A Transcriptional Enhancer Whose Function Imposes a Requirement That Proteins Track Along DNA

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Transcriptional regulation of the bacteriophage T4 late genes requires the participation of three DNA polymerase accessory proteins that are encoded by T4 genes 44, 62, and 45, and that act at an enhancer-like site. Transcriptional activation by these DNA replication proteins also requires the function of an RNA polymerase-bound coactivator protein that is encoded by T4 gene 33 and a promoter recognition protein that is encoded by T4 gene 55. Transcriptional activation in DNA constructs, in which the enhancer and a T4 late promoter can be segregated on two rings of a DNA catenane, has now been analyzed. The ability of an interposed DNA-binding protein to affect communication between the enhancer and the promoter was also examined. Together, these experiments demonstrate that this transcription-activating signal is conveyed between its enhancer and a T4 late promoter by a DNA-tracking mechanism. Alternative activation mechanisms relying entirely on through-space interactions of enhancer-bound and promoter-bound proteins are excluded.

The interaction of proteins that are attached to remote sites on DNA is a common theme in the regulation of transcription. Enhancers, which influence promoter activity from distant upstream or downstream locations, are common regulatory elements of eukaryotic gene expression, and they also regulate certain classes of prokaryotic genes (*1–3*). In principle, communication between proteins bound to distal DNA locations can proceed by (i) direct interaction through space by looping out intervening DNA, (ii) generation of topological change in intervening DNA, or (iii) linear tracking along DNA. The manner in which enhancers convey stimulatory signals to distal promoters has been exclusively attributed to direct, through-space interactions with DNA looping (*4–6*). The conveyance of a transcription-activating signal between an enhancer and a promoter by a DNA-tracking mechanism has not previously been described.

Our analysis of transcriptional activation from distant sites has focused on the development of bacteriophage T4, in which the expression of the so-called late genes does not start until after the onset of DNA replication and is severely depressed if ongoing replication is blocked (*7*). The transcriptional regulation of these late genes follows enhancer principles and requires the participation of three replication proteins, the T4-encoded DNA polymerase accessory proteins, encoded by T4 genes 44, 62, and 45 (gp44, gp62, and gp45) (*3*). These proteins constitute an active DNA-dependent adenosine triphosphatase (ATPase) that greatly increases the processivity of DNA chain elongation by the T4-encoded DNA polymerase (*8–10*). The DNA polymerase accessory proteins are thought to be part of the assembly that translocates the replication fork along the viral chromosome as it replicates DNA (9). These replication forks may act in vivo as "mobile enhancers" of T4 late transcription (*3*).

Transcriptional activation by the DNA polymerase accessory proteins is specific for the approximately 40 T4 late promoters, whose extremely simple 8–base pair (bp) consensus core sequence, TATAAATA, is centered approximately 10 bp upstream of transcriptional start sites (that is, it occupies the characteristic −10 position of prokaryotic promoters). Recognition of the simple T4 late promoters is conferred on the bacterial host cell's RNA polymerase core by the protein encoded by T4 gene 55 (gp55), which is the smallest of the σ family proteins.

Transcriptional activation by the three DNA polymerase accessory proteins requires a DNA-binding site that must be on the same DNA molecule as the active promoter, but may be located up to thousands of base pairs away, either upstream or downstream of the promoter. This binding site that generates transcriptional enhancement is a DNA structure, rather than a specific DNA sequence; in many of our experiments, it is a simple nick in the DNA duplex, although gaps can also serve. Transcriptional activation from the enhancer also requires the function of gp33, another T4-encoded RNA polymerase-binding protein. The gp33 is not required for promoter recognition or for basal, unenhanced transcription (*11*). Its properties fit perfectly to the definition of co-activator proteins (*12*); indeed, it might be regarded as the archetype of these proteins.

In the experiments described below, we have turned to an analysis of how the transcription-activating signal is conveyed between enhancers and promoters. We demonstrate that enhancement imposes an essentially absolute requirement for a DNA-tracking mechanism. Our findings stand in clear contrast to the DNA-looping, through-space mechanisms of transcriptional activation that have been postulated to explain transcriptional activation from a distance in other systems.

