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Biochemistry

DNA polymerase of bacteriophage T4 is an autogenous translational repressor

(T4 DNA polymerase/DNA replication/RNA footprinting/RNA-binding proteins/DNA-binding proteins)

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ABSTRACT **In bacteriophage T4 the protein product of gene *43* (gp43) is a multifunctional DNA polymerase that is essential for replication of the phage genome. The protein harbors DNA-binding, deoxyribonucleotide-binding, DNA-synthesizing (polymerase) and 3′-exonucleolytic (editing) activities as well as a capacity to interact with several other T4-induced replication enzymes. In addition, the T4 gp43 is a repressor of its own synthesis *in vivo*. We show here that this protein is an autogenous repressor of translation, and we have localized its RNA-binding sequence (translational operator) to the translation initiation domain of gene *43* mRNA. This mechanism for regulation of T4 DNA polymerase expression underscores the ubiquity of translational repression in the control of T4 DNA replication. Many T4 DNA polymerase accessory proteins and nucleotide biosynthesis enzymes are regulated by the phage-induced translational repressor regA, while the T4 single-stranded DNA-binding protein (T4 gp32) is, like gp43, autogenously regulated at the translational level.**

In bacteriophage T4, gene *43* is the structural gene for DNA polymerase (1, 2). This phage-induced enzyme is a multifunctional 105-kDa (896-amino acid) protein that is essential for phage DNA replication (see ref. 3 for discussion and references). It possesses two enzymatic activities, the DNA-synthesizing activity (polymerase) and a 3′-exonucleolytic activity that is particularly effective against single-stranded DNA (4). Together, these two activities are the major determinants of accuracy in DNA copying during replication (5). The gene *43* protein (gp43) functions in concert with several other essential phage-induced DNA replication proteins, some of which are known to interact directly with the gp43 and to affect its enzymatic activities and its role in control of fidelity (6, 7).

In addition to its known enzymatic functions in DNA replication, T4 gp43 regulates its own synthesis *in vivo* (8). Conditional lethal missense and nonsense mutants of T4 gene *43* overproduce their defective polypeptides when they are grown under nonpermissive conditions in *Escherichia coli* hosts. Some overproduce gp43 when grown under permissive conditions as well. The biological significance of this autogenous regulation and the level at which it occurs are not known, although it has been suggested that the T4 DNA polymerase is a repressor of its own transcription (9, 10). Such a mode of regulation would be consistent with the known DNA-binding properties of this protein; however, we have discovered that T4 DNA polymerase regulates its own translation rather than its own transcription. This report describes our findings, which include a demonstration that gp43 binds to a specific nucleotide sequence within the translation initiation domain of the mRNA. The ability of gp43 to bind a specific RNA target as well as DNA is reminiscent of another T4-induced DNA replication protein, the product of gene *32* (gp32, single-stranded DNA-binding protein), which is also an autogenous translational repressor. In addition, several other T4 replication enzymes are regulated by translational repression via the action of the T4 regA protein (11). Translational repression is thus a major mechanism for the control of T4 DNA replication in infected *E. coli* hosts.

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MATERIALS AND METHODS

Bacteria, Phage, and Plasmids. *E. coli* B-strain NapIV (12) was used as the host for plasmids and T4 phage infections. The T4 gene *43*–gene *44* double mutants *43amE4311–44amN82*, *43amE4301–44amN82*, and *43amB263–44N82* were constructed by standard phage crosses and their genotypes were verified by recombination and by gel electrophoresis as described (13, 14). In nonpermissive hosts (e.g., NapIV), all known T4 gene *43* nonsense mutants overproduce their mutant polypeptides 10- to 15-fold. The recombinant plasmids used are described in *Results*.

The methods used for measuring phage and plasmid gene expression *in vivo* have been described (14, 15). The RNA purification methods have also been described (16, 17).

