DNA polymerase (gp43) of phage T4 plays two biological roles, one as an essential DNA binding replication enzyme and the other as an mRNA-specific autogenous translational repressor. Binding of T4 gp43 to its mRNA target (translational operator RNA) interferes with gp43-DNA interactions, but it is unclear how the protein determinants for binding DNA are affected by the dynamics of gp43-mRNA interactions. We have used RB69 gp43, a natural variant of the T4 enzyme whose crystal structure has been determined to identify protein sites that respond to the interaction with specific RNA. We used protein phosphorylation markers, photocross-linking studies, protease sensitivity assays, and mutational analyses to examine the effects of operator RNA on the enzyme’s five structural domains (N, exo, palm, fingers, and thumb). Our studies suggest that this RNA affects gp43-DNA interactions through global effects on protein structure that occlude DNA-binding sites but leave the enzyme accessible to interactions with the sliding clamp (RB69 gp45) and possibly other polymerase accessory proteins. We discuss the possible biological significance of putative RNA-binding motifs in the N and palm domains of RB69 gp43.

The replicative DNA polymerase of bacteriophage T4, product of phage gene 43 (gp43), is a pol α-like (B-family) enzyme that displays a number of activities, all resident in the same 898-residue protein molecule (reviewed in Ref. 1). In addition to its two distinct catalytic functions (i.e. the polymerase (pol) and 3’-exonuclease (exo or editing) activities, T4 gp43 bears specific binding activities to deoxyribonucleoside triphosphates (dNTPs)), other proteins of the T4 DNA replication complex, and its own mRNA. In vivo, T4 gp43 functions as an autogenous translational repressor that interacts with a nucleotide sequence, termed “translational operator,” which overlaps the ribosome-binding site in the translation-initiation region of gene 43-encoded mRNA (2, 3). It is known that the RNA determinants of operator specificity to the protein include a propensity of the nucleotide sequence to form a specific stem-loop (hairpin) structure (2–6). Nucleotide sequence-independent determinants of operator specificity to the protein include a propensity of the nucleotide sequence to form a specific stem-loop (hairpin) structure (2–6). Nucleotide sequence-independent in- 

Experimental Procedures

Bacteria, Phages, and Plasmids—The Escherichia coli strain DH5α (F lacZAM15 lacY1 proAB supE44 thi-1 endA1 relA1 recA1 Δ(lac-proA)155) was used as a host for transformations with recombinant DNA in all protocols involving the cloning of DNA fragments in expression vectors and the introduction of specific mutations in cloned DNA through site-directed mutagenesis. DH5α was purchased as a frozen stock from Invitrogen (catalog no. 18265-17) and used as recommended by this supplier. The E. coli strain BL21(DE3) was used as the bacterial host for recombinant plasmids in experiments requiring in vivo transcription of cloned RB69 or T4 genetic sequences under control of a plasmid-borne T7 promoter. BL21(DE3) carries a chromosomally integrated T7 RNA polymerase gene that can be activated through lac operon inducers (11). The inducer used here was isopropyl-β-D-thiogalactoside. E. coli strain CAJ70 was used as host for
PB69 phage infections as previously described (7, 12).

Many of the plasmids we used were constructed as part of this study and are described below and under “Results.” Other plasmids used have been described in previous reports (7, 12, 13).

**Purification of gp43**—The purified T4 gp43 and PB69 gp43 used for photoreactive and in vitro experiments, in the case of T4 gp43, protease sensitivity assays were prepared by ion exchange and DNA-cellulose chromatography, as described previously (12, 14). For PB69 gp43 phosphorylation assays, we used N-terminally or C-terminally histidine-tagged derivatives of this protein. Optimal conditions for protein purification with such derivatives were obtained with three histidines added to the C-terminus of the protein and six histidines added to the N-terminus of the protein. The histidines were introduced through PCR-driven site-directed mutagenesis of pCW19R, a recombinant plasmid that carries the wild-type RB69 gene 43 under control of the T7 ϕ10 promoter (12, 13). Stratagene’s QuikChange protocol and reagents were used for the in vitro mutagenesis. We fused three histidine codons to the 5′-terminal end of the open reading frame of the gene by amplifying pCW19R in the presence of a synthetic DNA duplex in which the sense strand had the sequence 5′-gtggtagattgtcttcccACCAACCAAGTATATAATTGTGGCATTGC-3′ (RB69 gp43 codons in lowercase, histidine codons in uppercase, and vector residues italicized). This resulted in the isolation of pVPR43C-His3 (Table I). To add six histidine codons to the 5′-terminal side of the RB69 gene 43 open reading frame, we constructed pVPR43N-His6 (Table I) by amplifying pCW19R in the presence of a synthetic DNA duplex in which the sense strand had the sequence 5′-gtggtagattgtcttcccTTAATTGTGGCATTGC-3′ (RB69 gene 43 sequence in lowercase, initiation codon underlined, histidine codons in uppercase, and vector residues italicized). The two resulting plasmid constructs, pVPR43C-His3 and pVPR43N-His6, were isolated, and their nucleotide sequences were verified after transforming products of the respective QuikChange mutagenesis protocol into E. coli DH5α.

