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Research Articles

Enhancement of Bacteriophage T4 Late Transcription by Components of the T4 DNA Replication Apparatus

DANIEL R. HERENDEEN, GEORGE A. KASSAVETIS, JACK BARRY, BRUCE M. ALBERTS, E. PETER GEIDUSCHEK*

The expression of the late genes in bacteriophage T4 development is closely connected to viral DNA replication. Three T4-encoded DNA polymerase accessory proteins are shown to stimulate transcription at T4 late promoters in an adenosine triphosphate (ATP) hydrolysis-requiring process. The properties of the activation resemble those found for enhancers of eukaryotic transcription. However, the nature of the enhancer of T4 late transcription is novel in that it is a structure—a break in the nontranscribed DNA stand—to which the three replication proteins bind, rather than a sequence. Since the three DNA polymerase accessory proteins are carried on the moving replication fork as part of the replisome, we postulate that viral DNA replication forks act, in vivo, as the mobile enhancers of T4 late gene transcription. Whereas *Escherichia coli* RNA polymerase bearing the T4 gene 55 protein can selectively recognize T4 late promoters, it is only capable of responding to the transcription-enhancing activity of the three replication proteins on acquiring an additional T4-specific modification.

THE CONNECTION BETWEEN DNA REPLICATION AND REGULATED transcription in the development of bacteriophage T4 was established more than 25 years ago by the classic physiological-genetic work of Epstein and collaborators, and later supplemented by a direct analysis of transcription (*1*). One of the

D. R. Herendeen, G. A. Kassavetis, and E. P. Geiduschek are in the Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, CA 92093. J. Barry and B. M. Alberts are in the Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143.

*To whom correspondence should be addressed.

earliest attempts at in vitro analysis of developmental gene regulation also involved late transcription of the closely related T2 phage (*2*). A reasonably detailed understanding of the complex regulatory principles of T4 late gene expression subsequently emerged (*3, 4*). The essential facts are as follows: (i) T4 late gene expression is under the control of three phage genes, 33, 45, and 55, whose products (gp) bind to RNA polymerase; gp45, which binds least tightly, is also a component of the replication machinery. (ii) Late genes are not turned on unless viral DNA is replicated; when ongoing viral DNA replication is blocked, late gene expression is sharply diminished. (iii) Late gene expression can be at least partly uncoupled from DNA replication under certain circumstances that also lead to the introduction of breaks into viral DNA. However, even late gene expression that is not coupled to replication still depends completely on gp45. (iv) For all this regulatory complexity, T4 late promoters are among the simplest known. They consist of a TATAAATA sequence centered 10 base pairs (bp) upstream of the transcriptional start (*5, 6*); the sequence consensus extends more weakly to a few contiguous base pairs. (v) The ability to recognize these promoters in double-stranded DNA is conferred on the *Escherichia coli* RNA polymerase core by T4 gp55 (*7*), which is a protein of the σ family (*8*).

In the experiments described below, we analyze the function of the T4 replication apparatus in late transcription. We demonstrate that the accessory proteins of the T4 DNA polymerase holoenzyme, gp44/62 and 45, stimulate the opening of T4 late promoters. The complex formed by these three proteins is a DNA-dependent adenosine triphosphatase (ATPase) (*9*); its enhancing effect on late transcription requires ATP hydrolysis and enhancement can be generated from binding sites in DNA that are located hundreds of base pairs from a late promoter—either upstream or downstream. In a cell infected with phage T4, the principal DNA site to which these proteins bind is the replication fork. We propose that activation of late promoters in vivo is effected from moving replication forks acting as mobile enhancers. We also show that the *Escherichia coli* RNA polymerase must be modified, in some T4-specific manner, in order to be subject to the transcription-enhancing activity of the DNA polymerase-accessory proteins.

Enhancement of transcription from a late promoter by T4 replication proteins. T4 late genes are extensively transcribed in vitro on supercoiled DNA templates, but poorly transcribed on relaxed DNA (*10*). We therefore used relaxed DNA templates in searching for replication-associated activating functions of late transcription. The first indications of success came from experiments with single-stranded circular DNA that was undergoing transcription as it was being replicated (*11*). Eventually, we designed several plasmids (Fig. 1 and Table 1) containing (i) a short transcription unit composed of the T4 late promoter P_{23} upstream of a relatively efficient transcriptional terminator, and (ii) a site for cutting one DNA strand, that can be inserted into either strand. This nick site serves as a binding site for replication proteins and it defines the origin and direction of DNA synthesis catalyzed by the in vitro T4 DNA replication system relative to the direction of transcription. Two variant T4 late promoters (*6, 12*) have also been useful for our analysis. In plasmids pDHπ5, pDH310, and pDH410, the promoter P_{V450} is located 30 bp upstream of P_{23}; P_{V460} is located in the col EI origin region of all the plasmids, approximately 460 bp upstream of the transcription terminator T_{P4}.