***In Vitro* Repression Assays.** The cell-free S30 extracts from *E. coli* MRE600 and reagents used for *in vitro* translations were prepared as described by Pratt (18). Purified T4 DNA polymerase (1 μg/μl) was a generous gift from Navin Sinha (Rutgers University). Assays were conducted in 65 mM Tris acetate (pH 8.2) buffer containing 15 mM MgOAc, 15 mM KOAc, and 2 mM dithiothreitol. Each assay mixture contained 50 μg of RNA dissolved in 2.5 μl of water (either containing or lacking T4 DNA polymerase) and 12.5 μl of S30 extract plus the reagent cocktail for translation (18), including 10 μCi (1 Ci = 37 GBq) of [^{35}S]methionine. After incubation at 37°C for 20 min, translations were stopped by adding 80 μl of sodium dodecyl sulfate (SDS) extraction buffer, and the mixtures were heated in boiling water and analyzed by electrophoresis and autoradiography.

S1 Nuclease Analysis of RNA·RNA Hybrids and Reverse Transcriptase (RVT) Mapping of RNA 5′ Ends. For S1 nuclease mapping assays, a *Pst* I/*Xho* I T4 DNA fragment (MB292) containing a 3′-terminal segment [260 base pairs (bp)] of gene *62*, the entire *regA* gene (366 bp), the *regA-43* intergenic domain (78 bp), and a 5′-terminal segment (200 bp) of gene *43* was cloned in the RNA probe vector pGEM4 (Promega Biotec, Madison, WI) and used for *in vitro* synthesis of complementary RNA under the direction of added T7 or SP6 RNA polymerase. *In vitro* transcription was carried out at 37°C for 1 hr in 20 μl of 40 mM Tris·HCl (pH 7.5) buffer containing 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol; 20 units of RNasin (Promega

Abbreviation: RVT, reverse transcriptase.

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Biotec); 0.5 mM each ATP, CTP, and GTP; 0.05 mM UTP; 80 μCi of [α-^{32}P]UTP (800 Ci/mmol); 0.2 μg of DNA template; and 10 units of T7 RNA polymerase. The products of the reaction were treated with 0.5 unit of DNase I at 37°C for 15 min and purified by phenol/chloroform extraction and chromatography on an RNA grade Sephadex G50 pre-spun column (Boehringer Mannheim). The T4-induced RNA used for hybridization with the uniformly ^{32}P-labeled complementary RNA probe was purified from infected cells (15 min postinfection) as described (16). About 2 × 10^6 cpm of probe were mixed with 5 μg of total RNA in 30 μl of a mixture containing 80% formamide, 20 mM Pipes (pH 6.5), 400 mM NaCl, and 2 mM NaEDTA. The mixture was then heated at 90°C for 5 min and immediately transferred to 42°C. After overnight incubation at this temperature, 300 μl of S1 nuclease solution [800 units of S1 nuclease (Boehringer Mannheim)/100 mM NaCl/60 mM NaOAc, pH 4.5/2 mM $ZnSO_4$] was added. S1 nuclease digestion was performed at 20°C for 30 min and then at 4°C for an additional 15 min. The S1 nuclease-resistant nucleic acid was purified by phenol/chloroform extraction and ethanol precipitation and resuspended in 20 μl of 95% formamide containing 0.1% each of bromphenol blue and xylene cyanol in 10 mM NaEDTA. Samples were then subjected to electrophoresis in 6% polyacrylamide slab gels (1 mm thick) containing 7 M urea and TBE buffer (50 mM Tris borate, pH 8.3/1 mM NaEDTA) and the resolved ^{32}P-labeled bands were visualized by autoradiography.

The two RNA preparations used for RVT mapping assays originated from NapIV cultures that were infected with T4 *43*$^+$ (*44amN82*) and T4 *43*$^-$ (*43amB263–44amN82*) strains. In each case, 12 μg of RNA, isolated 15 min postinfection, was used as template with ^{32}P-end-labeled primer (at primer excess) for dideoxy sequencing reactions catalyzed by avian myeloblastosis virus RVT as detailed elsewhere (17).