His-tagged PB69 gp43 was purified from crude extracts of the appropriate clones following induction of transcription of recombinant plasmids in vivo. In each case, about 10 ml of E. coli BL21(DE3) culture (OD –0.5) containing the recombinant plasmid of interest was aerated with isopropyl-β-D-thiogalactoside (at 1 mM) for 2 h at 30 °C to effect elevated synthesis of gp43 under control of the plasmid-borne T7 promoter. Induced cells were harvested by centrifugation at 5000 × g for 10 min and washed in 5 ml of sonication buffer containing 20 mM sodium phosphate (pH 7.5), 400 mM NaCl, and 5 mM imidazole before being resuspended in 0.5 ml of the same buffer. The concentrated cell suspension was then sonicated (model W-225R sonicator; Heat Systems-Ultrasonic, Inc., Farmingdale, NY) for 2 min on ice and then centrifuged for 15 min at 15,000 × g for 30 min. The resulting 0.4–0.5 ml of crude extract was applied to a Ni2+–nitrilotriacetic acid spin column (Qiagen; catalog no. 30210). After washing the column with 20 volumes of 20 mM sodium phosphate buffer (pH 7.5) containing 20 mM imidazole and 400 mM NaCl, bound material was eluted with buffer containing 100 mM imidazole. The eluate was dialyzed against buffer containing 20 mM Tris-Cl (pH 7.0), 2 mM diethiothreitol, and 50% glycerol and then stored at –80 °C.

**Preparation of RNA Substrates for Photocross-linking Experiments**—Nucleotide sequences of the RNA substrates used for photocross-linking experiments are depicted in Fig. 1. The RNAs were synthesized by in vitro transcription of the appropriate DNA duplexes that had been cloned into the BL21(Smal) interval of the T7/SP6 expression vectors pSP73 and pSP72, sold by Promega (Madison, WI) (catalog no. P2191 and P2221, respectively). Nucleotide sequences of the sense strands of the cloned DNAs were as follows: i) 5′-GATCTTAAATATATACCG-GCTTAAACTCGGGCTATAAACTAAGGAATATCTATGAAAGAATTG-3′ and ii) 5′-GACCGTAAACAAAAGACCGAGTACGACCGAAGAATAAAGAGAAGACAGGACCAAGAATTG-3′, for cloning the T4 “CWTop” operator sequence (Fig. 1) under control of the SP6 promoter of pSP72, and (iii) 5′-GATCTTAAAACAAAAGACCGAGTACGACCGAAGAATAAAGAGAAGACAGGACCAAGAATTG-3′ for cloning the RB69 “RBop4” operator sequence (Fig. 1) under control of the T7 ϕ10 promoter of pSP72.

For in vitro transcription reactions, recombinant plasmids containing the desired DNA sequences were linearized by BamHI digestion, repurified, and then used in the RiboMax transcription mixture sold by Promega (catalog no. P1300; with either T7 RNA polymerase or SP6 RNA polymerase. Reaction mixtures (50 μl) contained 5 μg of DNA template, four ribonucleoside triphosphates (ATP, CTP, UTP, and GTP) at 300 μM each, and 90 units of the designated RNA polymerase. The mixes were incubated for 1.5 h at 37 °C and then quenched by the addition of 5 μl of RNase-free DNase (catalog no. P1300; Promega). After an additional incubation of 15 min at 37 °C, RNA products were purified through two phenol/chloroform (pH 4.5) extractions and one ethanol precipitation, dissolved in 100 μl of nuclease-free 10 mM Tris-Cl (pH 8.0) buffer, and separated from small DNA and RNA fragments and nucleotides on “Quick Spin G-50” columns (catalog no. 100406; Roche Molecular Biochemicals). Purity of the RNA products was evaluated by electrophoretic analysis on 10% polyacrylamide gels containing 7 M urea, and RNA concentrations were determined spectrophotometrically. For radiolabeling RNA, [α-32P]UTP (catalog no. NEG-307H, PerkinElmer Life Sciences) was added to transcription mixtures at 50 μCi/reaction (specific activity of 3000 Ci/mmol). To prepare photoactive RNA products for cross-linking experiments, the unlabeled CTP in these mixes was replaced with 5-iodo-CTP (catalog no. I5682; Sigma). All manipulations with photoactive RNAs were performed under reduced light conditions and 1 unit units of UV-sensitive bacterial RNA polymerase (Promega) and T4 UV-sensitive bacterial RNA polymerase (Promega) and then incubated at 37 °C for 15 min. The presence of operator RNAs exhibited a diminished affinity to gp43 (by ~10-fold) as compared with the unmodified counterparts; however, binding remained specific (i.e. 7–10-fold higher affinity than with RNA of non-specific nucleotide sequence (results not shown). Radiolabeled RNA substrates were examined for purity by electrophoresis on 10% polyacrylamide gels containing 7 M urea and visualized by the use of a...
**RESULTS**