Transcription at a promoter tethered by catenation to an enhancer. The enhancer of T4 late transcription must be located in cis to (that is, on the same plasmid as) the late promoter that it activates (*3*). This requirement could merely reflect a need to bring independent protein assemblies, the enhancer-bound DNA polymerase accessory proteins and promoter-bound RNA polymerase, into a common vicinity for their through-space interaction. Alternatively, there could instead be an intrinsic requirement for situating these two protein complexes on a continuous, common strand of duplex DNA (*13, 14*). We therefore constructed (*15–21*) two plasmids (pDH72 and pDH82) to allow the production of catenated DNA templates, in which a T4 late transcription unit and a "nick as enhancer" can be segregated on the two singly linked circles of a DNA catenane (Fig. 1). In plasmid pDH72, the enhancer is placed so that it activates transcription at late promoters that yield distinguishable 314- and 420-nucleotide (nt) transcripts. The nick that serves as enhancer must be in the nontranscribed strand of the activated transcription units (*3*). Enhancement of this transcription in monocircular plasmid also requires the three T4 DNA polymerase accessory proteins (Fig. 2, lanes 3 and 4). In the catenane, the P_{23}(420) transcription unit is in cis to the nick on one DNA circle (the smaller one), but the P_{23}(314) transcription unit is located on the other, unnicked DNA circle. Transcriptional activation of catenated pDH72 was clearly and almost completely confined to the transcription unit in cis to the enhancer, that is, to P_{23}(420) on the nicked DNA circle of the catenane (Fig. 2, compare lanes 6 and 4). The minor amount of activation of P_{23}(314) by the accessory proteins (Fig. 2, lanes 6 and 5) was attributable to adventi-

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tious random nicking that occurred during the multistep preparation of the catenated DNA template and placed a nick on the same DNA ring as P_{23}(314) in a minor proportion of templates (*22*).

It might be argued that the through-space interaction between protein complexes bound at the nick and at the T4 late promoter is perhaps incompatible with the particular stereochemistry of the DNA rings in pDH72. To that end, we also constructed pDH82 (Fig. 1), and used it in a similar experiment. In pDH82- and pDH72-derived catenanes, the spatial orientations of the apposed nick and the P_{23}(314) promoter differ in such a way that the two catenanes are mirror images of each other at the macromolecular level (Fig. 1, right side). The nick in pDH82 was placed in the nontranscribed strand of the P_{23}(420) transcription unit and the transcribed strand of the P_{23}(314) transcription unit. Accordingly, gp44/62 (*10*) and 45 preferentially activated P_{23}(420) in cointegrate monocircular DNA (Fig. 2, lanes 13 and 14). Placing P_{23}(314) on a linked circle in the catenane might conceivably have allowed activation from the nick in the smaller DNA circle, but that proved not to be the case (Fig. 2, lanes 15 and 16). The minor activation of P_{23}(314) by gp44/62 and 45 was, once again, almost quantitatively attributable to unintended random nicking of the relaxed DNA ring containing the P_{23}(314) transcription unit during preparation of the catenane (*22*).

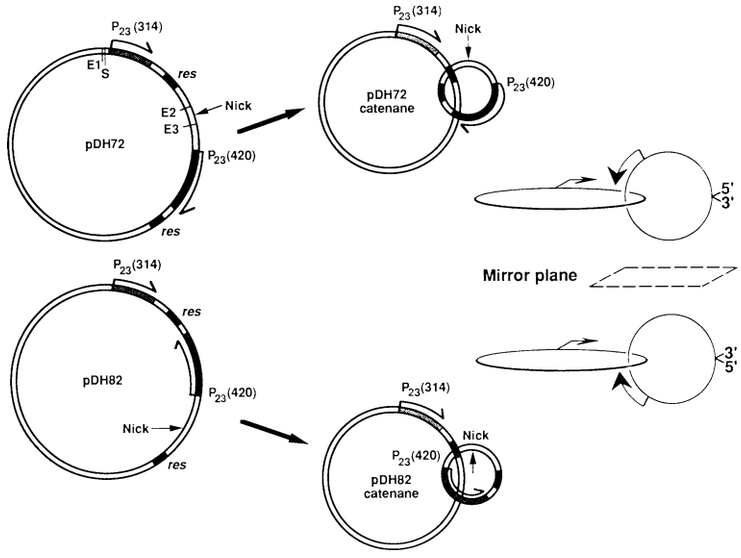

Fig. 1. DNA templates. Plasmids pDH72 and pDH82 (each 3882 bp) contain two γδ resolvase recognition sites in the same orientation (black, labeled *res*), one site at which the phage fd gpII endonuclease introduces a break into one DNA strand (Nick), and two copies of the T4 late promoter P_{23}, directing transcription of two RNA's that are distinguishable by their size, 314 and 420 nucleotides [stipples; P_{23}(314) and P_{23}(420)]. The arrows indicate the directions of transcription and are drawn nearest the nontranscribed strand (the strand that does not serve as template). In pDH72 the nick is on the nontranscribed strand of both P_{23}(314) and P_{23}(420) transcription units; in pDH82 the nick is on the nontranscribed strand of the P_{23}(420) transcription unit only. Treatment of these plasmids with γδ resolvase generates singly linked catenanes with P_{23}(420) in cis and P_{23}(314) in trans to the nick site. The stereochemical relation of the nick to the interlinked trans ring is opposite in the two catenanes. Deletion of Eco RI sites from pDH72 (E1, E2, E3) in various combinations generated pDH72ΔE1, -ΔE12, -ΔE13, -ΔE123, and -ΔE3. The Sma I site (S) used to linearize pDH72ΔE3 is also indicated. Plasmids are drawn to scale (*15*). The diagram at the right shows the spatial relations of the pDH72 and 82 catenanes. The larger circle is in the plane of the mirror; in the smaller circle, the nick is represented by its 5′ → 3′ polarity. On this macromolecular scale (although not at the level of chemical bonds), the two catenanes are mirror images.