These plasmids were transcribed in vitro in the presence of replication proteins with, or without, concurrent replication (Fig. 2). Transcription of supercoiled DNA (Fig. 2, lanes g and h) by T4-modified RNA polymerase, supplemented with gp55, generated RNA molecules that were assigned to individual late transcription units according to their lengths. In plasmids pDH310 and pDH410, the $P_{23} \rightarrow T_{T7}$ transcription unit (422 nucleotides) and the $P_{V450} \rightarrow T_{T7}$ unit (approximately 450 nt) share the same polarity, whereas the $P_{V460} \rightarrow T_{P4}$ unit (approximately 460 nt) is transcribed in the opposite direction. Since the T_{T7} and T_{P4} terminators are not fully efficient, transcriptions initiating at these promoters also yielded long RNA molecules. Transcription of this supercoiled DNA was unaffected by the presence of replication proteins (*13*). The nickable strand of plasmids pDH310 and pDH410 differs, so that leading strand DNA synthesis is codirectional with $P_{23} \rightarrow T_{T7}$ and $P_{V450} \rightarrow T_{T7}$ transcription in nicked pDH310, but codirectional with $P_{V460} \rightarrow T_{P4}$ transcription in nicked pDH410. In the absence of replication proteins, all three of these gp55-dependent transcription units were much less active on the nicked form of either plasmid than on supercoiled DNA (Fig. 2, lanes a and d). A high level of transcription issued from the vicinity of the nick in either direction; this was not gp55-dependent and could also be generated with *E. coli* RNA polymerase core (*13*).

In the presence of the six replication proteins that generate leading-strand DNA synthesis, the synthesis of $P_{23} \rightarrow T_{T7}$ RNA and

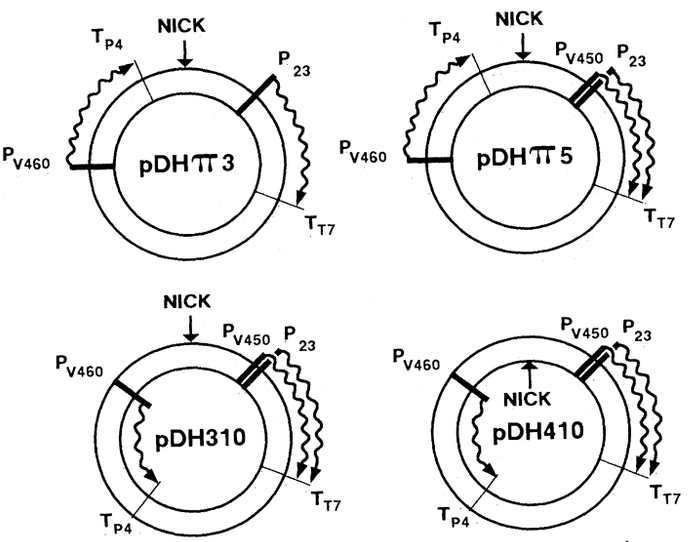

Fig. 1. Structure of plasmids for in vitro analysis of T4 late transcription. Each plasmid contains a T4 late promoter, P_{23}, upstream of the strong T7 phage early terminator, generating a readily identifiable gp55-dependent transcript in vitro; a filamentous phage M13 gpII endonuclease nick site specifies the starting point and polarity of DNA replication in vitro. The inner DNA strand runs counterclockwise 5′ → 3′, and is the template for P_{23}-initiated transcription. (Not drawn to scale. The term "transcribed strand" designates the DNA strand that serves as the template for RNA synthesis.) To construct pDHπ, a 459-bp EcoRI–Spe I fragment from pTE114 (*32*), which contains the P_{23} late promoter sequence extending from bp −18 to +17 only, and the T7 early terminator (T_{T7}), was inserted into πAN7 (*33*). To make pDHπ3, the gpII cutting site from M13mp18 was inserted into the indicated strand of pDHπ. To make pDHπ5, the $P_{23} \rightarrow T_{T7}$ transcription unit of pDHπ3 was replaced with the $P_{23} \rightarrow T_{T7}$ transcription unit from pTE110 with retention of transcriptional polarity (*32*). The P_{23} promoter from pTE110 contains T4 DNA sequence extending from bp −154 to +120; a variant T4 late promoter, P_{V450}, is located approximately 30 bp upstream of P_{23} and generates transcripts with the same polarity. To construct pDH310 and pDH410, an Eco RI fragment containing the gpII cutting site was inserted into the Eco RI site of pTE110. Both orientations of the insert were recovered: pDH310 and pDH410 are nicked by gpII in the nontranscribed and transcribed strand, for P_{23}-directed RNA synthesis, respectively. Each of these plasmids contains a vector-derived T4 late transcription unit defined by a variant T4 late promoter, P_{V460}, upstream of a transcription terminator, T_{P4} (Table 1). Plasmid DNA was purified essentially as described (*6*). The purification of pDHπ3 and pDHπ5 included a Bgl II digestion to linearize the P3 helper plasmid and permit separation on a subsequent CsCl–ethidium bromide gradient.