Operator RNA Affects RB69 gp43 Structure—Previous studies seemed to suggest that operator RNA makes multiple contacts with gp43, some at locations outside the protein regions that bind primer-template DNA (4, 12). We introduced phosphorylation markers at various positions in RB69 gp43 and scanned the protein molecule for those sites whose access to the protein kinase PKA could be affected by the interaction with operator RNA. Results are shown in Fig. 2. We observed two types of effects by operator RNA on phosphorylation within this set of RB69 gp43 markers: inhibition (site 382RRRS385; Fig. 2) and stimulation (sites 893RRAS895, 259RRRT262, 245RLS246, and 245RRK248; Fig. 2). Inhibition of phosphorylation at the 893RRAS895 site may reflect either a direct or indirect occlusion of the site by the RNA. We note that this site is within 25 Å of the primer-template junction in the crystal structure of RB69 gp43 (8–10). The RNA-mediated stimulatory responses at the other three gp43 sites are likely to be the result of RNA-induced conformational changes in the protein.

For some of the PKA targets that we introduced into RB69 gp43, we observed no effect by the RNA on phosphorylation of the respective gp43 construct. One of the unaffected sites, 295pGRAS295 (Fig. 2), is located in the C-terminal tail segment of this 903-residue polymerase (i.e. in a gp43 segment known to bind the phage-induced polymerase processivity factor gp45 (sliding clamp protein) (10)). As also shown in Fig. 2B, and perhaps as expected, phosphorylation of the 295pGRAS295 site is inhibited by purified RB69 gp45. The addition of operator RNA did not alter the inhibitory effect by RB69 gp45, suggesting that gp43-operator interactions do not overlap, or affect, the gp43 determinants for binding the sliding clamp. Interestingly,

[Diagram of RNA structures]

**DNA Polymerase Determinants of RNA Binding**

Fig. 1. The T4 and RB69 RNA substrates used in this study. The RNA substrates were prepared by *in vitro* transcription of cloned synthetic DNA duplexes as described under “Experimental Procedures.” Structure of the RNA hairpin for the T4 operator has been inferred from genetic, phylogenetic, biochemical (RNase sensitivity), and NMR studies (2, 5, 31, 32). Existence of the RNA hairpin for RB69 has been inferred from comparative studies with the T4 counterpart (6, 12). Position of nucleotides on the RNA sequences shown are marked in relation to the start codons of the respective mRNAs. Cloning vector-derived nucleotides in RNA products of *in vitro* transcription are displayed in lowercase letters.

phosphor imager (Fuji model FLA-3000). RNA stocks were stored at −80 °C and used within 3–4 days of preparation for radiolabeled RNAs or 4–5 weeks of preparation for unlabeled RNAs.

**RNA-Protein Cross-linking Methods**—The methods we used to identify RNA-linked gp43 peptides (Figs. 3 and 4) were based on the demonstrated utility of methylene blue as an enhancer of photocross-linking between proteins that bind specific RNA and base-paired residues in the RNA (15) as well as the utility of iodosubstituted pyrimidines as chromophores (16). In our experiments, we used 5 ng of methylene blue (catalog no. M9140; Sigma) per pmol of RNA. The reaction mixtures for photocross-linking contained 2000 μM methylene blue, 0.2% Coomassie Brilliant Blue R-250 (made in 50% methanol) and 5% DMSO. The reaction mixtures were subjected to both RNase and protease treatments following photocross-linking.