RNase Protection Assays. The RNA used for footprinting the binding site of T4 DNA polymerase was purified as described (19) from heat-induced pEM104-bearing *E. coli* NapIV. The T4 DNA polymerase used for these experiments was a generous gift from H. E. Selick and B. M. Alberts (University of California, San Francisco). An 8-μl mixture containing 1.7 μg of RNA and 1 μM DNA polymerase (or no polymerase) in binding buffer (50 mM Tris·HCl, pH 8.3/60 mM NaCl/10 mM dithiothreitol) was incubated at 37°C for 5 min, after which 1 μl of the desired RNase was added for 3 min. RNase A was used at a final concentration of 10^{-6} unit/μl and RNase T1 was used at 1 unit/μl. After the RNase treatments, the reactions were quenched with 10 μl of stop buffer [400 mM NaOAc/*E. coli* tRNA (2 μg/μl)/50 mM Tris·HCl, pH 8.3/20 mM NaEDTA/60 mM NaCl/10 mM dithiothreitol]. The nucleic acids were purified by phenol/chloroform extraction and ethanol precipitation and used with a ^{32}P-end-labeled 23-base synthetic oligodeoxynucleotide primer and avian myeloblastosis virus RVT in primer-extension sequencing assays to localize the sites of endonucleolytic cleavage by the RNases. The primer used was complementary to nucleotides 77–99 downstream from the A of the initiator AUG in gene *43*. The preparation of ^{32}P-labeled primers and the conditions for these assays have been described (17, 19).

RESULTS

Autogenous Repression of T4 DNA Polymerase Synthesis *in Vivo*. The results shown in Fig. 1 confirm the observations initially made by Russel (8) that T4 gene *43* nonsense mutants overproduce gp43 protein fragments (compare lanes E4311 and E4301 to lane 43$^+$) and further demonstrate that wild-type gp43 can repress the overproduction of mutant gp43 in trans (compare lanes 43$^+$ × E4311 and E4301 × E4311). Autogenous regulation of gene *43* expression resembles other

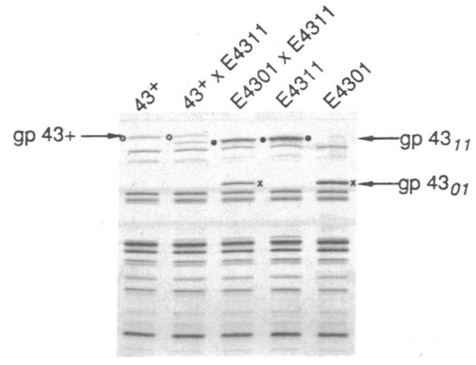

FIG. 1. Autoradiogram showing separation of ^{35}S-labeled T4-induced proteins by SDS gel electrophoresis and the ability of a wild-type gene *43* allele (*43*$^+$) to repress the expression of a mutant allele (*43amE4311*) in trans. Logarithmic-phase *E. coli* NapIV cultures (2 × 10^8 cells per μl) were infected with phage strains carrying the designated alleles of gene *43*. All the T4 strains used also carried the gene *44* nonsense mutation *44amN82*, which disallows T4 DNA replication and late gene expression in the NapIV host. The multiplicity of infection was 10 (5 of each phage in coinfections) and infection mixtures were labeled with [^{35}S]methionine (5 μCi/ml) for 10 min beginning 15 min postinfection. Extracts were then prepared and analyzed (13, 14). The *43*$^+$ (○), gp43$^+$, and 43 mutant [*43amE4311* (●), $gp43_{11}$ and *43amE4301* (×), $gp43_{01}$] polypeptides are noted by arrows.

systems of prokaryotic gene regulation that utilize diffusible repressor substances. Both transcriptional and translational repressor proteins have been described (20), and consequently we examined the mechanism by which gp43 regulates its own synthesis.