The effect of concurrent DNA replication. The availability of catenated DNA templates provided an opportunity to examine whether ongoing DNA replication changes the nature of the communication between the enhancer and the T4 late promoter. In catenated DNA, replication initiating at the nick and transcription on the un-nicked linked circle are mechanically independent, so that conceivable disruptions of elongating transcription complexes by the much more rapidly moving replication fork are avoided for the P_{23}(314) transcription unit. Finding experimental conditions that are optimal for concurrent leading- and lagging-strand DNA replication and for quantitatively maximal transcriptional enhancement required extensive exploration of reaction parameters. In the chosen experimental design, initiation of DNA replication at relatively low ionic strength at 37°C for a short time was followed by a brief period at 25°C (compatible with the continuation of already initiated DNA replication) during which RNA polymerase was allowed to bind and form open promoter complexes. Single rounds of transcription from these complexes were then generated by the addition of ribonucleoside triphosphates and heparin. In control experiments with monocircular pDH82, transcriptional enhancement by the DNA polymerase accessory proteins alone [quantitatively less pronounced than in Fig. 2 primarily because of the lower ionic strength of the experiment (*11*)] was principally confined, as expected, to the P_{23}(420) transcription unit, for which the DNA nick is in the nontranscribed strand (Fig. 3A, lanes 3 to 5); concurrent replication of the leading DNA strand did not change that preference (lane 6), as has been shown (*3*), but did substantially increase transcription during the brief allotted time interval. When both the leading and lagging DNA strands were replicated (lane 7) both the P_{23}(420) and the P_{23}(314) transcription units were approximately equally subject to

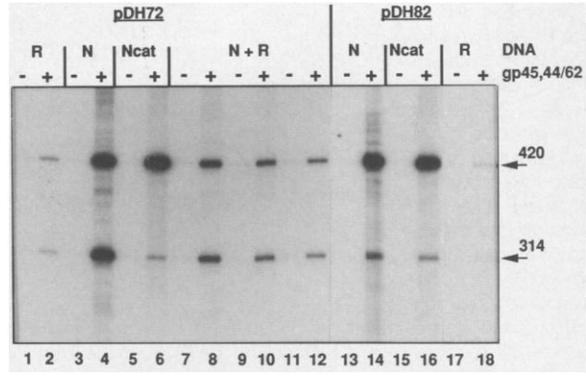

Fig. 2. The T4 DNA polymerase accessory proteins only activate promoters located in cis to a nick. Single rounds of transcription were executed by *Escherichia coli* RNA polymerase saturated with T4 gp55 and gp33 in the absence or presence of the T4 DNA polymerase accessory proteins (as indicated above each lane) (*33*). The state of templates pDH72 and pDH82 was either relaxed (R), nicked (N), or catenated, with the P_{23}(420)-containing ring nicked and the P_{23}(314)-containing ring relaxed (Ncat), as indicated. For lanes 7 to 12, nicked and relaxed pDH72 (N + R) were mixed in varying proportions and used for transcription to generate a calibration for enhanced transcription due to random nicks adventitiously introduced into the P_{23}(314)-containing ring, as explained in (*22*) and in the text. Sizes of transcripts are indicated at the right.

transcriptional enhancement. The activation of $P_{23}(314)$ by lagging-strand DNA synthesis confirms a previous conjecture that the complete replisome might function as an orientation-independent enhancer assembly at the replication fork (*3*).

When the above experiment was repeated with nicked catenated pDH82 DNA as template, only the $P_{23}(420)$ promoter in cis to the DNA nick was transcriptionally enhanced by the replication proteins (Fig. 3B, lanes 3 to 5) with or without concurrent leading strand—or leading- and lagging-strand—DNA replication (lane 6 and 7, respectively) (*23, 24*). Thus, even when the T4 DNA polymerase accessory proteins were operating in the context of the entire replisome, the transcriptional enhancement that they generated cannot be ascribed to interactions that allow distally bound proteins to interact through space by looping out of the intervening DNA.

Effects of roadblocks to tracking along DNA. When proteins that recognize each other from distal DNA sites are subject to the strict proviso of cis-location for their interaction, we can infer either that topological changes of the DNA are involved or that one or more proteins track linearly along DNA from one of the interacting sites to the other. The following experiments support a DNA-tracking model for protein interaction by showing that roadblocks to protein translocation can impede transcriptional activation. We used the mutant restriction endonuclease Eco RI Gln-111 as the roadblock to protein tracking, because this endonuclease-defective protein binds extremely tightly to the six-bp Eco RI recognition sequence and retains long residence times at this DNA site, even at elevated ionic strengths (*25*). The pDH72 plasmid has three Eco RI binding sites (Fig. 1); when the Eco RI Gln-111 protein occupies sites E2 and E3, it isolates the nick-enhancer from the two late promoters. Derivatives of pDH72 lacking various combinations of sites E1, E2, and E3 were also constructed as controls. Transcriptional enhancement by gp44/62 and gp45 was analyzed with these constructs in the presence and absence of the mutant Eco RI Gln-111 protein (Fig. 4, A to C).