**Limited Proteolysis of RB69 gp43 with Clostripain**—In experiments that assessed the effect of operator RNA on hydrolysis of RB69 gp43 by clostripain (protein protection by unmodified RNA; see Fig. 4B), 2.5 μg of the protein were incubated for specified time periods at 25 °C with 0.25 μg of preactivated clostripain in the presence or absence of 0.5 μg of RB69 RNA in 25 μl of solution containing 20 mM sodium phosphate (pH 7.5), 1 mM CaCl₂, and 20 μg/ml *E. coli* RNA. Proteolysis was arrested by the addition of 25 μl of electrophoresis sample buffer containing 2% SDS and 0.1 M mercaptoethanol. Then this solution was heated in a boiling water bath for 2 min, and aliquots were subjected to SDS-PAGE (10% gel) in duplicate sets. One set was stained for protein with Coomassie Brilliant Blue, and the other set was transferred to a polyvinylidene difluoride membrane for subsequent N-terminal sequencing of the separated peptides, as described above for samples that were subjected to both RNase and protease treatments following photocross-linking.

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the three sites whose phosphorylation is stimulated by operator RNA (Fig. 2) are clustered in the vicinity of a gp43 region that has been proposed to interact with the phage-induced single-strand-binding protein, gp32 (8); however, it is not yet known if operator RNA can affect gp32-gp43 interactions.

Specific Cross-linking between RB69 gp43 and the RB69 Operator—Recently, we demonstrated that iodocytosine-substituted RNA targets corresponding to the T4 and RB69 operator hairpins can be photocross-linked to the respective gp43 at high efficiency (up to 50%) in the presence of the dye methylene blue.2 Such cross-linking between proteins and RNA or DNA is known to be specific to base-paired regions of the nucleic acid (15). We also showed that the base-paired C at position −14 of RBop4 RNA (Fig. 1) can be photocross-linked to T4 gp43 as well as to RB69 gp43.2 In Fig. 3A, we compare the levels of gp43-RNA photocross-linked (XL) products obtained in experiments that utilized the two types of operator RNA substrates diagrammed in Fig. 1 as ligands for wild-type RB69 gp43 and T4 gp43. Conditions for photocross-linking were optimized for maximal yields of XL products, as described elsewhere,2 in order to attempt to isolate these products for further analysis. We also examined photocross-linking of the same RNA targets with an in-frame deletion mutant of T4 gp43 (T4 DelM; Fig. 3A) that we had previously observed to possess the specific RNA-binding activity (7). Although we have been able to visualize products of photocross-linking between gp43 and operator RNA substrates from both the T4 and RB69 phage systems, we have only been able to subsequently analyze products containing RB69-derived components. For unknown reasons, cross-linked products between RB69 gp43 and RBop4 could be solubilized in buffer lacking denaturants (SDS or urea), whereas similar products containing T4 gp43 were not similarly soluble. Fig. 3B shows electrophoretic separations of the XL products we used in the analyses described below.

In one type of analysis, photocross-linked material from the mixture of RB69 gp43 and RBop4 RNA (XL in Fig. 3B, lane 2) was incubated with a mixture of RNases T1 and A to effect complete hydrolysis of the RNA in the complex. Electrophoretic analysis of the hydrolysate (not shown), yielded an estimate of 1 mol of 32P-labeled nucleotide/mol of cross-linked product. This estimate is consistent with our previous findings,2 which also showed that the point of cross-linking is the iodocytosine-5'-diphosphate diol nucleotide at positions −14 to −1 of the RNA target depicted in Fig. 1.

In another type of analysis, a sample of the XL material in Fig. 3B, lane 2, was subjected to partial hydrolysis by clostripain, in addition to the complete prior hydrolysis of the RNA component with RNases A and T1 (B, lane 3). Methods are described under “Experimental Procedures.”

![Fig. 2. Effect of operator RNA on phosphorylation of markers in RB69 gp43. RB69 gp43 mutants bearing specific PKA phosphorylation sites were constructed, purified, and phosphorylated as described under “Experimental Procedures.” A, effects of RBop4 RNA (opRNA) on phosphorylation of these sites; “Cleft T” refers to the N-exo interdomain groove that binds the template DNA strand (see Fig. 6). B, the inhibitory effect of RB69 gp45 on phosphorylation of PKA sites whose phosphorylation is stimulated by operator RNA (Fig. 2) are clustered in the vicinity of a gp43 region that has been proposed to interact with the phage-induced single-strand-binding protein, gp32 (8); however, it is not yet known if operator RNA can affect gp32-gp43 interactions.](Image 322x405 to 540x728)

