nicked circular DNA. (■): linear DNA with 5′ overhanging (primer–template junction) ends; (●): linear DNA with 3′-tailed ends; (▲): nicked circular DNA; (△): unenhanced transcription (accessory proteins omitted). The distances from the relevant gp45 loading sites (see below) to the enhanced promoter are ~1520 bp, ~1070 bp and ~220 bp for the primer–template junction, 3′ tail and nick, respectively. (**b**) Effects of gp32 and *E.coli* SSB (SSB$_E$) on the efficiency of transcriptional activation. DNA with 5′ overhanging (closed symbols) and 3′ overhanging (open symbols) ends, without SSB (circles), with SSB$_E$, (triangles) or with gp32 (squares) was analyzed. (**c**) Effects of gp32 and SSB$_E$ on activation of transcription of DNA with 5′ overhanging ends, at one-fourth of the concentration of each of the DNA polymerase accessory proteins used in (b). Gp32 (closed symbols) and SSB$_E$ (open symbols) were used at 100 μg/ml (triangles) or 50 μg/ml (squares).

the transcription initiation inhibitor rifampicin. The assay measures the end-product of a complex reaction sequence, which requires an adequate measure of thermal equilibration, assembly of a gp44/62–gp45 complex at a loading site, entry of gp45 for tracking along DNA, interaction with RNA polymerase, promoter location and opening. Nevertheless, primer–template junctions generate half-maximal promoter opening within 1–2 min, at modest concentrations of the DNA polymerase accessory proteins (Figure 2a, closed squares). Promoter opening of nicked circular templates and of templates bearing recessed 5′ ends (i.e. 3′ tailed) is relatively slow, with 50% rise times of ~10 min and >30 min, respectively (Figure 2a, closed triangles and closed circles, respectively). Unenhanced transcription is negligible under these conditions (open triangles). Thus, the most effective of these enhancers (see below) is a primer–template junction that is located ~1.5 kbp from the activated promoter.

SSB proteins affect the ability of single-stranded DNA regions to generate enhanced transcription

The preceding experiments were carried out in the presence of gp32, the T4-encoded SSB. The effect of omitting gp32 or of substituting the *E.coli* SSB (SSB$_E$) on activation of transcription has also been examined (Figure 2b). In the absence of any SSB, 3′-tailed DNA does not generate activated transcription (open circles), and SSB$_E$ does not rescue activation (open triangles), implying that the lack of transcription is not merely due to sequestration of transcription components by single-stranded DNA. Significant rates of promoter opening with 3′ tails are only obtained in the presence of gp32 (open squares). In contrast, primer–template junctions generate modest rates of promoter opening in the absence of any SSB (closed circles), and the rate of promoter opening is greatly increased by providing SSB$_E$ (closed triangles). Gp32 further increases the rate of promoter opening (closed squares). SSB$_E$ and gp32 have no effect on transcription of nicked, circular DNA (data not shown). Thus, a single-stranded region of DNA is necessary for the SSBs to manifest their effects on activation of transcription.

The profound effect of both SSB$_E$ and gp32 on enhancement of transcription from a primer–template junction implies that simply masking exposed single-stranded DNA greatly improves loading of gp45. The next experiment (Figure 2c) looks for a more specific role of SSB in gp45 loading by examining quantitative differences in the ability of gp32 and SSB$_E$ to facilitate transcriptional enhancement. Since SSB$_E$ and gp32 differ greatly in their modes of DNA association (Lohman and Ferrari, 1994), differences in transcriptional activation could also reflect different degrees of saturation of single-stranded DNA. Rates of promoter opening were examined at limiting concentrations of the DNA polymerase accessory proteins (in order to slow down the otherwise rapid rate of open complex formation and accentuate differences between gp32 and SSB$_E$ in promoting enhanced transcription). At their respective saturation limits (excluding the possibility that quantitative differences in activation reflect different degrees of saturation of single-stranded DNA with SSB), gp32 is a substantially more effective co-factor than SSB$_E$ for enhancement of transcription from a primer–template junction (Figure 2c, compare closed symbols with open

symbols), perhaps reflecting the ability of the T4 DNA polymerase accessory proteins to interact specifically with gp32 (Formosa *et al.*, 1983; Richardson *et al.*, 1989; see also Tinker *et al.*, 1994b).

Single-stranded DNA activates transcription only from downstream of a T4 late promoter

It has been established that nicks in DNA can serve as the enhancer-like sites of gp45-activated T4 late transcription whether located upstream or downstream of a T4 late promoter (Herendeen *et al.*, 1989). The next experiments examine DNA templates bearing single-stranded extensions for the directionality of their activation, using the tightly DNA binding and DNA cleavage-defective mutant of *Eco*RI (*Eco*RI-Gln111) to block the DNA track between a gp45 loading site and a target T4 late promoter (Herendeen *et al.*, 1992) and thereby insulate particular promoters from individual gp45 loading sites.

Plasmid pDH72ΔE1 has two co-directional T4 late transcription units separated by two *Eco*RI sites (drawn at the top of Figure 3a). Addition of *Eco*RI-Gln111 to pDH72ΔE1 bearing gp45 loading sites at both ends isolates the left promoter from the right gp45 loading site, and the right promoter from the left gp45 loading site. Transcription from pDH72ΔE1 with a primer–template junction at each end is shown in Figure 3a, lanes 1–5. Without added *Eco*RI-Gln111, transcription from the right promoter (420 nt RNA) is strongly activated by the DNA polymerase accessory proteins, and the left promoter (314 nt RNA) is weakly activated (lanes 1 and 2). Addition of *Eco*RI-Gln111 preferentially inactivates the left promoter, as if access to its downstream primer–template junction were essential for activated transcription. Similarly, pDH72ΔE1 DNA with 3′ tails strongly activates the right promoter and weakly activates the left promoter (lanes 6 and 7). Addition of *Eco*RI-Gln111 inactivates the left promoter and has little effect on the right promoter (lanes 8–10), again suggesting that a downstream loading site is essential for activated transcription.

