RegA proteins from phage T4 and RB69 have conserved helix–loop groove RNA binding motifs but different RNA binding specificities

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ABSTRACT

The RegA proteins from the bacteriophage T4 and RB69 are translational repressors that control the expression of multiple phage mRNAs. RegA proteins from the two phages share 78% sequence identity; however, *in vivo* expression studies have suggested that the RB69 RegA protein binds target RNAs with a higher affinity than T4 RegA protein. To study the RNA binding properties of T4 and RB69 RegA proteins more directly, the binding sites of RB69 RegA protein on synthetic RNAs corresponding to the translation initiation region of two RB69 target genes were mapped by RNase protection assays. These assays revealed that RB69 RegA protein protects nucleotides –9 to –3 (relative to the start codon) on RB69 *gene 44*, which contains the sequence GAAAAUU. On RB69 *gene 45*, the protected site (nucleotides –8 to –3) contains a similar purine-rich sequence: GAAAUA. Interestingly, T4 RegA protein protected the same nucleotides on these RNAs. To examine the specificity of RNA binding, quantitative RNA gel shift assays were performed with synthetic RNAs corresponding to recognition elements (REs) in three T4 and three RB69 mRNAs. Comparative gel shift assays demonstrated that RB69 RegA protein has an ~7-fold higher affinity for T4 *gene 44* RE RNA than T4 RegA protein. RB69 RegA protein also binds RB69 *gene 44* RE RNA with a 4-fold higher affinity than T4 RegA protein. On the other hand, T4 RegA exhibited a higher affinity than RB69 RegA protein for RB69 *gene 45* RE RNA. With respect to their affinities for cognate RNAs, both RegA proteins exhibited the following hierarchy of affinities: *gene 44* > *gene 45* > *regA*. Interestingly, T4 RegA exhibited the highest affinity towards RB69 *gene 45* RE RNA, whereas RB69 RegA protein had the highest affinity for T4 *gene 44* RE RNA. The helix–loop groove RNA binding motif of T4 RegA protein is fully conserved in RB69 RegA protein. However, homology modeling of the structure of RB69 RegA protein reveals that the divergent residues are clustered in two areas of the surface, and that there are two large areas of high conservation near the helix–loop groove, which may also play a role in RNA binding.

INTRODUCTION

The bacteriophage T4 RegA protein controls the level of expression of a group of T4 early genes at the level of translation (1). It has been shown that RegA protein inhibits translation by competing with ribosomes for binding to the translation initiation region (TIR) of specific mRNAs (1–4). The fact that RegA protein binds to 15–30 different mRNAs including its own gene, *regA*, and distinguishes them from the numerous other T4 RNAs concurrently present in the cell makes it an exceptional paradigm for understanding protein–RNA recognition (1,5). Comparison of 12 known RegA-regulated mRNAs indicates that the TIRs are AU-rich, but they do not contain a highly conserved nucleotide sequence nor apparent similar secondary structures (6,7). However, the finding that the target mRNAs vary in their sensitivity to RegA repression *in vitro* (1,3) and *in vivo* (8) is consistent with the nucleotide variations present in the TIRs. The studies of Brown *et al.* (9) in which the systematic evolution of ligands by exponential enrichment (SELEX) technique was used to identify RNAs preferentially bound by RegA protein, suggest that an optimal target for RegA protein is 5′-AAAAUUGUUAUGUAA-3′. Previous studies (6) of the RegA recognition element (RE) in T4 *gene 44* indicate that in *gene 44* mRNA the binding site is a single-stranded sequence of 11–12 nt, which includes the Shine–Dalgarno region and an AU-rich element, which has some similarity to the SELEX high affinity RNA (specifically, the AAAUU sequence).

RB phage are T4-related bacteriophage that contain hydroxymethylated cytosine, which can complement some T-even phage mutations (10). To identify functional domains in RegA protein, Miller and Jozwik (11) examined the *regA* gene in a number of T-even and RB phages. They found that RegA protein was identical in all the phages examined (11) except for the distant phylogenetic relative RB69, where the encoded RegA protein was 22% divergent from T4 RegA protein (12). In T4 *regA⁻* infections of cells harboring plasmids carrying RB69 *regA*, RB69 RegA protein was found to be capable of repressing T4-encoded mRNAs, including T4 *gene 44*, *gene 45* and *rpbA* mRNAs (12). In two-plasmid assays in which RB69

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regA and T4 *gene 44*, *gene 45* and *rpbA* were expressed *in trans*, RB69 RegA protein appeared to repress the three T4 genes, and protein synthesis in general, more efficiently than T4 RegA protein (12).