Autogenous Repression of T4 DNA Polymerase Synthesis Occurs Post-transcriptionally. To test whether gene *43* mutant infections that overproduce gp43 also overproduce gene *43*-specific mRNA, we used two types of assays to quantitate this RNA: (*i*) RVT-catalyzed primer-extension assays that measured the total gene *43* mRNA as well as the relative abundance of mRNA 5′ ends (17), and (*ii*) S1 nuclease mapping assays with RNA probes that measured the levels of gene *43* RNA relative to RNA products of the T4 *regA* gene, which is known to be regulated independently of T4 gene *43* (21–23). Some results are shown in Fig. 2. Whereas mutant gene *43* protein was overproduced 6- to 10-fold relative to wild-type gp43 in such infections (Fig. 1; unpublished results), the levels of gene *43* mRNA were not similarly affected. In Fig. 2, the multiple RNA bands that were detected for genes *43* and *regA* in the S1 nuclease mapping assays reflect the overlapping modes of transcription and the posttranscriptional processing that characterize these two cistrons *in vivo* (ref. 17; unpublished results). The RVT mapping experiment shows the positions of 5′ ends on the gene *43* mRNA species that result from these transcriptional and posttranscriptional events. In both types of assays, we observed that, although the relative intensities of different mRNA bands can vary slightly with different infections, the overall amount of gene *43* mRNA was unaffected in *43*$^+$ versus *43*$^-$ T4 infections. In the S1 nuclease mapping assays described in Fig. 2, densitometric scans (data not shown) of the autoradiogram lanes yielded similar gene *43*/gene *regA* mRNA ratios in all three infections analyzed. Similarly, a comparison of the lower portions of the autoradiogram lanes in the RVT mapping experiment indicated that the total amount of gene *43* mRNA was the same in both *43*$^+$ and *43*$^-$ infections. Therefore, the observed derepression of gene *43* expression was not due to increased transcription.

Translational Repression of T4 gp43 Synthesis *in Vitro*. The heat-inducible λ*cI857P$_L$N* expression plasmid pEM104 (24),