If an unobstructed path from a downstream loading site were required for promoter activation, then one might expect weak activation of the left promoter of pDH72ΔE1, even in the absence of *Eco*RI-Gln111: open promoter complexes forming on the pathway from the downstream gp45 loading site might have the same blocking property as *Eco*RI-Gln111 itself. Alternatively, the weak activity of the left-hand promoter of Figure 4 could indicate sensitivity to non-specific inhibition. To resolve this issue, a similar set of transcription experiments was carried out on the DNA templates shown in panels b and c of Figure 3. Plasmid pGS722 has two convergent T4 late transcription units separated by a pair of *Eco*RI sites. Addition of *Eco*RI-Gln111 protein isolates each promoter

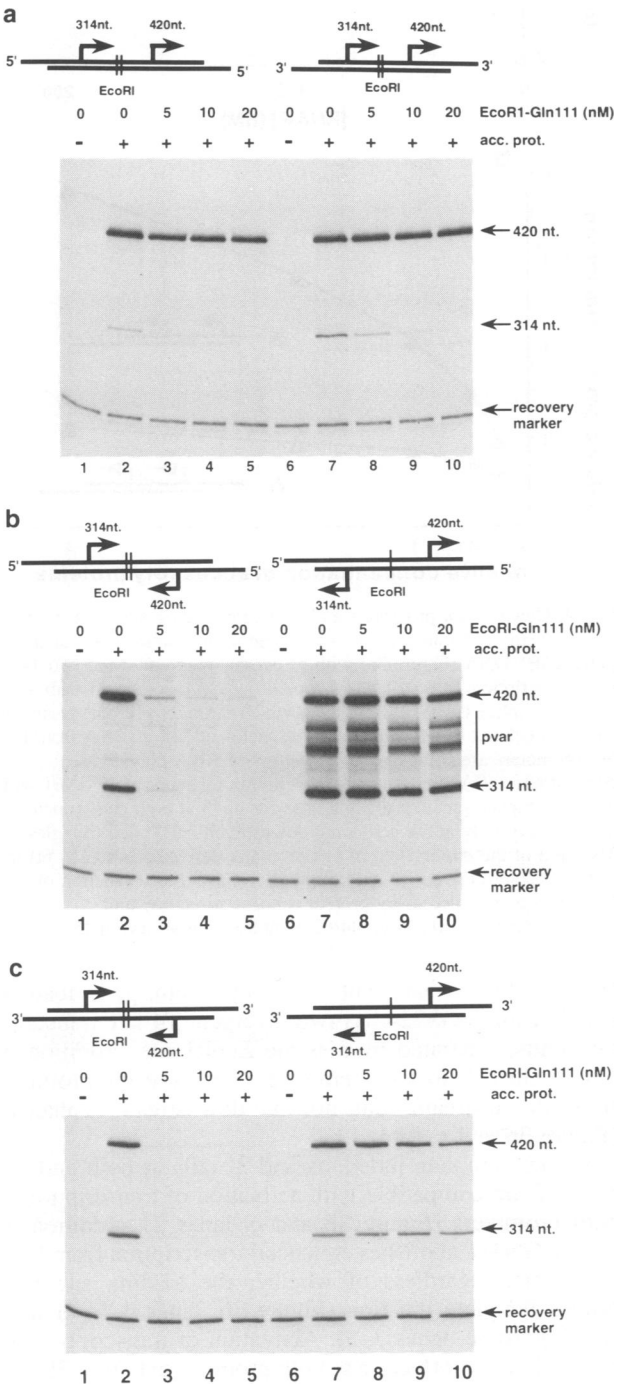

Fig. 3. The activation path: effect of *Eco*RI-Gln111 on activation of transcription of the co-directional transcription units of pDH72ΔE1 (a), and on the opposed transcription units of pGS722 (b) and pGS724 (c). (**a**) Two *Eco*RI sites separate the two co-directional T4 late transcription units of pDH72ΔE1; DNA-bound *Eco*RI-Gln111 separates the left-hand transcription unit (314 nt RNA) from its downstream gp45 loading site, and the right-hand transcription unit (420 nt RNA) from its upstream loading site, as shown at the top of the panel. DNA, gp32, dATP and *Eco*RI-Gln111 at the indicated concentrations were pre-incubated, transcription proteins were added, and a single round of transcription was analyzed. Lanes 1–5: DNA with 5′ overhanging ends; lanes 6–10: DNA with 3′ overhanging ends. (**b**) and (**c**) In pGS722, the two convergent T4 late transcription units are separated by two *Eco*RI sites. DNA-bound *Eco*RI-Gln111 separates both promoters from their downstream, but not their upstream, loading sites. In pGS724, the two divergent T4 late transcription units are separated by a single *Eco*RI site. DNA-bound *Eco*RI-Gln111 separates both promoters from their upstream, but not their downstream, loading sites. Pre-binding of *Eco*RI-Gln111 and single-round transcription was performed as specified for (a). Concentrations of *Eco*RI-Gln111 are specified in the figures. (b) DNA with 5′ overhanging ends; (c) DNA with 3′ overhanging ends. Lanes 1 and 6, all panels: unenhanced transcription (DNA polymerase accessory proteins omitted). (The additional transcripts that are marked by a vertical line at the side of panel b probably initiate at a variant T4 late promoter and terminate heterogeneously within the single-stranded DNA ends, as discussed in conjunction with Figure 1.)

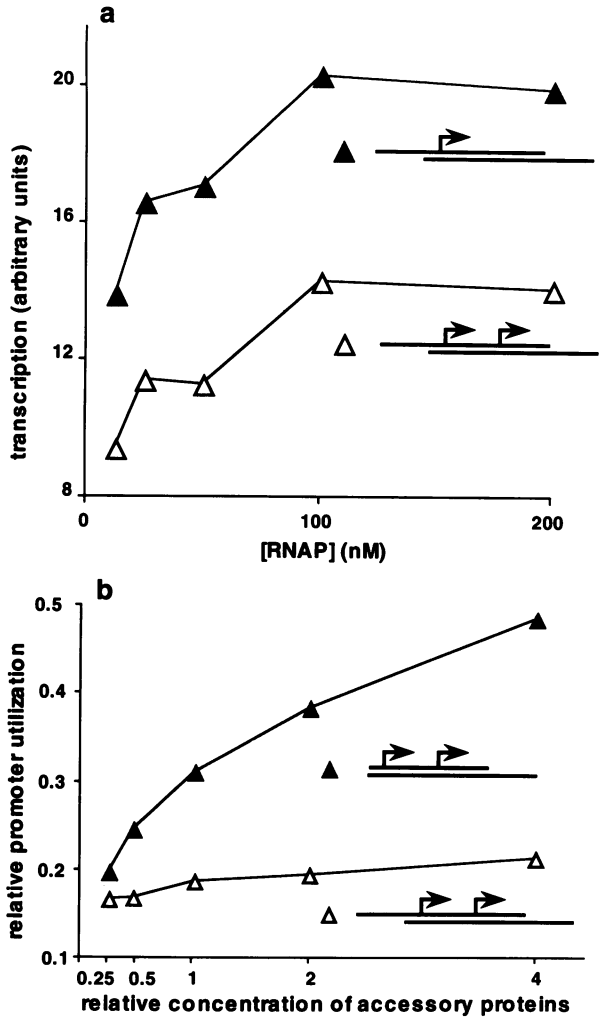

Fig. 4. Downstream promoters and upstream loading sites can interfere with activation of transcription. (**a**) Single-round transcription of linear pDH72ΔE1 DNA (Figure 3a) with 5′ overhanging ends, and two T4 late transcription units in tandem (open triangles), compared with a similar template (pDH72Δ420) from which the right (420 nt) promoter has been deleted (filled triangles). The yields of 314 nt RNA from the left promoters are compared as a function of RNA polymerase concentration. (**b**) Single-round transcription of linear pDH72ΔE1 with primer–template junction loading sites for gp45 at both ends (open triangles), or only at the activating downstream end (filled triangles). The ratio of the molar yield of left promoter-derived RNA (314 nt) to right promoter-derived RNA (420 nt) is compared as a function of DNA polymerase accessory protein concentration, one unit corresponding to 160 nM gp 44/62 complex with 90 nM gp45.