![Fig. 3. An analysis of gp43-operator interactions by methylene blue-mediated photocross-linking. A, comparison of photocross-linked products from several experiments that utilized different RNA substrates with either T4 gp43 or RB69 gp43 (lanes 1–4). Lanes 5 and 6 of A compare products of photocross-linking from experiments that utilized a deletion mutant of T4 gp43 (T4 DelM) that retains specific RNA-binding activity (7). B, lanes 1 and 2, separation of products of photocross-linking (on a urea gel) from an experiment that utilized RB69 gp43 and an iodocytosine-substituted RBop4 RNA substrate. B, lane 1, shows results from analysis of an RNA sample that was mixed with methylene blue and no protein. B, lane 2, the products of cross-linking after irradiation of the mixture with visible light in the presence of RB69 gp43 and methylene blue. B, lane 4, separated (on SDS gel) products of partial proteolysis of the XL material by clostripain after complete prior hydrolysis of the RNA component with RNases A and T1 (B, lane 3). Methods are described under “Experimental Procedures.”](Image 51x495 to 296x728)
DNA Polymerase Determinants of RNA Binding

Mutational Analysis of RNA Binding by RB69 gp43—Since all of the PKA site-bearing RB69 gp43 constructs we have described in this report (Fig. 2) were observed to bind operator RNA normally, we presume that none of the amino acid positions that were altered in these constructs are critical for operator recognition. We have targeted additional RB69 gp43 residues for mutational analysis of this protein, focusing on amino acid residues that have been implicated in utilization of primer-template DNA and nucleotide precursors for DNA replication (9, 13). Alkaline-scanning mutagenesis did not reveal any effects on the translesion repressor function of RB69 gp43 when residues in the polymerase and 3'-exonuclease catalytic centers of this protein were substituted with alanine. Examples of results are shown in Fig. 5, and a summary of the analysis of a larger set of RB69 gp43 mutants is presented in Table II. We have not yet encountered any single (or double) amino acid substitutions in gp43 that eliminate RNA binding without also affecting some aspects of the RNA replication function of the protein. In contrast, replication defective gp43 mutants that bind operator RNA normally are common and typically exhibit dominant lethal phenotypes when tested in plasmid-phage complementation assays (Table II). This phenotype ensues when a plasmid-encoded mutant gp43 represses the biosynthesis of wild-type gp43 from a T4 or RB69 phage that infects the plasmid-bearing E. coli host.

DISCUSSION

Fig. 6 summarizes our findings from the current study on a reproduction of the RB69 gp43 structure that appeared in the work of Franklin et al. (9). In this figure, we have highlighted the locations of selected landmarks on the ribbon diagram of the gp43 structure and also included a representation of the RNA hairpin structure for the gene 43 translational operator linking, but without the addition of methylene blue and without exposing the mixtures to high intensity visible light. Samples were then subjected to partial hydrolysis with clostripain, and the products of hydrolysis were resolved by SDS-PAGE. The results, shown in Fig. 4B, revealed a protection of the protein from the protease by RBop4 RNA. Three peptide fragments (~80, ~50, and ~28 kDa, respectively) were detected by Coomassie Blue staining of products from incubations containing tRNA that were either seen at much lower levels or not observed with treatments in the presence of RBop4. N-terminal sequencing of the 28- and 50-kDa proteolytic products recovered from gels indicated that cleavage of the unprotected RB69 gp43 occurred at Arg249 (Fig. 4B). The cleavage at Arg707 (or possibly Arg719) is inferred from the estimated peptide fragment sizes. In the crystal structure of RB69 gp43 (8), all three of the putative RBop4-protected sites map on the face of the protein molecule opposite from the location of the fingers domain (which includes amino acid residues 471–572 of RB69 gp43; Fig. 4A). In other words, the results of this experiment implicated the two palm domain peptide segments highlighted in Fig. 4A in the cross-link with operator RNA. The results also suggest that access to clostripain of the gp43 sites near amino acid positions Arg249 and Arg707 (or Arg719) is diminished in the presence of operator RNA. Arg249 resides in the RB69 gp43 N-exo interdomain groove, which binds the single-stranded DNA template, and Arg707/Arg719 reside in the pol-thumb interdomain groove, which binds double-stranded DNA (8, 9). We note that results of the protease sensitivity assays contrast with results of experiments that used PKA as a gp43-modifying reagent (Fig. 2), where no RNA-mediated inhibition of phosphorylation by PKA was observed near Arg249. The difference in responses may be related to differences in dimensions of the two gp43-modifying enzymes used, which could affect their access to Arg249.