$P_{V450} \rightarrow T_{T7}$ RNA was greatly enhanced in nicked pDH310 (Fig. 2, lanes b and c), whereas $P_{V460} \rightarrow T_{P4}$ RNA synthesis was barely stimulated. In contrast, with nicked pDH410, $P_{V460} \rightarrow T_{P4}$ transcription was greatly stimulated by the replication proteins, relative to $P_{23} \rightarrow T_{T7}$ and $P_{V450} \rightarrow T_{T7}$ transcription (Fig. 2, lanes e and f). Thus, the stimulation of transcription due to the T4 replication proteins was especially strong for transcription in the same direction as potential replication, the nick preferentially allowing promoter activation when it was on the nontranscribed strand. Concurrent DNA replication—with deoxyribonucleotides (dNTP) added—further stimulated transcription modestly but consistently: only 9 percent in lane c relative to that in lane b for the $P_{23} \rightarrow T_{T7}$ transcript of pDH310, but 68 percent on the average in seven experiments with various plasmids as template. The variables determining the extent of stimulation have not yet been analyzed (*14*). The replication proteins also lowered the rate of transcription initiating in the vicinity of the nick in a unidirectional manner (Fig. 2, lanes b and e). Anticlockwise transcription (with respect to Fig. 1) was diminished on nicked pDH310, whereas clockwise transcription was reduced on nicked pDH410. It is likely that replication proteins bound at the nick differentially block access to RNA polymerase binding.

Table 1. Plasmid constructs for in vitro analysis of T4 late transcription.

Plasmid	Size (bp)	Nickable strand*	Distance of upstream nick to P_{23}-start (bp)	$P_{23} \rightarrow T_{T7}$ transcript (nt)	Late promoter	
					Variant	Transcription direction†
pDHπ3	1428	Nontranscribed	88	316	P_{V460}	Same
pDHπ5	1668	Nontranscribed	222	422	P_{V450}	Same
					P_{V460}	Same
pDH310	3340	Nontranscribed	222	422	P_{V450}	Same
					P_{V460}	Opposite
pDH410	3340	Transcribed	232	422	P_{V450}	Same
					P_{V460}	Opposite

*With respect to P_{23}-initiated transcription. The transcribed strand is the one that serves as the DNA tempate for RNA synthesis. †Relative to the direction of $P_{23} \rightarrow T_{T7}$ transcription.

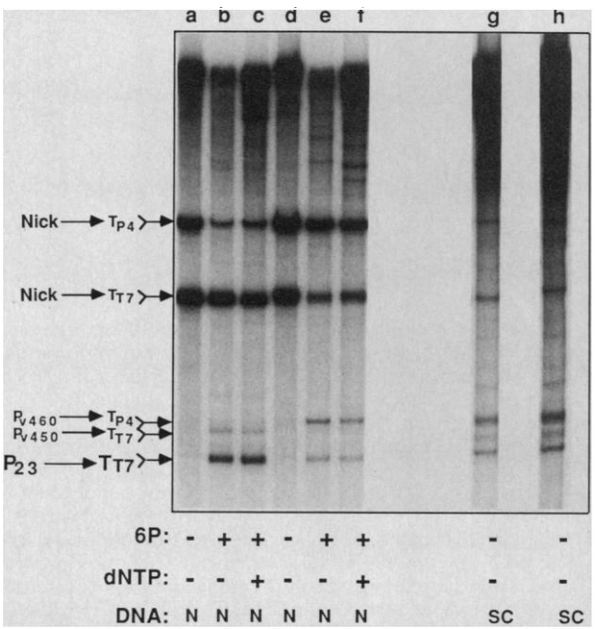

Fig. 2. Effects of T4 replication proteins on transcription. Transcription of pDH310 (lanes a, b, c, and g) and pDH410 (lanes d, e, f, and h) (each 5 n*M*, that is, 0.125 pmol) by T4-modified RNA polymerase, supplemented with gp55, proceeded for 15 minutes. When added, the replication proteins (6P) consisted of gp45, 44/62, 43, 41, and 32 (the last-named at 6.0 μ*M*, that is, 200 μg/μl). The condition of the DNA, whether nicked (N) or supercoiled (SC), and the presence or absence of dNTP's are indicated below each lane. The extent of incorporation of [α-^{32}P]dGTP into DNA was measured in parallel reactions (*34*) and was 6.7 (lane c) and 7.7 (lane f) pmol/μl (corresponding to 1.6 and 1.8 copies of the leading strand, respectively). Transcripts are identified at the side of the figure. Details are specified in (*34*). Quantities of $P_{23} \rightarrow T_{T7}$ RNA produced (in arbitrary units) were: lane a, 0.12; lane b, 2.40; lane c, 2.62; lane d, 0.14; lane e, 0.60; lane f, 0.46; lane g, 0.81; lane h, 1.36.

Activating function of the T4 DNA polymerase accessory proteins. Combinations of the replication proteins were tested for their ability to stimulate T4 late transcription in the absence of deoxyribonucleoside triphosphates (*13*). We used the smaller pDHπ3 plasmid as a template for the experiment because it has a lesser diversity of in vitro transcripts. All combinations of proteins stimulated $P_{23} \rightarrow T_{T7}$ transcription on this template provided that they included gp44/62 (*15*) and gp45, the DNA polymerase accessory proteins; however, neither gp45 nor gp44/62 separately, or in separate combination with other replication proteins, sufficed. The three DNA polymerase accessory proteins exhibited the same polarity of transcriptional activation as the entire collection of replication proteins: transcriptional enhancement was principally generated when the nontranscribed DNA strand was nicked. The DNA polymerase accessory proteins bind best to DNA template-primer junctions (*16*) but should also be capable of binding to the gpII endonuclease-generated nick in pDHπ3. As was the case for pDH310 (Fig. 2), nick-initiated anti-clockwise transcription of pDHπ3 was depressed by the replication proteins.