from its downstream, but not its upstream, gp45 loading site. Plasmid pGS724 has two divergent T4 late transcription units, separated by a single *Eco*RI site. Addition of *Eco*RI-Gln111 to this template isolates each promoter from its upstream, but not its downstream, enhancer (Figure 3b and c, top).

Primer–template junctions and 3′ tails at both ends of pGS722 are compatible with activation of transcription at both promoters (Figure 3b and c, lanes 2). Addition of *Eco*RI-Gln111 abolishes enhanced transcription from both promoters, regardless of whether the loading site is a primer–template junction (Figure 3b, lanes 3–5) or a 3′ tail (Figure 3c, lanes 3–5). Activation of transcription on pGS724 is also effective at both promoters (Figure 3b and c, lanes 7), but addition of *Eco*RI-Gln111 to pGS724 has little effect on enhanced transcription, again regardless of whether the loading site is a primer–template junction (Figure 3b, lanes 8–10) or a 3′ tail (Figure 3c, lanes 8–10). That a single *Eco*RI site is sufficient to block DNA tracking by gp45 has been shown previously (Herendeen *et al.*, 1992) and is verified here by the disappearance of an enhancement-dependent, higher molecular weight transcript upon addition of *Eco*RI-Gln111 (data not shown). We conclude that activation of transcription from a single-stranded DNA end only occurs from downstream of a T4 late promoter, and can be generated from primer–template junctions or 3′ tails.

In contrast, the results of Figure 1 suggest that only promoters with single-stranded extensions on their transcribed strands are activated (lanes 11–14, in particular). A solution to this apparent conflict lies in the orientation of the promoters of pDH82, the requirement for a clear and unobstructed pathway between loading site and promoter, and the relative efficiency of a primer template junction as an activator of transcription. Activation of each of the convergent promoters of pDH82 (Figure 1) by a downstream loading site necessitates gp45 tracking past the other promoter. Open promoter complexes should form roadblocks to enhancement of other promoters in *cis*. In the case of pDH82 (Figure 1) bearing an efficient primer–template junction loading site for gp45 at one end, and a relatively weak 3′ tail loading site at the other ('top-long' and 'bottom-long' DNA), the promoter upstream of the efficient loading site is expected to open quickly (Figure 2) and block enhancement of the promoter upstream of the inefficient loading site.

Traffic problems on the track: upstream enhancers and downstream promoters can interfere with activation

The preceding supposition has been tested directly by comparing open promoter complex formation on pDH72ΔE1 (Figure 4, top) with open promoter complex formation on a derivative template, pDH72Δ420, from which the potentially interfering downstream promoter has been deleted. Indeed, the yield of transcripts is increased by deletion of the downstream promoter (Figure 4a), at limiting or excess RNA polymerase, and is therefore unlikely simply to reflect competition for RNA polymerase by the additional late promoter on pDH72ΔE1.

To explain more fully the weak activation of the upstream promoter in Figure 3, we examined whether gp45 loaded at a non-activating site (upstream of a proximal promoter, the stream referring to the direction of transcription) can interfere with activation from a gp45 loading site located downstream of that promoter. The effect of accessory protein concentration on the relative yield of transcripts from tandem promoters was compared on DNA with a gp45 loading site only at the downstream end (the upstream end having been made blunt), and on DNA with gp45 loading sites at both ends, placing a non-activating gp45 loading site upstream of the left-hand promoter (Figure 4b). Increasing the accessory protein concentration increases the relative yield of transcripts from the left promoter on the template with only a downstream gp45 loading site (filled triangles), but not on the template with gp45 loading sites at both ends (open triangles). These results are consistent with the supposition

that the effect on transcription of increasing the density of activation-competent gp45 is countered by an increasing density of activation-incompetent (because improperly oriented, as we will discuss below) gp45 loaded from the upstream end. In conclusion, upstream enhancers and downstream promoters can interfere with activation of transcription by the DNA polymerase accessory proteins.

Gp45 efficiently traverses nicks, but not long single-stranded gaps

Nicks and single-stranded extensions generate transcriptional activation in distinctive ways: single-stranded protrusions strictly require the target promoter to be located upstream, while nicks are enhancer-like in that they can activate transcription at promoters that are located upstream or downstream. The next experiment examines whether single-stranded gaps share this activation property with nicks.

The two co-directional T4 late transcription units of pGS725 are flanked by *Eco*RI sites and separated by a gpII endonuclease site, which allows a nick to be placed into their non-transcribed strand. The transcription unit upstream of the nick yields a 314 nt transcript and the downstream transcription unit yields 420 nt RNA. Treatment of nicked DNA with exo III generates gapped DNA. *Eco*RI-Gln111 confines transcriptional activation by gp45 to the DNA segment shown at the bottom of Figure 5 and increases the efficiency of activation at higher ionic strength, presumably by preventing gp45 tracking to, and dissociating from, DNA ends (Herendeen *et al.*, 1992).

Nicked templates allow activation of both promoters, in the absence or presence of SSB (Figure 5, lanes 2, 5 and 8). Gapped templates only allow efficient activation of the upstream promoter (to the left of the gap), even in the absence of any SSB, and in spite of the continuous DNA strand linking the two transcription units (lanes 3, 6 and 9). Pre-incubation of gapped DNA with accessory proteins fails to rescue activation of the downstream promoter (data not shown). Thus, gaps seem to have essentially the same activation properties as single-stranded extensions: they must be located downstream of the promoter. This result and the experiments shown in Figures 3 and 4 suggest that gp45 tracks across a nick but fails to traverse an ~250 nt (on average) single-stranded region.