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FIG. 4. A, amino acid sequence of cross-linked 29-kDa peptide. Nine N-terminal residues of the 29-kDa peptide produced in the experiment for Fig. 3B, lane 4, were sequenced (see "Experimental Procedures"). The rest of the sequence was inferred from the approximate size of the peptide and cleavage site specificity of clostripain. Boldface residues are identical to corresponding positions in T4 gp43 (7). Shaded segments were implicated in the cross-link with RNA (see "Results"). Italicized residues correspond to a segment of the fingers domain that can be deleted (DelM mutants; Fig. 3) without loss of the RNA-binding function. Underlined residues were substituted with alanine and tested for biological activity in plasmid-phage complementation assays as described previously (Ref. 12; see Table II and Fig. 5). B, RNA-mediated protection of RB69 gp43 from cleavage with clostripain. The upper part shows electrophoretic separation (SDS-PAGE) of products from incubations of RB69 gp43 with clostripain in the presence (lanes 7–9) and absence (lanes 1–6) of RBop4 RNA (see "Experimental Procedures"). Interpretations of the results are diagrammed in the lower part, with the photocross-linked 29-kDa gp43 fragment marked in the middle of the bar representing the length of the RB69 gp43 molecule. The cleavage at position Arg249 was determined by N-terminal sequencing of the ~50-kDa fragment. The cleavage at Arg249/Arg719 was inferred from the sizes of peptide products separated by SDS-PAGE after partial hydrolysis with clostripain and the specificity (Arg-X) of this protease.

Table II. Time (min) 1 2 4 8

| RBop4 RNA | + | + | + | + |
|-----------|---|---|---|---|
| gp43      | - | + | + | + |
| clostripain | + | + | + | + |
| heavy chain | + | + | + | + |
| Size marker (kDa) | 116 | 97.2 | 66.4 | 55.5 |

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Phages T4 and RB69 are phylogenetically related to each other and encode similar sets of DNA replication proteins from similarly organized and regulated genes on the two phage genomes (7, 23–25). Nevertheless, several of the homologous gene products from the two phage systems can be distinguished from each other, not only by sequence, but also on the basis of biological specificity of the respective gene functions (7, 12, 25). In particular, the T4 and RB69 DNA polymerases, which are ~61% identical (~74% similar) in amino acid sequence (7), differ in their relative abilities to service genomes of the two phages when tested in cross-species complementation assays (12). On the other hand, although T4 gp43 appears to be more narrowly specific toward its own genome and T4 genome products, the RB69 enzyme can replicate T4 genomes and interact with the T4 gene 43 translational operator just as effectively as T4 gp43 (7, 12, 13, 26).2 Also, a variety of biologically active RB69-T4 gp43 chimeras can be constructed through segmental exchanges between the two related proteins (7, 12). Thus, we presume that the structures of T4 gp43 and RB69 gp43 are very similar to each other in the global context but differ at the atomic level, especially with regard to the protein determinants that recognize the specificity elements of translational operators. Unlike operator RNA, primer-template DNA appears to be recognized solely through nucleotide sequence-independent interactions (9, 10). In vitro, the affinity of T4 gp43 to operator RNA ($K_d = 1–2$ nm) is $30–100$-fold higher than to DNA or RNA of nonspecific sequence ($K_d = 70–100$ nm) (4, 6, 12). In addition, the specific RNA is a potent inhibitor of DNA binding by the protein (4), suggesting that the RNA and DNA binding activities of gp43 are functionally linked to each other. Based on the work described here, we surmise that the inhibition of gp43-DNA binding by operator RNA is mediated through two types of effects, RNA-induced conformational changes in the protein and direct occlusion of some DNA-binding sites by the specific RNA. RNA-induced effects on global structure of the protein are perhaps best reflected in the results of experiments that utilized protein phosphorylation markers in RB69 gp43. The phosphorylation of some of these markers was stimulated by operator RNA (Fig. 2; see also Fig. 6), suggesting that the respective gp43 sites were made more accessible to PKA through the gp43-operator interaction. Also, our photocross-linking studies (Fig. 3A) do not directly implicate the DNA binding grooves of this DNA polymerase in operator binding (Fig. 6). Thus, it is possible that occlusion of DNA-binding sites by operator RNA occurs entirely as a consequence of RNA-induced effects on the structure of RB69 gp43. Interestingly, these effects by the RNA do not seem to interfere with interactions between the polymerase and its sliding clamp (Fig. 2).