Transcriptional enhancement was essentially completely blocked when the Eco RI–binding sites flanking the nick (E2 and E3) were bound by the mutant Eco RI Gln-111 protein (Fig. 4A, lanes 1 and 2, Fig. 4, B and C, filled squares). The amount of transcription on this construct was reduced by Gln-111 protein to the background level on relaxed DNA (Fig. 4A, lanes 9 and 10), which was due to a small fraction of adventitiously and randomly placed nicks. In the absence of Eco RI binding sites, enhancement of transcription was unaffected by the mutant Eco RI protein (Fig. 4A, lanes 7 and 8; Fig. 4, B and C, open squares). Constructs containing only the E3 or the E2 site showed complementary asymmetries of inhibition. Protein interposed along the shortest path between enhancer and promoter partly blocked transcriptional enhancement. Thus, the Eco RI Gln-111 protein preferentially blocked enhancement of $P_{23}(420)$ from site E3 and of $P_{23}(314)$ from site E2 (Fig. 4A, lanes 3 to 6, Fig. 4, B and C, triangles). The fact that the occupancy of either the E2 or the E3 site did not inhibit transcription from both promoters indicates that protein bound to these sites did not prevent the complex of gp45 with gp44/62 from interacting at the nick site. These experiments also show that the transcription-enhancing effect of tracking along DNA is bidirectional: the bound Eco RI Gln-111 protein at site E3 allowed effective enhancement of the upstream $P_{23}(314)$ transcription unit, while occupancy of site E2 allowed effective enhancement of the downstream $P_{23}(420)$ transcription unit.

Striking confirmation of a DNA-tracking mechanism was provided by a similar transcriptional enhancement experiment with linear rather than circular DNA. It has been shown that a DNA nick can serve as an enhancer in linear DNA at low ionic strength (*3*), and in circular DNA at low and high ionic strengths (*11*). When it was subsequently observed that transcriptional enhancement in linear DNA fails at the elevated salt concentrations that are optimal with circular DNA, we considered whether this peculiar discrimination might be explained by a DNA-tracking model for