Activation of transcription from a recessed 5′ end may require a gp32-mediated structural change at the double strand–single strand DNA junction

Gp45 loading in a specific orientation that is determined by the 5′→3′ polarity of single-stranded DNA at the loading site accounts for the observed polarities of transcriptional enhancement at nicks (strand specificity) and at primer–template junctions (downstream loading), as discussed further below, but fails to explain the ability of 3′ tails to act as enhancers of transcription when located downstream of a T4 late promoter. Since 3′ tails specifically require gp32 to activate transcription (Figure 2b), double strand–single strand DNA junctions were examined for gp32-dependent structural changes. Figure 6 shows the DNase I footprints of accessory protein complexes formed in the presence and absence of gp32 at the two

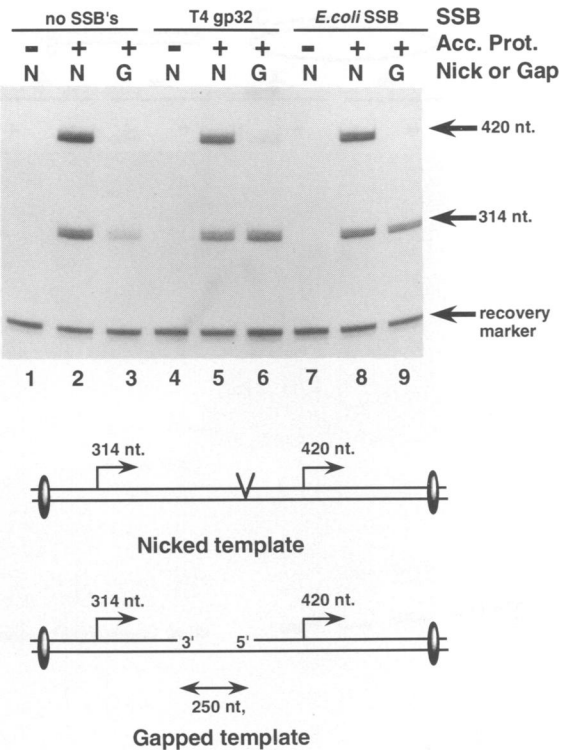

Fig. 5. The ability of gp45 to traverse single-stranded DNA. The co-directional T4 late transcription units of pGS725 are separated by a gpII endonuclease site, and flanked by two *Eco*RI sites. Supercoiled DNA was nicked or treated to generate an ~250 nt single-stranded gap, then linearized, as diagrammed at the bottom of the figure (the ovals indicating bound *Eco*RI-Gln111). Single-round transcription was done as specified in Materials and methods. Lanes 1, 4 and 7: unenhanced transcription of nicked linear DNA in the absence of any SSB, with gp32 and with SSB_E respectively. Lanes 2, 5 and 8: enhanced transcription of nicked linear DNA without SSB, with gp32 and with SSB_E respectively. Lanes 3, 6 and 9: enhanced transcription of linear gapped DNA without SSB, with gp32 and with SSB_E respectively.

types of termini. The DNA probes for lanes 1–4 and 9–12 have primer–template junctions; probes for lanes 5–8 and 13–16 have recessed 5′ ends. Lanes 1–8 show the 'top' strands of the respective termini, as drawn at the top of the figure, and lanes 9–16 show the 'bottom' strands. In the presence of gp32, strong protection of primer–template junctions by the DNA polymerase accessory proteins extends well into the double-stranded region (compare lanes 4 and 12 with lanes 2 and 10), similar to the protection attributed by Munn and Alberts (1991a) to a complex of the DNA polymerase accessory proteins. At 5′-recessed ends, the formation of comparable complexes is barely perceptible, at best (even to the prepared mind; compare lane 16 with lane 15, and lane 8 with lane 7). Gp32 binds to duplex DNA asymmetrically: only the strand with the single-stranded extension is protected from DNase I digestion. The effect is most clearly seen with recessed 5′ end DNA (contrast lanes 15 and 13 with lanes 7 and 5, respectively). At the primer–template junction, the gp32 footprint has the same general characteristics (lanes 1, 3, 9 and 11), but is partly obscured by a background in the undigested probe for lanes 9–12. We interpret the asymmetry of protection as being due to cooperative gp32 binding to the contiguous single strand