In one experiment, we transcribed a 1:1 molar mixture of relaxed but covalently closed pDHπ3 DNA and nicked pDHπ5 DNA in the presence of the DNA polymerase accessory proteins (*13*). The P_{23}-initiated transcripts of pDHπ3 and pDHπ5 can be distinguished by their different sizes (316 and 422 nt, respectively). Transcriptional stimulation at P_{23} by the replication proteins was confined to the nicked pDHπ5 DNA. Accordingly, under these conditions, the nick only activated transcription in cis.

Quantitative effects of the other replication proteins on transcriptional activation by gp44/62 and 45 have not been extensively analyzed, but we have checked for the effects of two replication proteins that influence the binding of gp44/62 and 45 to template-primer junctions (*16, 17*). The gp43, which competes with gp44/62 and 45 for such binding sites, diminished the activation by these proteins. Gene 32 protein, which makes gp44/62 and 45 bind more tightly, modestly increased the transcriptional activation (less than two-fold at 3 μ*M*), and also counteracted the gp43-dependent inhibition. Other replication proteins added one at a time were without effect.

ATP-dependent promoter opening. The DNA-dependent ATPase activity of the DNA polymerase accessory proteins is optimal at primer-template junctions and requires ATP or dATP (deoxyadenosine triphosphate) as a substrate (*9*). The ATP analog, ATP-γ-S, is a nonhydrolyzable competitive inhibitor, while AMP-PNP (βγ-imidoadenosine 5′-triphosphate) is a nonhydrolyzable, noninhibiting analog (*18*); both analogs, and AMP-PCP (βγ-methyleneadenosine 5′-triphosphate), are suitable substrates for RNA synthesis. The DNA polymerase accessory proteins greatly stimulated P_{23} transcription from nicked pDHπ5 only in the presence of ATP (Fig. 3, compare lanes a and b); the nonhydrolyzable analogs were ineffective (compare lanes c and d, f and h, and j and k). Addition of 1 m*M* dATP to reactions containing AMP-PNP or AMP-PCP sufficed to generate transcriptional activation (lanes e and i). However, equimolar amounts of dATP and ATP-γ-S did not permit substantial transcriptional enhancement (lane 1), consistent

with the higher affinity of accessory proteins for ATP-γ-S than for dATP (*18*). The gene 44/62 and 45 proteins bind more tightly to DNA in the presence of ATP-γ-S than with ATP present (*16*). Thus, our data show that the ATPase function of the accessory proteins, and not merely their DNA or nucleotide binding, was required for stimulation of T4 late transcription on a nicked template. Half-maximal transcriptional activation was generated by 140 μ*M* ATP (*13*), consistent with the K_m (Michaelis-Menten constant) of the DNA-dependent ATPase activity, previously determined to be 130 to 180 μ*M* (*9*).

The lack of substantial transcriptional activation by the accessory proteins on closed circular DNA (*13*) is consistent with the known importance of DNA ends for protein binding and for the ATPase activity (*9*). The accessory proteins also stimulated transcription from the nick in the clockwise direction in an ATP-dependent manner (Fig. 3, lanes a and b). The degree to which this stimulation occurred varied, as did the basal level of transcription from the nick in the absence of accessory proteins. The cause of this variability has not been further analyzed.

We measured the rate of promoter opening at P_{23} by T4-modified RNA polymerase supplemented with gp55 by allowing interactions of all components at the P_{23} promoter to occur in the absence of ribonucleoside triphosphates for specified durations. Transcription was then started by the addition of a mixture of nucleotides and heparin. Under these circumstances, only promoter complexes bearing tightly bound RNA polymerase initiate transcription, and each complex completes only one round of transcription because loosely bound and recycling RNA polymerase molecules are sequestered and inactivated by heparin. Thus, the number of open promoter complexes at P_{23} could be quantified by densitometric scanning of $P_{23} \rightarrow T_{T7}$ transcripts on autoradiograms (*19*). The RNA polymerase alone formed open promoter complexes much more rapidly and readily on supercoiled than on nicked pDHπ5 (Fig. 4). However, with the accessory proteins and 1 m*M* dATP present, the rate of open promoter complex formation on nicked DNA exceeded even the rate on supercoiled DNA. Stimulation required the ATPase activity of the accessory proteins. We conclude that the ATP-requiring step in transcriptional enhancement occurs before the initiation of transcription.

Enhancing late transcription from either side of the promoter. To distinguish unequivocally between the effects of upstream or downstream placement of activating proteins on transcription, nicked pDHπ5 DNA was linearized either with Acc I endonuclease, positioning the nick upstream of P_{23}, or with Sma I, placing it downstream of P_{23} (Fig. 5A). Unnicked pDHπ5 was linearized by each endonuclease as controls. (Acc I generates a 2-nt 5′ overhang and Sma I generates blunt DNA ends.) Stimulation of promoter opening on these templates required the accessory proteins (Fig. 5B). With no nick in the DNA (lanes c, d, g, and h) the stimulation of P_{23} opening by the accessory proteins was 1.5 times or less. The presence of the nick (on the nontranscribed strand) sufficed to make the template activable by gp45, 44, and 62. The location of the nick in relationship to P_{23} did not seem to be qualitatively crucial, since nicked circular (Fig. 5B, lanes a and b), upstream-nicked (lanes e and f), and downsteam-nicked (lanes i and j) templates all were well stimulated by the accessory proteins.