The level of amino acid sequence identity between T4 gp43 and RB69 gp43 (~61%) contrasts with the 90–94% identity that has been observed among the catalytic subunits of the pol $\alpha$-like DNA polymerase $\delta$ in mammals and ~58% identity between the mammalian $\delta$ subunits and their counterparts in Drosophila. Also, we note in comparison that the pol I-like (A-family) replicative DNA polymerases of phages T7 and T3 are ~97% identical. These comparisons (based on alignments from GenBank) may underscore the importance of a conserved RNA binding function in the two diverged (by sequence) gp43 variants we have studied here. The RB69 gp43 structural framework and many of the protein’s amino acid sequence motifs are shared by at least three other B-family DNA polymerases whose crystal structures have also been determined.

![In vivo RNA-binding assay of RB69 gp43 mutants. E. coli CAJ70 cultures (at 2 × 10^6 cells/ml) expressing plasmid-borne mutant RB69 gene 43 (lanes 2–6) or without plasmid (lane 1), were infected with an RB69 gene 43 phage mutant (12) at a multiplicity of infection of 10 and then labeled with [35S]methionine (5 Ci/ml) for 10 min beginning at 10 min postinfection. Samples of infected cultures were subsequently analyzed by SDS-PAGE. Position of the truncated gp43 fragment (gp43f) expressed from the RB69 43sacd mutant is marked with an arrow. The plasmid-encoded RB69 gp43 mutants used to test for repression of phage induced gp43f synthesis carried the following amino acid substitutions, respectively: D621A (lane 2), delM (lane 3), D411A (lane 4), D623A (lane 5), and D621A/D623A (lane 6) from RB69. We propose that the RNA hairpin binds RB69 gp43 within the protein cavity that harbors the intersection between the dNTP binding site and the primer-template junction near the polymerase catalytic center (10, 18–20). RB69 gp43 residues that are essential for dNTP binding and the two catalytic functions of this enzyme do not appear to be essential for operator recognition, since they can be substituted with alanine without loss of repressor activity (Fig. 5 and Table II). Assays for protection of RB69 gp43 from clostripain by operator RNA (Fig. 4B) suggest that the interaction with specific RNA affects access of the protease to sites in the binding grooves for both double-stranded DNA (near Arg$^{227}$; Fig. 6) and single-stranded DNA (near Arg$^{248}$; Fig. 6) of this DNA polymerase. Previously, in binding studies that utilized T4 gp43 and a variety of RNA targets, we estimated that an RNA length of at least 26 nucleotides of nonspecific sequence, in addition to the sequence-specific hairpin (~18 nucleotides), is included in the gp43-operator complex during translational repression (6). An RNA length of ~26 nucleotides 3’ distal to the operator RNA hairpin structure may correspond to as much as 88 Å, which would be sufficient to reach any other part of the gp43 molecule if the RNA hairpin were to be anchored in the region bounded by the sites mapped in our current study. Thus, it is possible that all five domains of this protein contribute determinants for RNA binding. In essence, we can rule out the possibility that gp43 harbors a structural domain exclusively for the RNA binding function, although our photocross-linking studies implicate the enzyme’s palm domain in contacts with the base-paired segment of the RNA hairpin component of the operator (Fig. 4). Possibly, the palm domain bears a cluster of determinants for RNA sequence recognition.

The multiplicity of protein-RNA contacts (this work) and degeneracy of RNA sequences that can be repressed by a gp43 (5, 6) make it impractical to probe a multifunctional protein of this size for mutations that selectively or preferentially affect the RNA binding function. Nevertheless, it has been possible to demonstrate that knocking out the catalytic activities of the RB69 gp43 does not knock out either the RNA or DNA binding function of this protein (Table II). In contrast, all of the known T4 gp43 missense mutants that exhibit defects in RNA binding (i.e. exhibit derepressed gp43 synthesis) appear to be also defective in aspects of the DNA binding function (e.g. fidelity of DNA synthesis or protein stability (21, 22)). A crystal structure of the gp43-RNA complex would be helpful in establishing whether DNA (or nonspecific nucleic acid) and operator RNA share contact points on the protein but may be insufficient to elucidate dynamic aspects of the gp43-operator interaction.
The high degree of divergence between the mRNA targets for T4 gp43 and RB69 gp43,2 in contrast to conservation of the gp43 structural framework in nature (12, 27–29), may mean that most gp43-like DNA polymerases have had opportunities to evolve specific RNA binding functions. It will be important to find out whether these other polymerases interact with naturally occurring specific RNA ligands and whether such ligands have diverse physiological roles or are always associated with translational control.