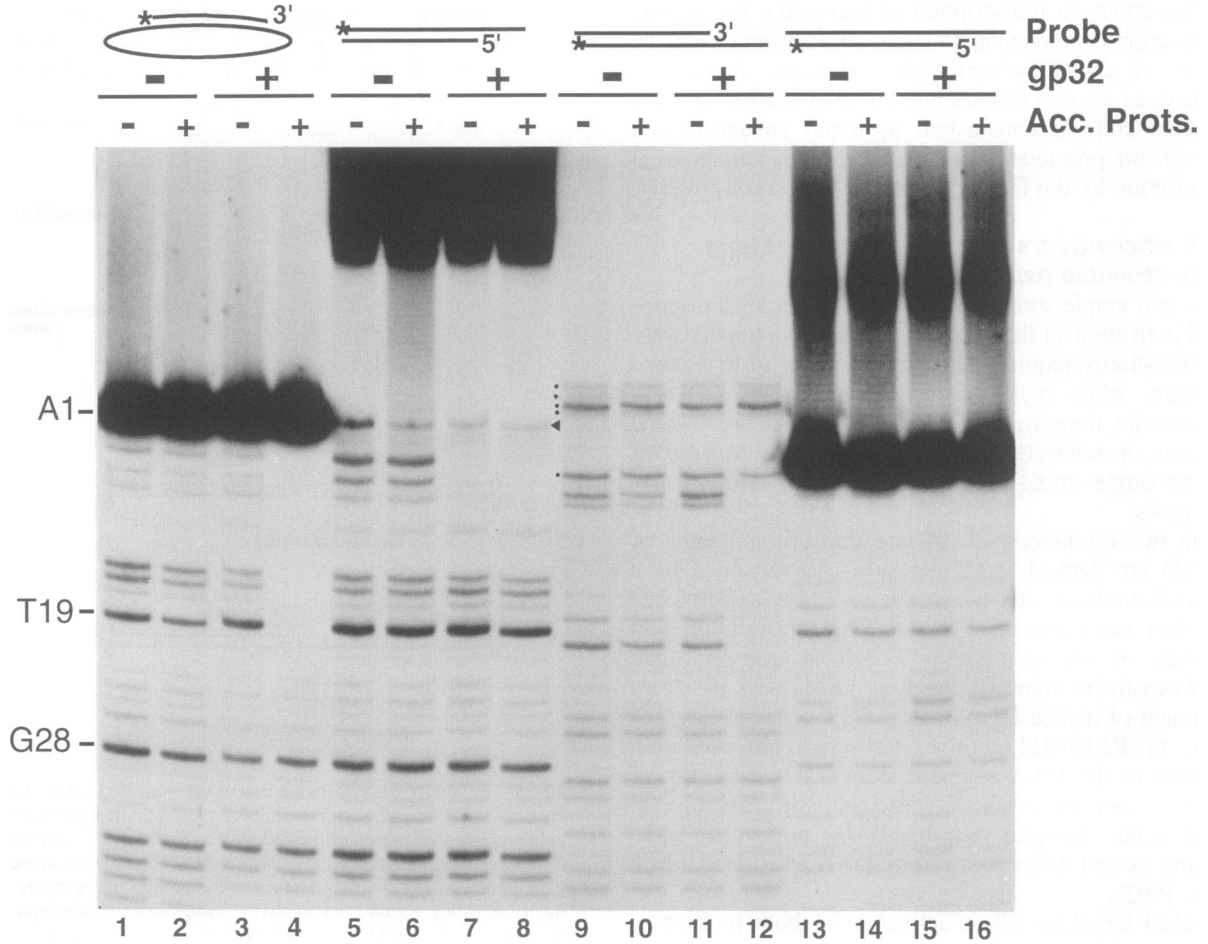

Fig. 6. DNase I footprints of protein complexes at double strand–single strand junctions. Probes for DNase I footprinting, diagrammed at the top of the figure, were incubated with the indicated proteins, in the presence of 200 μM ATP-γ-S, then digested with DNase I, and analyzed as described in Materials and methods. Small amounts of labeled fragments contaminating probes for lanes 5–8 and 9–12 are marked with a triangle and black dots, respectively, and are excluded from the analysis. Lanes 1–4: primer–template junction probe, 5′ end-labeled in the short strand; lanes 5–8: 3′-tailed DNA labeled in the long strand; lanes 9–12: primer–template junction probe labeled in the short strand; lanes 13–16: 3′-tailed DNA labeled in the short strand. Lanes 1, 5, 9 and 13: no-protein controls; lanes 2, 6, 10 and 14: with DNA polymerase accessory proteins alone; lanes 3, 7, 11 and 15: with gp32 alone; lanes 4, 8, 12 and 16: with accessory proteins and gp32.

that allows partial invasion of the duplex region. The failure to protect the complementary short strand is consistent with the high cooperativity of gp32 binding to single-stranded DNA. The length of single-stranded DNA that is exposed at the end of the complementary DNA strand would accommodate only one molecule of gp32, which would bind relatively inefficiently (Kowalczykowski *et al.*, 1981). Nevertheless, melting of this short section of the 5′-recessed strand at the double strand–single strand junction could render it capable of being loaded with gp45 in an orientation that is compatible with transcriptional activation. The efficiency of gp45 loading at this site is at least an order of magnitude lower than at the primer–template junction (Figure 2a), consistent with the lack of a clear-cut footprint of DNA polymerase accessory proteins in lanes 8 and 16.

Discussion

A model to explain the transcriptional enhancement properties of diverse DNA structures

Nicks in DNA, gaps and single-stranded extensions of either polarity all serve as loading sites for the DNA polymerase processivity factor and transcriptional activator gp45, but with greatly different efficiencies (Figures 1 and 2). Interference with transcriptional activation can arise in two ways: gp45 tracking along DNA in the activation-incompatible orientation diminishes the activity of properly oriented gp45 (Figure 4b), and open promoter complexes can block the track to gp45 (Figure 4a).

The seemingly disparate properties of different gp45 loading sites and of differently organized and oriented transcription units can be reconciled by a single model with the following features (Figure 7): (i) the loading enzyme, gp44/62, (Kaboord and Benkovic, 1995) recognizes the 5′→3′ polarity of the continuous DNA strand at the loading site. (ii) The gp44/62–single-stranded DNA interaction determines the polarity of loading of gp45. (iii) Once the orientation of gp45 on DNA is determined, it is not reversed subsequently (Tinker *et al.*, 1994a). (iv) When gp45 enters its complex with gp44/62 at the DNA loading site, it is situated on the side of gp44/62 that faces the 3′ end of the continuous or longer DNA strand. At a primer–template junction, this places gp45 over double-stranded DNA (Figure 7, line b), consistent with the prior footprinting and photocrosslinking analyses

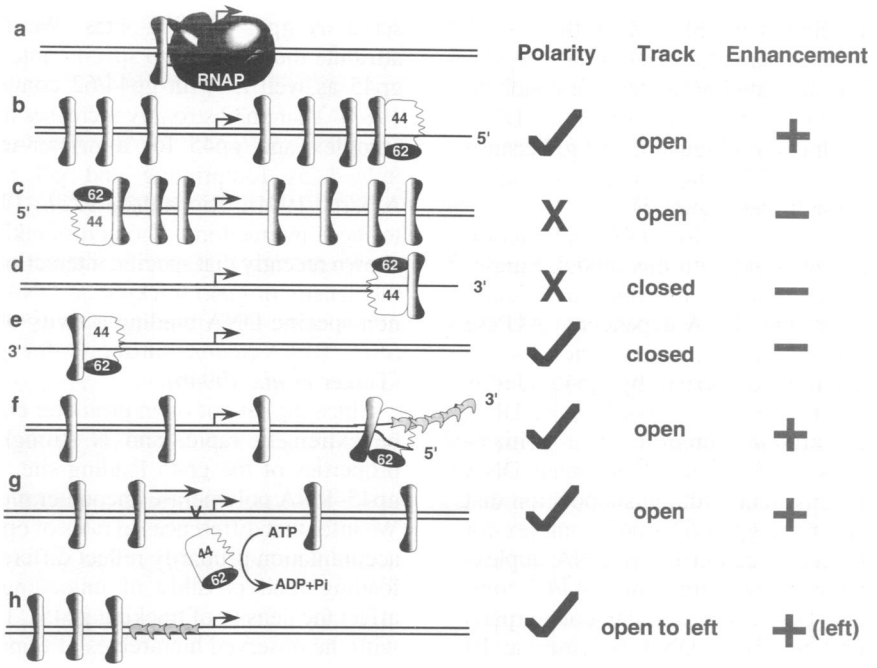

Fig. 7. A model of gp45 loading, tracking and transcriptional activation. (**a**) Only one lateral face of the supposedly toroidal gp45 interacts productively with RNA polymerase. (**b**) The orientation of gp45 on DNA is determined by its interaction with the loading enzyme gp44/62, which recognizes the 5′→3′ polarity of single-stranded DNA at the loading site; ATP hydrolysis releases gp45 from the accessory protein complex, and gp45 can then diffuse onto duplex DNA; when a primer–template junction is located downstream of a T4 late promoter, gp45 thus loaded is in the proper orientation to interact productively with RNA polymerase. (**c**) When the primer–template junction is located upstream of a T4 late promoter, gp45 is loaded in the wrong orientation to interact productively with RNA polymerase. (**d** and **e**) Assembly of accessory protein complexes at 3′ tails places gp45 in the single-stranded region, interposing barriers to tracking regardless of gp45 orientation. (**f**) 3′ tails serve as effective loading sites when asymmetrically invaded by gp32, creating a pseudo-primer–template junction; only the downstream loading site imposes the proper orientation on gp45 for transcriptional activation. (**g**) gp45 can traverse a nick; recognition of the continuous DNA strand by gp44/62 at the loading site as shown on line (b) generates the requirement that the nick must be in the non-transcribed strand of the activated T4 late transcription unit; gp45 can track across the nick, from left to right, whenever gp44/62 falls off the DNA. (**h**) gp45 cannot traverse a gap; it is released to the left, as at a primer–template junction, but cannot track to the right.