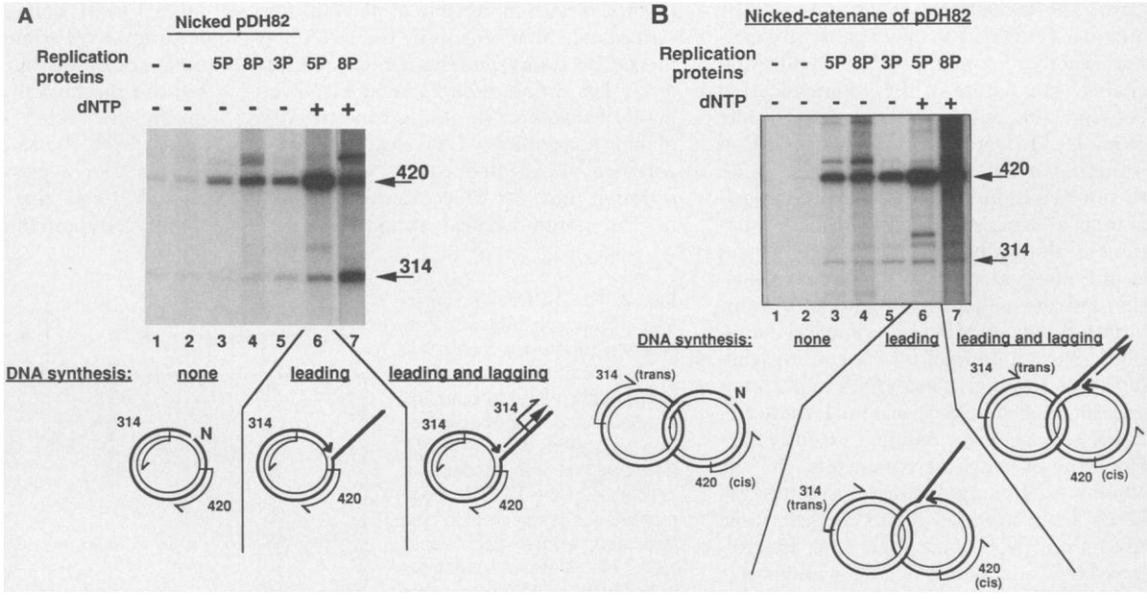

Fig. 3. Concurrent DNA replication does not activate the trans promoter in a catenane. (**A**) In vitro transcription was performed in the presence or absence of T4 DNA replication proteins and concurrent DNA synthesis (*34*). Nicked monocircular pDH82 was incubated with the three DNA polymerase accessory proteins (3P), with DNA polymerase, gp43, and single-stranded DNA-binding protein, gp32, added (5P), or with all the above plus the components of the primasome, gp41, gp59, and gp61 (8P). The presence of all four dNTP's at the start of reaction is indicated above the appropriate lanes; however, all reaction mixtures contained dATP from the start (to impair digestion of DNA by the exonuclease activity of gp43). All four rNTP's were added at the start of the reaction analyzed in lane 7 as substrates for RNA primer synthesis, and at the start of reactions analyzed in lanes 2 and 4, as controls. Replication was allowed to initiate (where applicable) before the gp55- and gp33-containing RNA polymerase holoenzyme was added. The template structures and type of replication are indicated by the cartoon (not to scale) at the bottom of the figure. Transcripts are identified at the right. (**B**) The nicked catenane of pDH82 served as the template for transcription and replication. The extent of DNA synthesis was determined in parallel reactions with labeled dNTPs. After 1 minute of the 2-minute RNA polymerase binding period, the average replication fork movement was: (A) lane 6, 1980 nt; (A) lane 7, 1514 nt; (B) lane 6, 2140 nt; (B) lane 7, 2153 nt.

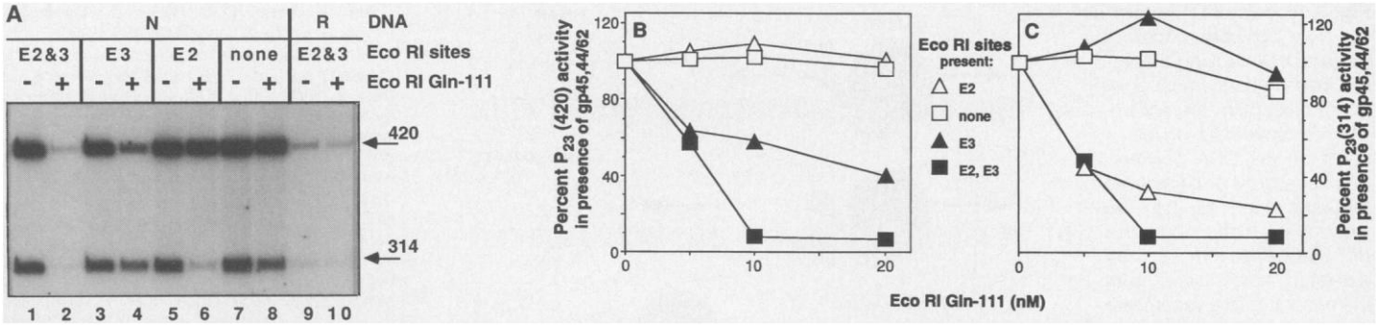

Fig. 4. Transcriptional activation is blocked by binding of the mutant Eco RI Gln-111 protein between the enhancer and the promoter. (**A**) Single-round transcription, enhanced by gp44/62 and gp45, was analyzed with nicked pDH72ΔE1 (lanes 1 and 2), nicked pDH72ΔE12 (lanes 3 and 4), nicked pDH72ΔE13 (lanes 5 and 6), nicked pDH72ΔE123 (lanes 7 and 8), and relaxed pDH72ΔE1 (lanes 9 and 10). The condition of the DNA (N or R), the Eco RI sites that are present on each template and the presence (+) or absence (−) of 30 nM Eco RI Gln-111 protein are indicated above each lane. The details were as specified in (*35*). (**B** and **C**) The effect of the concentration of Eco RI Gln-111 protein on promoter activity. Experiments as in (A), except that the final concentration of Eco RI Gln-111 was 0, 5, 10, or 20 nM. The abundance of 420-nt and 314-nt RNA is plotted in (B), and (C), respectively. The template was nicked pDH72ΔE1 (closed squares), nicked pDH72ΔE12 (closed triangles), nicked pDH72ΔE13 (open triangles), or nicked pDH72ΔE123 (open squares). Transcript abundance is expressed relative to transcription in the absence of Eco RI Gln-111, set at 100 percent. Transcription attributable to nonspecific nicks (on relaxed templates) has not been subtracted.

transcriptional enhancement provided that the tracking proteins fall off the ends of linear DNA only at higher electrolyte concentrations. This hypothesis implies that transcriptional enhancement in linear DNA at high ionic strength might be obtained under conditions in which the Eco RI Gln-111 protein forms a barricade between the nick-enhancer and the DNA ends. To test this prediction, nicked pDH72ΔE3 DNA was linearized with Sma I endonuclease, which puts sites E1 and E2 at the opposite ends of a linear DNA segment containing the nick enhancer and the P_{23}(420) transcription unit (Fig. 1). The dependence of transcriptional enhancement by gp44/62 and gp45 on potassium acetate concentration was tested on this template in the presence or absence of Eco RI Gln-111 protein. Unenhanced transcription was sensitive to electrolyte concentration in the presence and in the absence of the mutant Eco RI protein (Fig. 5, lanes 1 to 6) and with both circular DNA (lanes 13 to 15) and linear DNA. Transcriptional activation by gp44/62 and 45 was resistant to high concentrations of potassium acetate with nicked circular DNA (lanes 16 to 18) but not nicked linear DNA (lanes 7 to 9). With the Eco RI Gln-111 protein bound to sites E1 and E2, transcriptional enhancement at P_{23}(420) became as resistant to high concentrations of electrolyte with nicked linear DNA (lanes 10 to 12) as with nicked circular DNA (in the absence of Eco RI Gln-111 protein; lanes 16 to 18). The outcome of this experiment confirms our explanation for the peculiar salt sensitivity of enhancement in linear DNA and proves that transcriptional enhancement involves the tracking of protein between the enhancer and the promoter.