We performed an experiment (similar to the one shown in Fig. 5)

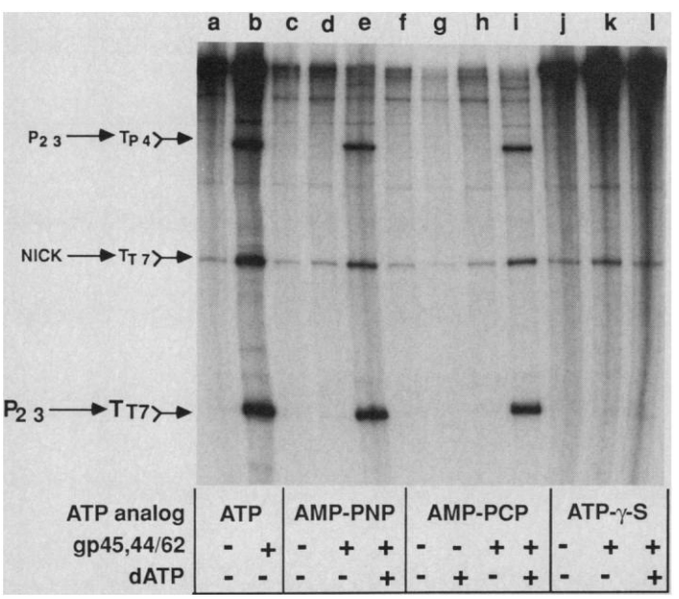

Fig. 3. Transcriptional role of the accessory protein ATPase activity. Transcription by T4-modified RNA polymerase (supplemented with gp55) of nicked pDHπ5 (5 n*M*; 5.5 μg/ml) proceeded for 15 minutes in the presence or absence of gp45 and gp44/62, as indicated below each lane. The concentration of rNTP's and proteins was as stated in (*34*) for lanes a and b. For the other lanes, 1 m*M* ATP was replaced with an equal concentration of the following analogs: AMP-PNP for lanes c to e, AMP-PCP for lanes f to i, and ATP-γ-S for lanes j to l. dATP (1 m*M*) was added to reaction mixtures as indicated. Transcription that was initiated at P_{23} and at the nick is indicated.

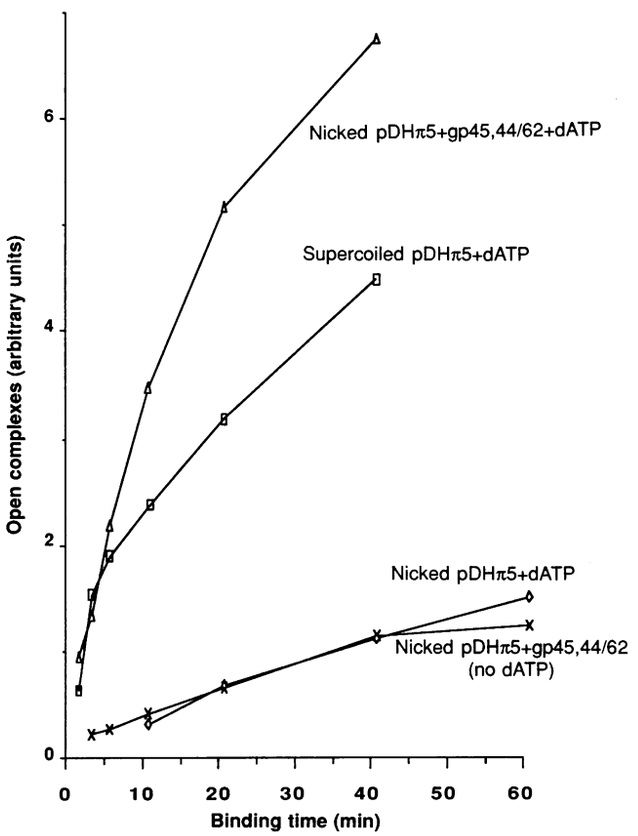

Fig. 4. Open promoter complex formation enhanced by gp45 and gp44/62. T4-modified RNA polymerase (supplemented with gp55) was allowed to bind to pDHπ5 (5 n*M*) in 20 μl of transcription buffer for the indicated times. Transcription was started by the addition of ATP, GTP, CTP, UTP, and heparin to 150, 150, 25, and 25 μ*M*, and 100 μg/ml, respectively, in 5 μl of transcription buffer, and continued for 10 minutes. Synthesis of $P_{23} \rightarrow T_{T7}$ RNA was quantified as indicated (*34*). Supercoiled pDHπ5 + 1 m*M* dATP (—□—); nicked pDHπ5 + 1 m*M* dATP (—◇—); nicked pDHπ5 + gp45 (13.4 μg/ml) + gp44/62 (54 μg/ml) (—X—); nicked pDHπ5 + gp45 (13.4 μg/ml) + gp44/62 (54 μg/ml) + 1 m*M* dATP (—△—).

with pDH310 and pDH410 to decide whether the strand placement of the nick would affect promoter activation from a relatively distant (3118 and 3108 bp, respectively) downstream site. Somewhat surprisingly, the nick on the P_{23}-conjugate nontranscribed strand (in pDH310) activated $P_{23} \rightarrow T_{T7}$ transcription selectively and did not activate $P_{V460} \rightarrow T_{P4}$ transcription. Placing the nick on the P_{V460}-conjugate nontranscribed strand (in pDH410) activated $P_{V460} \rightarrow T_{P4}$ transcription selectively (*13*).