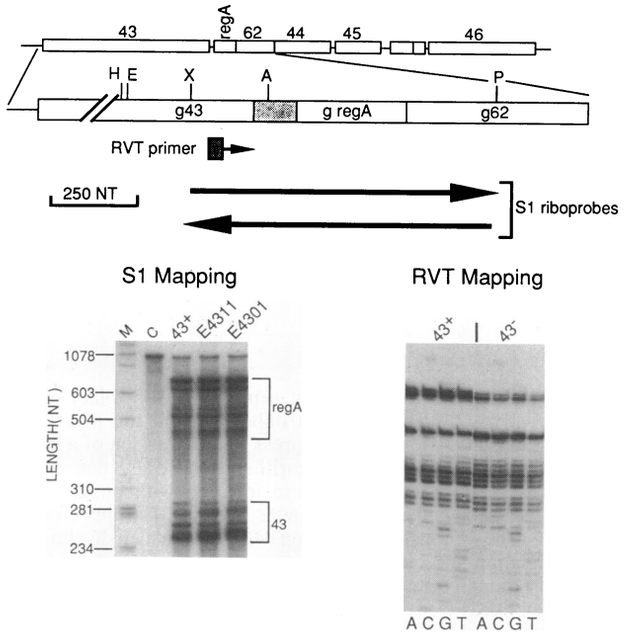

FIG. 2. RNA analyses showing the insensitivity of T4 gene *43* mRNA synthesis to mutations in the structural gene. (*Upper*) T4 genomic segment for which the analyses were carried out. The direction of transcription for this region in T4 infections is from right to left. The autoradiograms show the results of RNA quantitations by S1 nuclease analysis of RNA·RNA hybrids (S1 Mapping) and 5′-end mapping by oligodeoxynucleotide primer extension with avian myeloblastosis virus RVT (RVT Mapping). The sizes and polarities of the RNA probes used for S1 nuclease mapping and the location of the primer used for RVT mapping are shown. The RNA used for S1 nuclease mapping was from infections with T4 *43amE4311–44amN82* (lane *E4311*), T4 *43amE4301–44amN82* (lane *E4301*), and T4 *44amN82* (lane 43^+), respectively (see Fig. 1). RNA for RVT mapping was from infections with T4 *43amB263–44amN82* (lanes 43^-) and *44amN82* (lanes 43^+). The dideoxynucleotide used in each RVT reaction is indicated below each lane. The control (lane C) in the S1 nuclease mapping experiment was an S1 nuclease-digested RNA·RNA hybrid that was prepared by hybridizing the two complementary *in vitro* transcriptional products (S1 nuclease RNA probes) of the DNA cloned between the SP6 and T7 promoters of the pGEM4 RNA probe vector—i.e., it represents the size of full-length protected RNA spanning the region of interest. Hybridizations to T4-induced RNA utilized the S1 nuclease RNA probe (→). Lane M, size markers obtained by ^{32}P-end labeling of *Hae* III-digested ϕX174 replicative form DNA. NT, nucleotides; H, *Hin*III; E, *Eco*RI; X, *Xho* I; A, *Ava* I; P, *Pst* I.

which is diagrammed in Fig. 3, was placed in *E. coli* NapIV and used to produce a polycistronic mRNA capable of synthesizing T4 gpregA and an amino-terminal T4 gp43 fragment ($gp43_{EM}$ in Fig. 3). Identity of the pEM104-generated polycistronic RNA was verified by RNA·RNA hybridization and primer-extension assays similar to those described in Fig. 2 (data not shown). The purified RNA was used with cell-free extracts from *E. coli* (S-30 system) in translation assays that measured the effects of added purified wild-type T4 DNA polymerase on *in vitro* production of the two pEM104-encoded T4 proteins. As shown in Fig. 3, the added enzyme inhibited $gp43_{EM}$ synthesis and did not affect gpregA synthesis from the plasmid-generated polycistronic mRNA. *In vitro* expression from this RNA was insensitive to inhibitors of transcription (results not shown), indicating that DNA contamination was not a factor in the observed effects. These results suggest that T4 DNA polymerase is a repressor of its own translation and that it binds to an mRNA site distal to the *regA* control region, since *regA* expression is not affected by T4 DNA polymerase either *in vivo* (21–23) or *in vitro* (Fig. 3).