The role of tracking. This appears to be the only instance in which transcriptional activation from distant sites is known to require tracking of proteins along DNA. Previous experiments to determine whether an enhancer can activate a promoter on noncontiguous DNA (with the use of catenanes or protein-bridged DNA fragments) have directly shown or implicated activation by direct protein-protein interaction through "space", with no apparent role for the intervening DNA beyond bringing otherwise diffusely distributed components into a common vicinity (*14*, *26*). Direct protein-protein interactions are also involved in many instances of negative transcriptional regulation (*27*).

Our experiments do not distinguish whether it is the promoter-recognition proteins or enhancer-recognition proteins that must track or slide along DNA in order to generate the observed transcriptional activation (Fig. 6). RNA polymerase tracking cannot be ruled out, inasmuch as this enzyme slides along duplex DNA in searching for promoters (*28*). However, the T4 DNA polymerase accessory proteins, which act as a "sliding clamp" to increase the processivity of DNA chain elongation (*8*), must be able to slide in either direction relative to DNA primer 3′ ends as they stimulate both the 5′ → 3′ polymerization and the 3′ → 5′ exonuclease activities of T4 DNA polymerase (*29*). Such a bidirectional mobility corresponds to the ability of DNA tracking to activate a T4 late promoter situated on either side of an enhancer (Fig. 4). Although it has not yet been proved that the T4 accessory proteins slide along DNA in the absence of T4 DNA polymerase, their functional homologs in the *Escherichia coli* replication apparatus have the strikingly suggestive properties of being able to slide along DNA, with a nick as a site of entry. The *E. coli* γ complex and the β protein establish a preinitiation complex with DNA polymerase III holoenzyme at a primer-template junction. The γ complex is a DNA-dependent ATPase (as is gp44/62), whose activity is stimulated by the β protein (similar to gp45). Loading of the β protein onto nicked circular DNA depends on the ATPase activity of the γ complex. Thereafter, the β protein tracks bidirectionally along DNA independently

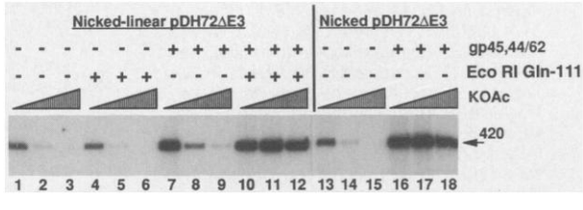

Fig. 5. Eco RI Gln-111 protein can prevent tracking proteins from falling off the ends of linear DNA. Nicked pDH72ΔE3 was linearized with Sma I (lanes 1 to 12), which places the Eco RI sites E2 upstream, and E1 downstream of both the nick and the P_{23}(420) transcription unit (*35*). Control reactions with nicked, circular pDH72ΔE3 are shown in lanes 13 to 18. DNA was incubated with or without 30 nM Eco RI Gln-111 protein (as indicated above each lane) for 10 minutes at 37°C. The potassium acetate concentration was either 100 mM (lanes 1, 4, 7, 10, 13, and 16), 173 mM (lanes 2, 5, 8, 11, 14, and 17), or 247 mM (lanes 3, 6, 9, 12, 15, and 18). After the initial incubation period, core RNA polymerase, gp55, and gp33 were added, with or without gp45 and gp44/62 (as indicated above each lane). Ten minutes later, rNTP's and heparin were added to initiate a single round of transcription.

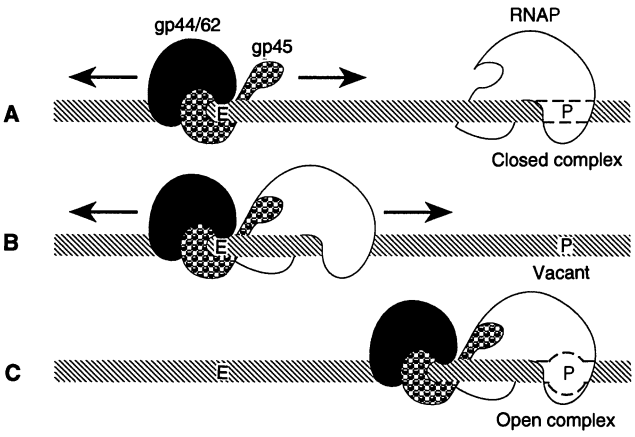

Fig. 6. Models of DNA tracking. Two possible modes of protein DNA tracking that result in the enhancement of T4 late transcription are shown: (**A**) the movement of (all or some of) the DNA polymerase accessory proteins from the enhancer (E) to the closed promoter complex (P); (**B**) the movement of an activated holoenzyme from the enhancer to a vacant late promoter. Both models draw attention to the bidirectional (arrows) and orientation-dependent functions of the T4 late enhancer. The enhanced promoter complex (**C**) is arbitrarily drawn to contain all three DNA polymerase accessory proteins associated with RNA polymerase, although that is not an intrinsic requirement of the model and there is some evidence that it may not be so (*36*).

of the γ complex and its ATPase (*30, 31*).