of Munn and Alberts (1991a,b) and Capson and co-workers (1991). (v) ATP hydrolysis releases gp45 from its complex with gp44/62 at the loading site; when it is situated over double-stranded DNA, gp45 can now undergo one-dimensional diffusion along a double-stranded DNA track (Herendeen *et al.*, 1992; c.f. *Gogol et al.*, 1992). (vi) The same face of gp45 interacts with the gp44/62 complex, tethers the gp43 DNA polymerase (probably the C-terminus of the latter; Nossal, 1994, referring to work by Goodrich *et al.*, 1992; see also Capson *et al.*, 1991; Munn and Alberts, 1991) to its template, *and* interacts with RNA polymerase to generate transcriptional activation. (vii) The gp45-facing end of RNA polymerase is located at the upstream end of the T4 late promoter complex (Tinker *et al.*, 1994a).

Thus, a primer–template junction located downstream of a T4 late promoter (the 'stream' referring to the flow of transcription) loads gp45 in the proper orientation for its interaction with RNA polymerase and, counterintuitive though it may seem, ultimately places gp45 at the upstream end of an open promoter complex (Figure 7, line a). A primer–template junction located upstream of a late promoter loads gp45 in the orientation that is incompatible with the required polymerase interaction at that particular promoter (line c). On 3′-tailed, 5′-recessed DNA (line d), the same polarity of gp44/62 binding and assembly of gp45 places the latter over single-stranded DNA, its path to tracking along double-stranded DNA blocked by the interposed gp44/62 complex, by its inability to traverse single-stranded DNA (Figure 5), and possibly because gp45 cannot be loaded efficiently onto single-stranded DNA. [For example, the inner surfaces of *E.coli* β and yeast PCNA are basic, and thought to interact with the phosphate backbone of the DNA duplex (Kuriyan and O'Donnell, 1993; Krishna *et al.*, 1994). If those interactions are important for assembly of the sliding clamp, their perturbation by single-stranded DNA could interfere with DNA loading.] Enhancement of transcription from a 3′-tailed DNA end located upstream of the late promoter is blocked by that occlusion (Figure 7, line e). When the 3′-tailed DNA end is located downstream of the promoter, the orientation of gp45 is, moreover, incompatible with activation (line d). We propose that the ability of a 3′-tailed DNA end to activate transcription at all requires a different process: the highly cooperative gp32 partly melts a short segment of duplex DNA at the double-stranded–single-stranded junction and provides a marginally stable binding site for a gp44/62–gp45 assembly that has the proper polarity to release gp45 for tracking along double-stranded DNA. Only the downstream-located 3′-tailed DNA end confers the proper orientation for transcriptional activation (line f).

The DNA nick is unique as an enhancer because it allows gp45 to activate transcription at promoters located on either side (Herendeen *et al.*, 1989). This is due to the ability of gp45 to track across the nick. The proper

orientation of gp45 loading is established by the 5′→3′ polarity of the continuous strand, which must be the transcribed strand of the activated promoter. We conclude that gp45 is first released from its loading site at the DNA nick toward the left, as drawn on Figure 7, line g, because converting the nick into an ~250 nt gap confines activation by gp45 to that side (line h and Figure 5).

The known properties of the T4 DNA polymerase accessory proteins are consistent with this model. Single-stranded DNA and primer–template junctions are equally effective co-factors for the DNA-dependent ATPase activity of gp44/62 complex alone; a preference for the primer–template junction is conferred by gp45 (Jarvis *et al.*, 1989b). A photocrosslinking analysis of the DNA polymerase accessory protein complex at a primer–template junction places gp45 on double-stranded DNA (Capson *et al.*, 1991), consistent with the supposition that the specific preference of the gp44/62–gp45 complex for a primer–template junction is conferred by DNA duplex-confined gp45 stabilizing the placement of gp44/62 complex on single-stranded DNA. The cellular counterparts of gp45, the β subunit of *E.coli* DNA polymerase III holoenzyme and PCNA are tori (Kong *et al.*, 1992; Krishna *et al.*, 1994). Anticipating that gp45 will also turn out to be toroidal, one can rationalize readily that the central hole of gp45 might be too small for tracking along gp32-laden single-stranded DNA and that DNA secondary structure, as well as direct interactions of nucleotides in single-stranded DNA with the external faces of the protein catenane, would also obstruct tracking along bare single-stranded DNA. We do not know the size of the smallest gap that forms an effective barrier to gp45 tracking, but would not be surprised to find gp45 able to cross a short gap such as might be created at an Okazaki fragment junction by digestion of the RNA primer (Nossal, 1994). If the gp45 trimer (Jarvis *et al.*, 1989a) is a PCNA-like torus with a 3-fold axis down its central cavity (Krishna *et al.*, 1994), then its lateral faces must be non-identical, capable of different specific protein–protein interactions and of manifesting the polarity that is required to account for the properties of transcriptional enhancement at late promoters exhibited in this and prior work (Herendeen *et al.*, 1989; Tinker *et al.*, 1994a).

Efficiencies of transcriptional activation

Extraordinarily effective transcriptional activation, reflected in very rapid promoter opening, is afforded by primer–template junctions in the presence of gp32 (Figure 2). Activation, as measured by the rate of accumulation of open promoter complexes, is relatively inefficient in the absence of any SSB (Figure 2), probably due to non-productive sequestration of proteins on single-stranded DNA tails. This can be prevented by adding (the heterologous) SSB_E. SSBs probably also prevent tracking gp45 from falling off the ends of linear DNA. The effect is comparable with that of confining tracking gp45 by using a circular DNA template, or by blocking the ends of its linear DNA with tightly bound protein (cf. Stukenberg *et al.*, 1991; Herendeen *et al.*, 1992).