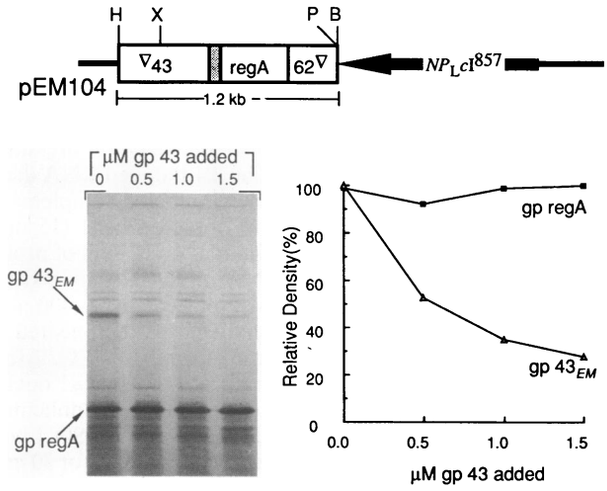

FIG. 3. Translational repression by T4 DNA polymerase *in vitro*. Cell-free extracts from *E. coli* MRE600 were used to translate an RNA mixture enriched for polycistronic mRNA from heat-induced pEM104 (*Upper*). The pEM104-generated mRNA encodes *regA*-encoded protein (gpregA) and an amino-terminal gene *43* protein fragment ($gp43_{EM}$). Translations were carried out in the absence and in the presence of 0.5, 1.0, and 1.5 μM purified wild-type T4 DNA polymerase and [^{35}S]methionine and were analyzed by SDS gel electrophoresis and autoradiography. Densitometric scans of the autoradiogram on the left were used to prepare the plots on the right. Note the inhibition of $gp43_{EM}$ synthesis relative to gpregA synthesis with T4 DNA polymerase additions. At concentrations >1.5 μM (data not shown), T4 DNA polymerase exhibited inhibitory effects on the translation of all *in vitro* products.

Location and Nucleotide Sequence of the mRNA Site for Autogenous Translational Repression by T4 DNA Polymerase. We used RNase protection (RNA footprinting) assays (25, 26) to localize the sites of gp43 binding on pEM104-generated RNA (Fig. 4). In these experiments, purified wild-type T4 DNA polymerase (at 1 μM) was incubated with the RNA in a binding buffer and the mixture was treated with either pancreatic RNase (which cleaves between U and A and between C and A residues) or with RNase T1 (which cleaves between G and N residues). After the RNase treatments, the nucleic acids were purified and sequenced by the RVT-catalyzed primer-extension technique (refs. 17 and 19; Fig. 2). Cleavages by the RNases were detected as interruptions in the nucleotide sequence as visualized on autoradiograms of sequencing gels. As shown in Fig. 4, each RNase generated its own characteristic cleavage pattern and the addition of T4 DNA polymerase strongly protected specific residues, all of which were located upstream of the initiator AUG for the gene *43* message. These footprint patterns were reproduced with concentrations of T4 DNA polymerase as low as 0.1 μM (data not shown). Some RNase T1-resistant residues were observed in incubations with and without T4 polymerase, suggesting the existence of a secondary structure in the leader segment of gene *43* mRNA. Similar conclusions were derived from results with RNase T2 and dimethyl sulfate treatments, and identical results were obtained with RNA purified from phage-infected cells (data not shown). Also, other assays showed that added T4 DNA polymerase does not protect any part of the *regA* ribosome-binding domain in the same RNA preparations. We conclude that T4 DNA polymerase inhibits its own synthesis by specifically binding to its own mRNA in the translation initiation region.

DISCUSSION

We have shown that autoregulation of bacteriophage T4 DNA polymerase biosynthesis occurs at the translational