Although RB69 RegA represses T4 mRNAs, it is not known if T4 RegA represses homologous RB69 mRNAs. Interestingly, RB69 and T4 DNA polymerases (which are 61% identical) both function as RNA binding, autogenous translational repressors of *gene 43* (13). However, T4 DNA polymerase binds only T4 *gene 43* mRNA, while RB69 DNA polymerase can bind and repress operator RNAs from both phages (13). Given this difference in RNA binding specificity in the homologous T4 and RB69 DNA polymerases, we wondered whether similar differences in RNA discrimination exist in the phage RegA proteins. Also, since T4 and RB69 RegA proteins are 22% dissimilar in amino acid sequence and they appear to exhibit differences in RNA binding properties (12), the correlation of structural and functional properties of the two proteins may yield new insights into how RegA protein recognizes specific RNAs.

As a first step in probing potential structure–function differences between RB69 and T4 RegA proteins, we have examined the *in vitro* RNA binding properties of the two phage proteins. The RB69 RegA protein binding site has been mapped on RB69 *gene 44* and *gene 45* mRNAs by RNase protection assays and compared to the known T4 RegA binding sites on the respective T4 mRNAs (6,14). The affinities of the two RegA proteins for synthetic RNAs corresponding to both T4 and RB69 REs were then measured. These studies revealed that T4 and RB69 RegA protein bind to the same sites on RB69 *gene 44* and *gene 45* RNAs. However, the two proteins differ in their overall RNA binding affinities and in their RNA binding specificities. To explore structural relationships in the proteins, molecular modeling has been used to predict the structure of RB69 RegA protein, based on the known structure of T4 RegA protein (15). These models revealed regions of conserved and divergent residues on the surfaces of the two proteins that potentially may contribute to the observed differences in RNA binding specificities and affinities of these proteins.

MATERIALS AND METHODS

Reagents and strains

Oligodeoxyribonucleotides were synthesized on an Expedite (Model 8909) nucleic acid synthesizer by the MUSC Oligo synthesis facility. Oligoribonucleotides were synthesized by the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT). *Escherichia coli* AR120 (λ cI+, N+) was obtained from A.Shatzman (Smith Kline Beecham Pharmaceuticals). RNase A and RNase I were purchased from Ambion.

Cloning of RB69 RegA protein

Construction of the pAS1 vector containing T4 *regA* was described previously (3). The RB69 *regA* gene was subcloned from pEM141 (12) into pAS1 by PCR amplification of *regA* and ligation into *Bam*H1/*Sal*I cleaved pAS1. The primers used for sub-cloning RB69 *regA* into the pAS1 vector contained the following sequences: forward, 5′-CGC GGA TCC GGA ATG

GTA AAA TGA TTG AAA TTA AAT TG-3′; reverse: 5′-CGC GTC GAC CCA TTG CTT TAA TTA CCA ATT GTA TAT TTT GC-3′. Verification that the cloned *regA* gene was wild-type (WT) was obtained by DNA sequence analysis of the entire *regA* coding region.

RegA protein purification

WT T4 RegA protein was purified from AR120 cells containing the pAS1-*regA* plasmid following induction of transcription from the phage λ P_L promoter by nalidixic acid treatment, as described previously (16,17). Purified T4 RegA protein was used to generate concentration standard curves for quantitation of RegA protein in cell supernatants (see below). The concentrations of purified RegA protein were determined by triplicate OD_{280} measurements or amino acid analyses (W. M. Keck Foundation).

Preparation of induced cell supernatants

Cell supernatants were prepared by the freeze–thaw method of Johnson and Hecht (18), with slight modifications, as described previously (19). Cell supernatants were stored in 80 µl aliquots at –20°C. Evaluation of the protein content of both the supernatant and the pellet resuspension was carried out by SDS–PAGE. RegA protein concentrations in cell lysates were determined by quantitation of protein fluorescence in gels stained with Sypro Orange (Molecular Probes) compared to a standard curve of known concentrations of purified RegA protein, using a Molecular Dynamics Storm imager. Protein concentrations had an error range of 1–16% (average of 7%).