TABLE II

| Structural domain | Residues replaced by alanine | Location of residues | Replication function | Repressor function | Other phenotypes |
|-------------------|-----------------------------|----------------------|---------------------|--------------------|-----------------|
| N                 | Lys48                        | Exo catalytic center | +                   | +                  | Mutator<sup>a</sup> |
|                   | Tyr49                        |                      | +                   | +                  | Mutator<sup>a</sup> |
|                   | Asp222, Asp327               | dNTP-binding pocket  | +                   | +                  | Mutator<sup>a</sup> |
| Fingers           | Lys560                       | PT junction          | –                   | –                  |                 |
|                   | Asp563, Tyr567               |                      | –                   | –                  |                 |
| Palm<sup>b</sup>  | Phe395, Val396               | Pol catalytic center | –                   | +                  | Dominant negative |
|                   | Asp411, Asp421               |                      | –                   | +                  | Dominant negative |
|                   | Asp482, Asp563               |                      | –                   | +                  | Dominant negative |
| Thumb<sup>b</sup> | Lys705, Arg707, Tyr708, Asn711, Asp714, Met715, Arg719 | Double-stranded DNA-binding | –                   | +                  | Dominant negative |

<sup>a</sup> Ref. 13.
<sup>b</sup> Alanine substitutions at the following residues in the palm and thumb domains were also tested and observed to have normal replication and repressor function: Glu614, Tyr619, Glu716, and Thr718.
<sup>c</sup> Partial repression of mRNA

![Fig. 6. Representations of the structures of RB69 gp43 (multicolored ribbon diagram to the left; from Ref. 9) and the operator hairpin for RB69 gene 43 (blue diagram to the right). Orientations of the five gp43 domains (N, exo, thumb, palm, and fingers) are shown in the “closed” form of the protein structure (9). The primer (yellow) and template (gray) DNA strands are shown occupying the Palm-Thumb interdomain groove (for double-stranded DNA) and the N-Exo interdomain groove (for the single-stranded DNA template). Marked onto the gp43 structure are the approximate locations of sites protected from clostripain by operator RNA (Arg249 and Arg707; Fig. 4), the polymerase catalytic residues (pol active site) and the locations of putative RNA-binding motifs (RNP1 and RNP2) of the palm. The fingers (blue) domain of RB69 gp43 protrudes behind the structure (relative to the orientation shown in the figure) and includes the dNTP-binding residues near the fingers-palm junction (9, 20). Also marked are the PKA target sites that we engineered (Table I) to test for gp43 phosphorylation in the presence and absence of operator RNA (Table II). For these sites, the effect of operator RNA on PKA-mediated phosphorylation is marked either in green (for stimulation), red (for inhibition), or blue (for lack of an effect).](image-url)
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binding proteins (16). Recently, it was pointed out that the corresponding region in the N-terminal domains of the archaeal polymerases also bear this architectural feature (28, 29). Whereas these observations are intriguing, it remains to be seen whether the archaeal enzymes also bind RNA or whether the putative rGMP binding region in RB69 gp43 is involved in RNA binding by this protein. Amino acid substitutions that disrupt the putative RNP motif of the N domain do not seem to affect gp43-operator interactions (K48A, Y49A, and D95A; Table II). We have examined the structure of RB69 gp43 for additional clues to the existence of protein folds that resemble the RNA-binding motifs of other proteins that bind specific RNA. In Figs. 4 and 6, we point out the approximate location of RNA binding by this protein. Amino acid substitutions that the putative rGMP binding region in RB69 gp43 is involved in the putative RNP2 structural arrangement usually occurs within modules consisting of 30–40 amino acids (30), whereas the RNP1- and RNP2-like sequences of the gp43 palm are separated by ~200 residues, including the entire fingers domain of this polymerase (Fig. 4). Amino acid substitutions in RB69 gp43 that disrupted the similarity to the RNP1 consensus did not affect the replication and repressor functions of the protein (E614A and Y619A; Table II). Mutations in the RNP2-like sequence of RB69 gp43 knocked out both the replication and repressor functions of the protein (F395A and V396A; Table II). There are yet additional folds in RB69 gp43 that exhibit similarity to RNA binding motifs. In particular, a structural motif in a looped segment of the thumb domain (residues 711–720) resembles motif E of some RNA replicases and protrudes within 10–18 Å of the pol catalytic center of RB69 gp43. A crystal structure of the gp43-operator complex is needed to shed more light on the relevance of these putative RNA-binding motifs to translational operator recognition by this DNA polymerase.

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