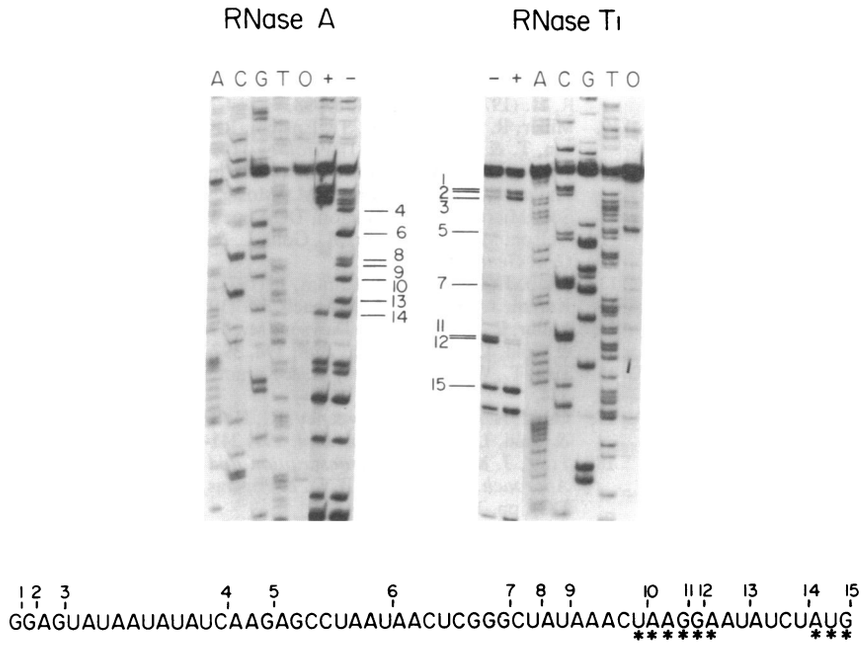

FIG. 4. The regions of gene *43* mRNA protected by purified T4 DNA polymerase from RNase digestion. A portion of gene *43* mRNA is shown with the Shine–Dalgarno sequence and initiator AUG (*). The positions of the numbered autoradiogram bands correspond to vertical lines on the RNA sequence. Autoradiogram lanes showing RNase digestion in the presence (+) and absence (−) of gene *43* protein are also indicated. The A,C,G,T designations refer to the dideoxynucleotides used in the sequencing reactions. Lanes O, results from primer-extension reactions in which dideoxynucleotides were omitted.

level. Infected cells overexpressing mutant DNA polymerase contain amounts of gene *43* mRNA similar to the amounts in cells expressing wild-type DNA polymerase at normal levels (Fig. 2). In addition, purified DNA polymerase differentially represses gene *43* mRNA translation *in vitro* (Fig. 3) and binds to this mRNA at a site within the ribosome-binding region (Fig. 4), two important characteristics of the best-studied translational repressors (27). By these criteria, gene *43* protein regulates its own synthesis translationally, as deemed plausible by Russel (8) when she discovered this regulatory system. Two papers published after Russel's work assert that gene *43* autogenous regulation is transcriptional (9, 10). In fact, those published results show that gene *43* mRNA is unstable but do not address directly the regulatory mechanism. In addition, the mutant infections used to conclude that regulation is transcriptional were not paired so as to isolate the derepression of gene *43* expression from alterations of either DNA synthesis or general transcription (9, 10). The infections used here (Fig. 2) achieve that isolation by the inclusion of amN82 (gene 44^-) in each phage, such that no infection yields DNA replication or late transcription.

Our other studies (ref. 17; unpublished data) indicate that after T4 infection most gene *43* mRNA originates from sites far upstream of the *regA–43* intergenic region. About half of this polycistronic mRNA is processed just 5′ to the DNA polymerase binding site. A minor promoter specific for gene *43* transcription is activated by the motA protein (refs. 17 and 28; unpublished data). In Fig. 5, we diagram the binding of the DNA polymerase to the translational operator based on the footprinting data from Fig. 4 and show the position of the 5′ ends of the overlapping species of gene *43* mRNAs. As depicted, all classes of gene *43* mRNA are substrates for repression by DNA polymerase. We also correct the sequence encoding the CUUCGG hairpin between genes *regA* and *43* (see ref. 29). Band compression artifacts of this stable hairpin contributed to sequencing errors (15, 30) that have been extensively cited (17, 24, 26, 28, 29, 31–33). The correct sequence (5′-UUAACGAAGGGGCUUCGG-3′) contains the two additional underlined Gs and increases the size of the intercistronic RNA hairpin from a 3-base-pair helix to one of at least 7 base pairs, which is closer to the average size of the many other CUUCGG hairpins found in T4 (29). In addition to clarifying transcriptional termination events that occur 3′ to this very stable hairpin (T.H., unpublished results), the new sequence also reveals a *mot* promoter box that contains only 5 of the 9 bases seen in the *motA* consensus sequence