There is, in addition, a specific quantitative effect of gp32 on the enhancer efficiency of primer–template junctions (Figure 2) and an absolute requirement for gp32 that is not filled by SSB_E when recessed 5′ DNA ends serve as gp45 loading sites. We think it plausible to attribute these effects to specific interactions of gp32 with gp45 as well as with gp44/62 complex (Formosa *et al.*, 1983). That gp32 strongly increases the affinity of gp44/62 complex and gp45 for a primer–template junction, as judged by footprinting and gel filtration (Munn and Alberts, 1991a; Richardson *et al.*, 1989), is probably due to these interactions. Photocrosslinking experiments have shown recently that specific interaction with gp32 increases the density of gp45 tracking on DNA, and that the general non-specific DNA binding activity of SSB_E or the human SSB, RPA, cannot substitute for gp32 in this regard (Tinker *et al.*, 1994b).

Since the rate of open promoter complex formation can be extremely rapid, and is strongly dependent on the properties of the gp45 loading site, events subsequent to gp45–RNA polymerase encounter must also be very rapid. We infer that differences in rates of open promoter complex accumulation primarily reflect differences in rates of gp45 loading (and possibly of unloading, which would also affect the density of tracking gp45). This view is consistent with the observed hierarchies of transcriptional activation. Optimal activation of transcription is achieved with primer–template junctions; nicks and 3′ tails are less efficient (Figure 2), because they are sub-optimal as binding sites for the gp44/62 complex (e.g. Figure 6) and consequently load gp45 less efficiently. Current experiments (T.-J.Fu, personal communication) are directed at exploring the dynamics of gp45 loading and tracking.

In closing, we want to point out that the most efficient enhancer in this resolved, highly purified and simplified *in vitro* system is not necessarily the primary contributor to T4 late transcription *in vivo*. Primer–template junctions are sites of assembly of DNA polymerase holoenzyme and of active DNA chain elongation, both expected to compete with gp45 loading. (Preliminary experiments confirm, for example, that stalled DNA polymerase holoenzyme blocks T4 late transcriptional enhancement by gp45; G.M.S. unpublished observations.) On the other hand, rapid promoter opening (Figure 2) allows a variety of gp45 loading sites to contribute incrementally to T4 late gene activity, and may also permit a single gp45 trimer to activate several rounds of transcription initiation during a single episode of tracking along DNA.

Materials and methods

Labeled and unlabeled nucleoside triphosphates, bovine serum albumin (BSA), terminal deoxynucleotidyl transferase (TdT), exonuclease III (exo III), DNA ligase, DNase I, proteinase K, restriction enzymes and rifampicin were purchased from various commercial suppliers.

Plasmids pDH82, pDH310 and the pDH72 series have been described (Herendeen *et al.*, 1989, 1992; Herendeen, 1991). Plasmid pGS722 is a derivative of pDH72ΔE1 in which the 420 nt T4 late transcription unit was inverted by excision with *Aat*II and *Nsi*I restriction enzymes, digestion of the 3′ overhanging ends with T4 DNA polymerase, and re-ligation of the fragments. Plasmid pGS724 was generated by inserting the *Sma*I–*Acc*I fragment of pTE110 containing the 420 nt T4 late transcription unit, into the *Sma*I site of pTE114 (Elliot and Geiduschek, 1984). Plasmid pDH72Δ420 (Figure 4a) was generated by deleting the 150 bp *Nsi*I–*Dra*III fragment of pDH72, which contains the 'right' T4 late promoter that yields 420 nt RNA, but retaining the T4 late transcription unit that yields 314 nt RNA. Plasmid pGS725 was generated by adding *Eco*RI linkers to the *Aff*III and *Eco*109 sites of pDH72ΔE123. (Complete sequences of these plasmids are available from the authors on request.)

Nicked circular DNA templates were prepared as described (Herendeen *et al.*, 1989). Linear DNA was prepared by digestion with *Sca*I (Figure 1, lanes 3 and 4) or *Afl*III (Figure 1, lanes 5 and 6) restriction endonucleases. DNA bearing 5′ overhangs was prepared as follows: supercoiled plasmid DNA was linearized with *Sca*I, purified and digested at 333 μg/ml in 66 mM Tris–HCl, pH 8, 6.6 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 50 μg/ml BSA with 16.7 U/μl exo III for 30 s at 37°C. Nuclease digestion was stopped by adding 1 volume of 40 mM Na_3EDTA, 40 mM Tris–HCl, pH 8, 0.4% (w/v) SDS, and protein was degraded by digestion with proteinase K (66 μg/ml). The extent of exo III digestion was assayed by monitoring the change in electrophoretic mobility of a small restriction fragment after treatment with S1 nuclease. Exo III treatment under these conditions removed ~100–200 nucleotides, on average.

The DNA with 3′ overhangs for Figure 1, lanes 9 and 10 was prepared as follows: supercoiled plasmid DNA was linearized with *Bgl*I restriction endonuclease, purified and digested at 333 μg/ml in 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$ with 6 U/μl T7 gene 6 exonuclease for 2 min at 0°C. Nuclease digestion was stopped, and the template was purified and analyzed as described above for exo III-treated templates.

DNA templates composed of one longer strand and one shorter strand (the 'top-long' and 'top-short' templates of Figure 1) were prepared from double-stranded restriction fragments that had been biotinylated at an appropriate single end and immobilized on streptavidin–agarose beads (Stahl *et al.*, 1988). The unbiotinylated single strands were obtained by elution with 0.15 M NaOH. Templates were prepared by annealing isolated complementary strands at 63°C for 2 h in transcription buffer with 300 mM K acetate. Gapped DNA templates were prepared by site-specifically nicking supercoiled DNA with the filamentous phage gpII endonuclease, then treating the nicked DNA with 50 U/μl exo III as described above, removing ~250 nt, on average, in the 3′→5′ direction.

3′-tailed DNA templates (except for Figure 1, as noted above) were prepared by cutting supercoiled plasmid DNA at the *Bgl*I site (plasmids in the pDH72 series, pDH82 and pGS722) or *Aat*II site (plasmids pDH310 and pGS724), purifying the linear DNA by standard methods, and reacting it in 200 mM K cacodylate, 25 mM Tris–HCl, pH 6.6, 250 μg/ml BSA, 0.5 mM $CoCl_2$, with 0.8 U/μl TdT and 250 pmol dTTP per pmol DNA ends for 30 min at 37°C. Nucleotide addition was stopped with 1 volume of 40 mM Na_3EDTA, 40 mM Tris–HCl, pH 8, 0.4% (w/v) SDS and 50 μg/ml proteinase K. The mixture was incubated for 30 min at 37°C, and DNA was purified by standard methods (Maniatis *et al.*, 1982). The average length of the poly(dT) tails added was ~150 nt, as judged by the change in electrophoretic mobility of a restriction fragment of the templates thus generated. DNA concentrations were determined by UV absorbance or by Hoechst dye spectrofluorimetry.