Dependence of transcriptional activation by the replication proteins on a T4-specific modificaiton of RNA polymerase. *Escherichia coli* RNA polymerase undergoes multiple modifications in the phage T4-infected cell (*20*). Although gp55 alone confers late promoter specificity on unmodified *E. coli* RNA polymerase core in vitro (*7*), we wanted to know whether anything else was required in order for the RNA polymerase to be responsive to the T4 replication proteins. The T4-modified RNA polymerase, supplemented with gp55, permitted P_{23} in nicked pDHπ3 to be activated by the six replication proteins (Fig. 6, lanes a to c). In contrast, unmodified *E. coli* RNA polymerase supplemented with gp55 was not activated (lanes d to f). P_{23} recognition and synthesis of $P_{23} \rightarrow T_{T7}$ RNA by both RNA polymerases was comparable on supercoiled pDHπ3 (lanes h and i). Thus, in this multiround assay, in the presence of gp55, the T4-modified RNA polymerase responded to the transcription-enhancing action of the replication proteins while the unmodified RNA polymerase was, at best, poorly activated even though it was equally capable of late promoter recognition. In a separate experiment, we showed that the same requirement for T4-modified RNA polymerase held true when only gp45, 44, and 62 were used to enhance transcription in single- or multiple-round transcription assays (*13*). We conclude that, although *E. coli* RNA polymerase bearing gp55 can recognize late promoters, it must incur an additional T4-induced modification in order to be susceptible to the enhancing effect of the DNA polymerase accessory proteins (*21*).

The mobile enhancer. Our working model of how transcription at T4 late promoters is enhanced by replication proteins includes the following elements: (i) The DNA-dependent ATPase of the T4 DNA polymerase holoenzyme, composed of gp45, 44, and 62, enhances transcription by increasing the rate of open promoter complex formation. In view of the requirement for ATP binding and cleavage, the transcriptionally active form probably is a DNA-bound complex containing gp44/62, 45, ADP, and P_i as also proposed for the form of these replication proteins that creates the active "sliding clamp" for the T4 DNA polymerase (*16*). (ii) The gp44/62–45 complex is transported around the genome during DNA replication (*22*) and also is presumed to be positioned on the genome during the DNA synthesis that is associated with recombination and DNA repair. Thus, we suggest that the replication fork acts as a mobile enhancer. Unlike conventional enhancers and upstream activation sites, this one lacks the signal of a characteristic sequence. We think that explains why the cis-acting sites of replication-dependent T4 late transcription are so simple, consisting only of the late promoter TATAAATA consensus sequence at −10. (iii) Another novel element of this transcriptional regulation is that RNA polymerase must be modified in a T4 infection–dependent manner to effectively receive the transcription-enhancing signal of replication proteins.

In substituting the replication fork with a nicked DNA template for the key experiments, we have deferred consideration of whether other DNA structures might have greater enhancer strength. Structures that include single-stranded DNA might also recruit other replication proteins for transcriptional activation. For example, the T4 single-stranded DNA-binding protein, gp32, increases the affinity of the gp44/62–45 complex for a DNA primer-template junction (*16*). The observation that certain gene 32 temperature-sensitive mutants are much more defective in late gene expression than in DNA replication has suggested a model in which gp32 alone exerts the replication-coupled transcriptional activation from promoter-