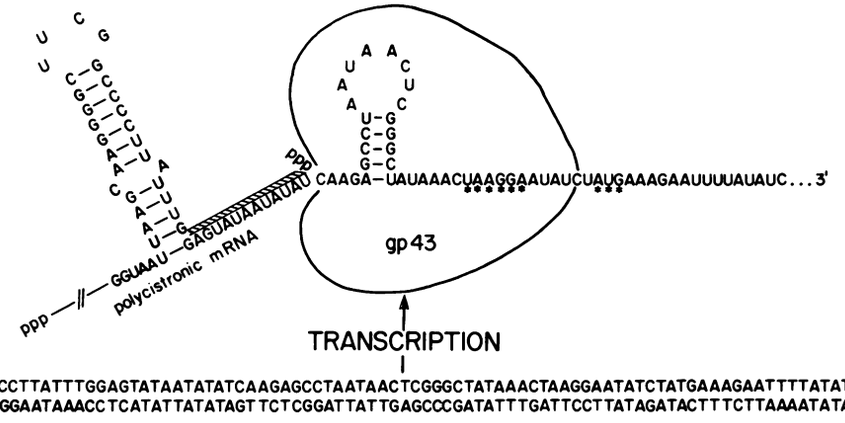

FIG. 5. Model for interaction of T4 DNA polymerase with its mRNA. Gene *43* protein is shown bound to its message in a manner consistent with the footprinting data in Fig. 4. The 5′ triphosphate ends for the *motA*-dependent transcript and the polycistronic transcript are denoted ppp. The Shine–Dalgarno sequence and the initiator AUG for gene *43* are shown by asterisks below the mRNA. The *motA* consensus sequence for gene *43* is boxed on the DNA. The location of processed ends ( ) on the gene *43* messenger RNA (17) is also shown.

(17). This may explain why transcripts from this promoter constitute only a minor fraction of the total gene *43* mRNA (17).

Our findings underscore the widespread use by bacteriophage T4 of posttranscriptional regulation of specific gene expression. In addition to DNA polymerase, two T4-induced translational repressors have been described: the regA protein, which is a translational repressor that acts on many target mRNAs, including its own transcript (26) and the single-stranded DNA-binding protein encoded by gene *32*, which represses only its own translation (34). Also, at least four T4-encoded mRNAs contain interruptions that are likely to be targets for posttranscriptional regulation: gene *60*, which encodes a subunit of the T4 topoisomerase (35), contains an "intron" that is simply skipped by the elongating ribosome (36), while three other early T4 genes have group I introns that are removed before translation (37). In addition, T4 encodes an endonuclease that inactivates several mRNAs by cleavage within the ribosome-binding site (E. Brody, personal communication; J. Ruckman, personal communication). These various posttranscriptional regulatory loops have not been integrated into a coherent physiological picture; however, we imagine that large lytic phages make "burst size" decisions that are analogous to the lysogeny/lytic decision made by λ (38) and that posttranscriptional regulation of gene expression in prokaryotes must offer some unknown advantages over regulation of mRNA synthesis.

We conclude with a speculation. A protein that binds to an RNA target sequence is likely to bind to the same site on DNA (39). For example, ribosomes are able to locate translation initiation regions on single-stranded DNA (34, 40–42), and a single-stranded DNA copy of a tRNA molecule can be recognized by a tRNA synthetase (B. Roe, personal communication). Also, *fd* gene *V* protein binds to an mRNA operator (43); the same sequence in DNA is near the *fd* replication origin and has been postulated (44) to be the binding site for the gene *V* protein, which is involved in the switch from double-stranded to single-stranded DNA synthesis. Since gene *43* autogeny involves recognition of a specific mRNA target, the same sequence in DNA may be used as a preferred entry site for DNA polymerase (and, subsequently, its associated replication proteins). Although this region has not been mapped as an origin for T4 DNA replication (45), it exhibits several features that are consistent with models for the assembly of prokaryotic "primosomes" and "replisomes" (31, 46, 47). Cooperation between the T4 motA protein, *E. coli* host RNA polymerase, T4 DNA polymerase and the other T4 replication proteins, and even endonucleolytically processed mRNA may allow the region of T4 DNA between *regA* and *43* to serve as a target for the assembly of the T4 replication complex.

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