The ability of enhancer-binding or promoter-binding proteins to track along DNA should not a priori exclude them from being able to interact productively directly through space by a DNA-looping mechanism. However, proteins that track along DNA may also generate topological changes in DNA, or they may deliver a protein to a site that is so configured as to be otherwise inaccessible. DNA tracking might also maintain a protein in an otherwise rare conformational state. An essential role for supercoiling is inconsistent with transcriptional activation in linear DNA, particularly with the almost complete dependence of enhanced transcription at P_{23}(420) on the binding of Eco RI Gln-111 protein to sites flanking the nick and promoter (Fig. 5). We suggest, instead, that the strict requirement for DNA tracking in enhancement of T4 late transcription implies a requirement for a specific stereochemistry of protein delivery, possibly combined with a requirement for a specific conformation of one of the interacting proteins that is only sustained during tracking. For example, an on-DNA alignment between at least one of the DNA polymerase accessory proteins and the gp33- and gp55-containing RNA polymerase may be necessary for their productive interaction (Fig. 6, C).

The demonstration that a DNA-bound protein can blockade transcriptional activation of a T4 late promoter (Fig. 4) provides a further clue to the requirement of DNA replication for late gene expression in vivo. The assembled replication proteins at the replication fork are likely to provide a preferred and particularly stable binding site for the DNA polymerase accessory proteins. Substantially increased late promoter opening is, in fact, generated by concurrent DNA replication under the conditions of the Fig. 3 experiments. In addition, the ability of replication to clear the DNA template of bound proteins that might otherwise block transcriptional enhancement (*32*) is likely to be an important component of T4 late gene expression in the virus-infected cell. It is conceivable that late promoters are only activated behind an advancing replication fork and, more generally, that transcriptional enhancement by DNA-tracking proteins is generally coupled to a mechanism of DNA clearance.

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15. These plasmids were constructed according to standard methods (*16*). Sites for γδ resolvase (*res*) on 116-bp Pvu II fragments were provided by N. D. F. Grindley. All *res*-containing plasmids were transformed into RR1031 (γδ$^-$, recA1 *E. coli*) (*17*) by electroporation (*18*). The *res* sites were cloned in tandem orientation into a vector containing the 314 nt P_{23} transcription unit of pTE114 (*19*). A fragment with the 420-nt P_{23} transcription unit and the phage fd gpII nick site from pDHπ5 (*3*) was cloned in both orientations between the *res* sites to create pDH72 and pDH82. The transcription units P_{23}(314) and P_{23}(420) are composed of the T4 gene 23 late promoter (P_{23}) upstream of the strong transcriptional terminator from phage T7 (*19*). Both P_{23}(314) and P_{23}(420) are oriented in the same direction in pDH72, but they are in opposing directions in pDH82. The locations (clockwise relative to Eco RI site E1 at bp 1) of the nick site, the P_{23}(314) start site, and the P_{23}(420) start site are base pairs 776, 39, and 999, respectively, in pDH72; and base pairs 1287, 39, and 1064, respectively, in pDH82. Catenation of these plasmids with γδ resolvase yields a 1065-bp ring containing the nick site and P_{23}(420), interlinked with a 2817-bp ring containing P_{23}(314). The three Eco RI sites of pDH72, E1, E2, and E3 (located at bp 1, 710, and 826, respectively) were deleted by standard methods to produce templates that contained various assortments of these sites. pDH72ΔE3 has a ~7 bp deletion, which fortuitously removes a nearby Sma I restriction site, rendering the Sma I site downstream of E1 unique. Singly linked catenanes were produced from pDH72 and pDH82 by reaction with γδ resolvase (provided by N. D. F. Grindley), essentially as described (*17*). To reintroduce negative supercoils to the catenanes (for recognition by phage fd gene II protein, gpII), ethidium bromide (to 3 μg/ml) and calf thymus topoisomerase I (to 1.5 units per microgram of DNA) were added, and incubation was continued for 1 hour at 37°C in the dark. Monocircular and re-supercoiled catenane DNA molecules were nicked with gpII [purified as described (*20*), from the overproducing *E. coli* strain K1073, provided by P. Model] as described (*3*). To relax the unnicked ring of the catenane and other unnicked DNA species, calf thymus topoisomerase I (BRL-Gibco; 1.5 unit per microgram of DNA) was added to the nicking reaction mixture. Relaxed closed circular DNA was prepared in the same buffer. The desired singly nicked catenane was purified by electroelution from an agarose gel slice purified on a NACS 52 column (BRL-Gibco), extracted with phenol, precipitated with ethanol, and resuspended in 10 mM tris-acetate, pH 7.8, 0.1 mM EDTA. Concentrations of the gel-isolated DNA were determined by fluorimetry, with 4′,6-diamidino-2-phenylindole (DAPI) as stain (*21*). Preparations of nicked monocircular DNA generally did not include a gel isolation step (except for the experiment shown in Fig. 2); these preparations contained about 70 to 85 percent of nicked DNA, with 30 to 15 percent of relaxed DNA. The fraction of nicked DNA was determined by Southern blot analysis (*16, 17*), and quantified by scintillation counting.
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relaxed pDH72 (Fig. 2, lanes 2, 4, 8, 10, and 12), and by a quantitative determination of the fraction of catenated DNA bearing at least one nick in each DNA circle (*15*). The transcription analysis of the known mixtures provided a standard curve of the synthesis of 314-nt RNA as a function of the fraction of DNA molecules that were nicked on the appropriate strand in cis. The standard curve provided an expected value for the synthesis of 314-nt RNA based solely on random DNA nicking. The observed enhanced synthesis of 314-nt RNA in Fig. 2, lane 6, was entirely attributable to nicking of catenated pDH72 DNA. With regard to the enhanced synthesis of 314-nt RNA in Fig. 2, lane 16, 70 percent was attributable to random nicking of catenated pDH82 DNA.