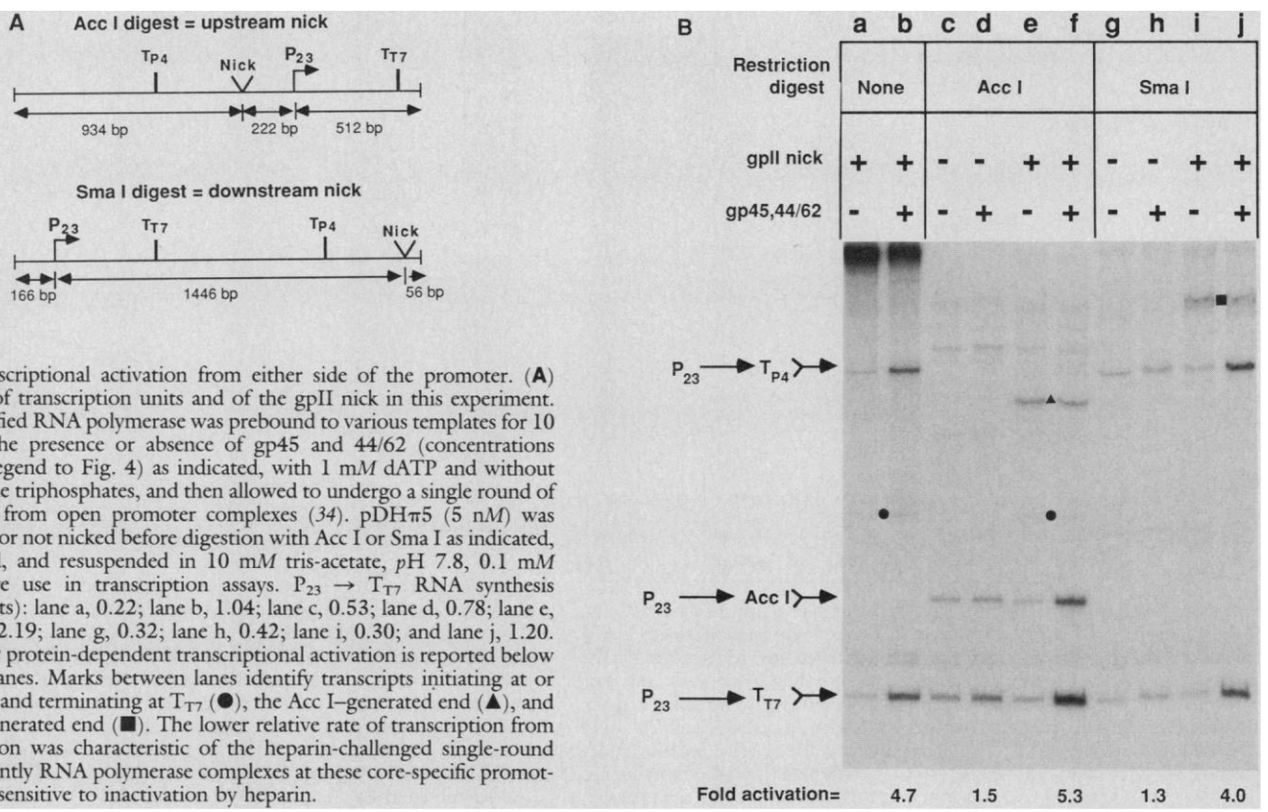

Fig. 5. Transcriptional activation from either side of the promoter. (**A**) Disposition of transcription units and of the gpII nick in this experiment. (**B**) T4-modified RNA polymerase was prebound to various templates for 10 minutes in the presence or absence of gp45 and 44/62 (concentrations specified in legend to Fig. 4) as indicated, with 1 m*M* dATP and without ribonucleoside triphosphates, and then allowed to undergo a single round of transcription from open promoter complexes (*34*). pDHπ5 (5 n*M*) was either nicked or not nicked before digestion with Acc I or Sma I as indicated, deproteinized, and resuspended in 10 m*M* tris-acetate, *p*H 7.8, 0.1 m*M* EDTA before use in transcription assays. $P_{23} \rightarrow T_{T7}$ RNA synthesis (arbitrary units): lane a, 0.22; lane b, 1.04; lane c, 0.53; lane d, 0.78; lane e, 0.41; lane f, 2.19; lane g, 0.32; lane h, 0.42; lane i, 0.30; and lane j, 1.20. The accessory protein-dependent transcriptional activation is reported below appropriate lanes. Marks between lanes identify transcripts initiating at or near the nick and terminating at T_{T7} (●), the Acc I–generated end (▲), and the Sma I–generated end (■). The lower relative rate of transcription from the nick region was characteristic of the heparin-challenged single-round assay. Apparently RNA polymerase complexes at these core-specific promoters are more sensitive to inactivation by heparin.

vicinal single-stranded DNA (*23*). While the evidence presented above makes it unlikely that gp32 is the sole transcriptional activator, the participation of gp32 as a component of transcriptional activation is not excluded. We have, in fact, found that gp32 somewhat increases the accessory protein-dependent activation, perhaps reflecting a gp32-dependent stabilization of polymerase accessory protein binding to the nick in our transcription templates. Similarly, the observed inhibitory effect of gp43 on transcriptional enhancement may be due to its competition with the gp44/62–45 complex for the same binding site in nicked DNA (*16*), and the mitigation of that inhibition by gp32 may reflect the association of all five of the replication proteins at the nick (*16, 17*).

The observed "polarity" of nick placement, the nontranscribed strand being strongly preferred, presents an interesting puzzle and challenge concerning one aspect of the mechanism of action of the DNA polymerase accessory proteins. Arguments can be put forward against what might appear to be the two most plausible interpretations of this result: (i) Strong strand-specificity of nick placement more than 3 kilobases away from a promoter argues against a purely steric restriction on the interaction between the replication proteins and the promoter complex, because DNA is not that stiff. (ii) If the replication proteins were required to translocate along DNA from the enhancer to the promoter in a unidirectional, ATP-driven way, the strand preference should switch for nicks placed on either side of the promoter; in fact it does not. This leaves as a formal possibility the specific entry of the accessory proteins onto one strand at a nick, followed by diffusion along that strand in either direction. However, the issue probably is not crucial for understanding enhancement from the replication fork in vivo, which would place the DNA polymerase accessory proteins on both DNA strands (*22, 24*).