Proteins used in transcription reactions were prepared as described (Herendeen *et al.*, 1992; Sanders *et al.*, 1994). The gp45 used in the experiments for Figure 6 was additionally chromatographed on single-stranded DNA cellulose to remove a minor contaminating nuclease activity. Filamentous phage gpII endonuclease was prepared by D.R. Herendeen and *Eco*RI-Gln111 protein was the kind gift of P.Modrich.

Single-round transcription assays shown in Figure 2 were performed as follows: 10 pmol T4-modified RNA polymerase, 50 pmol of gp55, 40 pmol of gp33 and, where appropriate, 36 pmol of gp45 trimer and 33 pmol of gp44/62 complex were combined in 50 μl transcription buffer (33 mM Tris acetate, pH 7.8, 10 mM Mg acetate, 200 mM K acetate, 1 mM DTT, 150 μg/ml BSA) at 0°C. This mixture was added to 1 pmol of DNA, 20 μg of gp32 and 2 nmol of dATP in 150 μl transcription buffer pre-equilibrated at 25°C. Aliquots (20 μl) were withdrawn at the indicated times and added to 5 μl of 5 mM GTP, 5 mM ATP, 0.5 mM [α-^{32}P] UTP (sp. act. 4000 c.p.m./pmol), 0.5 mM CTP and 125 μg/ml rifampicin in transcription buffer, pre-equilibrated at 25°C. Transcription was stopped 6 min later by adding 4 volumes of 20 mM Na_3EDTA, 40 mM Tris–HCl, pH 8, 250 mM NaCl, 250 μg/ml yeast RNA. Nucleic acids were purified by standard methods and resolved on 5% polyacrylamide gels containing 7 M urea; transcription products were quantified and analyzed using a Fuji BAS1000 radio-analytic scanner and by autoradiography. The experiments shown in Figure 2b and c differ only in omission of gp32, substitution of gp32 by an equal weight of SSB_E, and changing concentrations of gp44/62, gp45 and SSB, as indicated.

Single-round transcription assays shown in Figure 1 were performed in essentially the same manner, but with the following exceptions: initial reaction mixes (DNA and proteins in transcription buffer) were down-scaled 10-fold to 20 μl total volume, incubations were at 37°C rather than 25°C, the transcription buffer contained 300 mM in place of 200 mM K acetate, pre-incubation of DNA and proteins was for 30 min, and single-round transcription was continued for 10 min. Transcription assays involving *Eco*RI-Gln111 (Figures 3 and 5) were performed as described by Herendeen *et al.* (1992). Briefly, DNA (52 fmol) the indicated amount (50–200 fmol) of *Eco*RI-Gln111, dATP (20 nmol) and gp32 (1 μg) were combined in 15 μl transcription buffer and pre-incubated for 10 min at 25°C. For the experiment shown in Figure 5, 100 fmol of *Eco*RI-Gln111 was included in each reaction mixture. Transcription proteins (1 pmol of T4-modified RNA polymerase, 4 pmol of gp55, 4 pmol of gp33 and, where appropriate, 7.2 pmol of gp45 and 13.2 pmol of gp44/62 complex) in 5 μl transcription buffer were added and the incubation continued for 20 min. Ribonucleotides and rifampicin were then added in 5 μl transcription buffer, single-round transcription was allowed to proceed for 6 min, the reaction was stopped and reaction products purified and analyzed, as described for Figure 2.

Single-round transcription assays shown in Figure 4 were performed essentially as for Figure 2 with the exception that reactions were down-scaled 10-fold, and that transcription proteins and DNA were pre-incubated for 20 min before addition of ribonucleotides and rifampicin: assays shown in Figure 4a contained the indicated amount of RNA polymerase and proportionally increasing amounts of gp33 and gp55; assays shown in Figure 4b contained the indicated amount of DNA polymerase accessory proteins, expressed as multiples of 1.8 pmol gp45 and 3.2 pmol gp44/62.

Probes for DNase I footprinting (Figure 6) were prepared as follows. For the primer–template junction probe ^{32}P-labeled on the 'top' strand, a single-stranded, 5′ end-labeled *Bam*HI–*Nsi*I fragment of pDH310 was isolated from a denaturing gel and combined with an equimolar amount of single-stranded M13 DNA containing the T4 DNA insert from pDH310, in transcription buffer with 200 mM K acetate. The mixture was boiled for 3 min, annealed at 63°C for 2 h, then slow-cooled to room temperature. The primer–template junction probe labeled on the 'bottom' strand was prepared by isolating a single-stranded *Eco*RI–*Nsi*I fragment of pDH310 from a denaturing gel, then annealing the single-stranded fragment to the above-mentioned M13 derivative of pDH310. The resulting DNA was then cleaved at the *Bam*HI site, 3′ end-labeled by filling in the *Bam*HI site with Klenow fragment DNA polymerase I and [α-^{32}P]dGTP, and purified on Sepharose CL2B. The 3′-tailed probe labeled on the 'top' strand was prepared by dC-tailing the 5′ end-labeled and gel-isolated *Bam*HI–*Nsi*I fragment of pDH310 with TdT and 150 pmol of dCTP per pmol of DNA ends, and was purified as described above for 3′-tailed transcription templates. The 3′-tailed probe labeled on the 'bottom' strand was prepared by filling in the *Bam*HI site of the *Bam*HI–*Nsi*I fragment of pDH310 with [α-^{32}P]dGTP and dideoxy ATP, using Klenow fragment DNA polymerase. The labeled fragment was isolated from a native polyacrylamide gel, dC-tailed and purified as just described.

For DNase I footprinting, 7.2 pmol of gp45 trimer, 13.2 pmol of gp44/62 complex and 10 fmol of ^{32}P-labeled DNA probe, with or without 60 pmol of gp32, were combined in 20 μl of transcription buffer, 5% (w/v) polyethylene glycol (PEG; mol. wt 3300) and 200 μM ATP-γ-S (Munn and Alberts, 1991a). The mixture was incubated for 20 min at 25°C, then 4 ng of DNase I was added in 5 μl transcription buffer and the mixture incubated for 30 s at 25°C. The reaction was terminated by the addition of 4 volumes of transcription stop mix, then nucleic acids were purified by standard methods. Reaction products were resolved on 10% polyacrylamide–7 M urea gels, then analyzed by autoradiography and with a PhosphorImager, as specified above.

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