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34. Reaction conditions were as stated in (*33*), with the following exceptions. DNA, potassium acetate, rATP, gp41, gp43 (U.S. Biochemical), gp45, gp44/62, gp59, and gp61 were used at 2.5 nM, 125 mM, 1 mM, 40 μg/ml, 7 U/ml, 3.4 μg/ml (45 nM of the trimer), 13.5 μg/ml (82 nM of the complex), 0.5 μg/ml, and 2 μg/ml, respectively. Gp32 (U.S. Biochemical) was at 100 μg/ml, or at 50 μg/ml in the presence of the gp41 helicase. The T4 gp41, gp61, and gp59 proteins were provided by J. Barry and B. M. Alberts. Deoxyribonucleoside triphosphates (dNTP's) were at 2 mM dGTP, 200 μM dCTP, 200 μM dTTP, and 2 mM dATP (or 1 mM dATP when the other three dNTP's were omitted). When primase, gp61, was present, rNTP's were added at the start of the reaction. DNA replication was initiated in a 10-μl reaction mixture by incubation for 4 minutes at 37°C, then 2 minutes at 25°C. RNA polymerase core, gp55, and gp33 (in 5 μl) were then added to 33 nM, 330 nM, and 132 nM, respectively. Incubation continued for 2 minutes at 25°C, then heparin at 400 μg/ml and the remaining 3 rNTP's (if not already present) were added (in 5 μl). Reactions were stopped after 4 minutes, and RNA was purified and analyzed by methods explained in (*3*). DNA synthesis was quantified in parallel reactions with [α-^{32}P]dCTP (NEN-Dupont) at 2365 cpm/pmol and unlabeled rNTP's.
35. These experiments were done as described in (*33*) with the following variations: The concentrations of DNA, gp55, gp45, and gp44/62 were 2.6 nM, 200 nM, 360 nM, and 680 nM, respectively. The concentration of potassium acetate was 200 mM for the experiment shown in Fig. 4, or as stated in the legend to Fig. 5. Eco RI Gln-111 (*25*), provided by P. Modrich, was diluted from a 40-μM stock (in 200 mM NaCl, 20 mM(P), potassium phosphate (pH 7.8), 0.5 mM DTT, 0.2 mM EDTA, BSA at 200 μg/ml, 10 percent glycerol) and added to concentrations stated in figure legends, occupying 13 percent of the reaction mixture volume. For the experiment shown in Fig. 4, Eco RI Gln-111 protein was allowed to bind to DNA for 8 minutes at 20°C, before the gp55- and gp33-containing holoenzyme (with or without gp45 and 44/62) was added. Open promoter complexes were allowed to form for 16 minutes at 20°C before rNTP's were added in the presence of heparin (400 μg/ml) to initiate transcription, unless otherwise stated in the figure legends. Transcription was terminated after 8 minutes (Fig. 4) or 10 minutes (Fig. 5) by a modification of a previously described procedure (*37*): one-fifth volume of 0.1 M EDTA, 2 percent SDS, proteinase K at 5 mg/ml (Beckman) was added to reaction mixtures and incubated 30 minutes at 37°C. One-third volume of 8 M urea and 15 percent sucrose (containing dyes) was then added to these samples. All samples were heated 3 minutes at 100°C and subjected to electrophoresis on 5 percent polyacrylamide, 7 M urea gels.
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