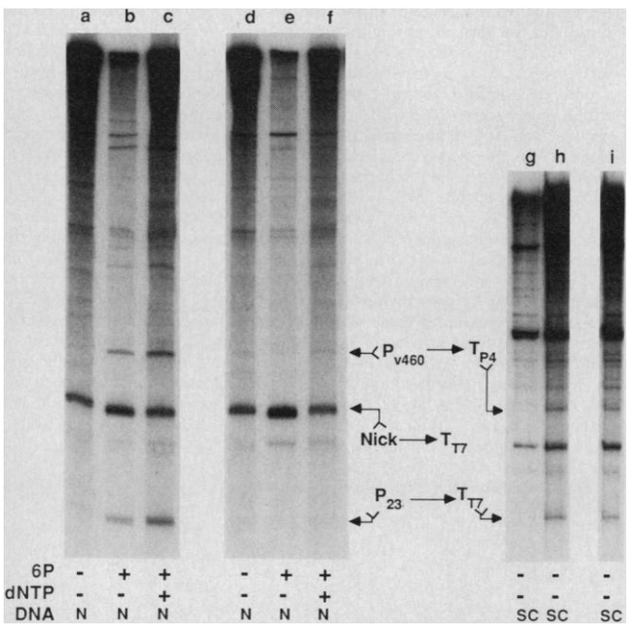

Fig. 6. Activity of modified and unmodified RNA polymerases. RNA was synthesized (30 minutes at 35°C) under standard conditions, but without RNasin. The concentration of pDHπ3 was 5 n*M*; its state, nicked (N) or supercoiled (SC) and the presence of replication proteins (6P) and deoxyribonucleotides is indicated below each lane. The concentration of gp32 was 3.0 μ*M*. Transcription was with 0.5 pmol T4-modified RNA polymerase (20 n*M*), supplemented with gp55 (lanes a, b, c, and h), 0.5 pmol of *E. coli* RNA polymerase core, supplemented with gp55 (lanes d, e, f, and i), or 0.5 pmol of *E. coli* RNA polymerase core (lane g). The autoradiogram of lanes a to f was exposed 3.5 times longer than that of lanes g to i. The amount of replication in reactions c and f was measured by incorporation of [methyl-^{3}H]dTTP (33 and 40 pmol/μl, corresponding to 18.5- and 22.3-fold replication of the leading strand, respectively). Transcripts are identified with arrows. $P_{23} \rightarrow T_{T7}$ RNA (in arbitrary units): lane a, 0.12; lane b, 0.78; lane c, 1.39; lane d, 0.09; lane e, 0.14; lane f, 0.12; lane h, 1.77; lane i, 1.33.

Significance for phage T4 and other gene expression. The above experiments establish a direct role for three replication proteins in T4 late gene expression: gp45, 44, and 62. The involvement of gp45 protein has been surmised on the basis of diverse genetic, physiological, and biochemical evidence (*25, 26*), but the involvement of gp44 and 62 has not been appreciated previously. Since they harbor the DNA-dependent ATPase activity of the gp44/62–45 complex, gp44 and 62 must play an active role in the enhancement (*27*).

Our experiments also provide at least qualitatively plausible interpretations of the coupling between T4 DNA replication and late transcription. We suppose that transcription diminishes greatly if replication is allowed to start and is then turned off (by thermally inactivating temperature-sensitive mutant replication proteins) (*28*) because complexes of replication forks with replisomes containing temperature-sensitive proteins fall apart at elevated temperatures, and replisomes whose forward progress has been blocked similarly dissociate (*16*). It seems likely that the DNA replication dependence of late transcription is relieved under conditions favoring the introduction and stabilization of many nicks and gaps into T4 DNA (*19, 21*) because these DNA structures also attract the 44/62 and 45 proteins and thereby have enhancer activity.

In order to demonstrate the transcription-enhancing effect of the gp44/62–45 complex in vitro, it is necessary to remove DNA supercoiling or otherwise lower the intrinsic strength of T4 late promoters to transcription by RNA polymerase consisting only of the core enzyme and gp55. Conversely, we suggest that conditions of the intracellular milieu and structural features of phage chromosomes that abolish or greatly diminish the intrinsic transcribability of T4 late genes by such a "minimal" polymerase in vivo are an intrinsic part of making T4 late gene expression replication-dependent. The absence of torsional strain in T4 genome DNA due to negative supercoiling at late times after infection (*29*) probably is important. Other contributions may exist.

The ATP hydrolysis requirement for the long-range transcriptional activation by the gp44/62–45 complex is reminiscent of the mode of action of the nitrogen regulatory NtrC protein, which also binds to promoter-distal enhancer sites in activating transcription at the *glnA* promoter. The wild-type NtrC protein requires phosphorylation (by the NtrB kinase) in order to be transcriptionally active. A constitutive mutant form of the NtrC protein, which is active in the absence of phosphorylation, nevertheless requires ATP binding and hydrolysis for its transcription-activating function (*30*).

In closing, we want to point out that bacteriophage T4 late genes and some herpes simplex virus 1 late genes share three properties: (i) expression depends on DNA replication; (ii) promoters are very compact and simple; and (iii) late genes on plasmids (in the HSV1 case, tested in transient expression assays) partly escape the replication dependence of genes on viral chromosomes (*31*). These similarities suggest a possible role for the replication fork or a replicative origin as a cis-acting element, and for replication proteins as trans-acting factors, in enhancing HSV1 late gene transcription.

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