RNA purification

Synthetic oligoribonucleotides were deprotected by treatment with tetra butyl ammonium fluoride (TBAF) overnight (20) and then purified by perfusion chromatography using Poros™ HQ and R1 columns (PerSeptive Biosciences) (20). RNA purity was assessed by analysis of ^{32}P-labeled RNA on 6% polyacrylamide gels. For RNase footprinting and gel shift assays, purified RNAs were 5′-^{32}P-end-labeled by treatment with T4 polynucleotide kinase and [γ-^{32}P]ATP (21).

RNase footprinting

Purified 18mer RB69 *gene 44* RE RNA (5′-AUGAGGAAAA-UUACAUGA-3′) and *gene 45* (5′-UGAAAGGAAAUAAA-AUGA-3′) RE oligomers were 5′-^{32}P-end-labeled and then repurified by 6% polyacrylamide gel electrophoresis. ^{32}P-RNAs (10 nM; 20 000 c.p.m.) were incubated with cell supernatants containing 20 and 50 nM T4 and RB69 RegA proteins. RNase A digestions were carried out in 10 µl of 100 mM Tris–HCl pH 6.5, 1 mM EDTA, at 4°C for 5 min at an enzyme concentration of 10^{-7} U/µl. RNase I digestions were performed in 10 µl of 10 mM Tris–HCl pH 8.0, 50 mM NaCl, at room temperature for 15 min at an enzyme concentration of 0.01 U/µl. Following enzymatic digestions, equal volumes (10 µl) of gel loading buffer (95% formamide, 0.05% xylene cyanol and bromophenol blue) were added to each reaction and samples were heated at 75°C for 3 min. RNAs were then electrophoresed on 15% polyacrylamide–8 M urea gels. Following electrophoresis, gels were fixed in 10% acetic acid–10% methanol for 1 h and dried. RNA fragments were visualized by

phosphorimaging using a STORM™ imager (Molecular Dynamics).

RNA gel mobility shift assays

^{32}P-labeled RNAs (10 nM) were incubated with increasing volumes of induced cell supernatants to generate titration curves. Binding was carried out in 10 mM Tris–HCl pH 7.5, 50 mM NaCl and 1 mM EDTA at 4°C for 15 min. Binding reactions were performed in 10–20 µl final volume with concentrations of 2.5–40 nM RegA protein. A freshly thawed aliquot of cell supernatant was used for each experiment and then discarded. Reaction products were analyzed by electrophoresis on a native 8% polyacrylamide gel in 0.5× TBE (89 mM Tris, 89 mM boric acid, 4 mM EDTA, pH 8.3) at 4°C. Gels were dried and analyzed by autoradiography and ^{32}P-RNA was quantitated by phosphorimaging on a Molecular Dynamics Imager Model 425.

Apparent association constants (K_{app}) for mutant proteins were determined from gel shift assays in a manner similar to that of Rebar and Pabo (22). Association constants were calculated from PhosphorImager data as follows:

$$K_{app} = [\text{protein:RNA complex}]/([\text{protein}_f] \times [\text{RNA}_f])$$

where [protein:RNA complex] = fraction ^{32}P-RNA bound × [total RNA]; [RNA$_f$] = fraction ^{32}P-RNA free × [total RNA]; and [protein$_f$] = [total protein] – [bound protein] (assuming one protein per bound RNA) (17).

K_{app} was calculated at four points on the titration curve. The mean of the four values was calculated and K_{app} values from two to four experiments were averaged. Standard deviations (calculated using the non-biased or 'n – 1' method using Excel) ranged from K_{app} (Av)/2.9 to K_{app} (Av)/9.5.

RESULTS

Overexpression of RB69 RegA protein

To facilitate *in vitro* studies of the RNA binding properties of RB69 RegA protein, plasmids were constructed which overexpress RB69 RegA protein. For this purpose, the RB69 *regA* gene was subcloned from pEM141 (12) that expresses low levels of the protein, into the pAS1 vector (23), which has been used previously for inducible high level expression of T4 *regA* (16). In the resulting construct, pAS1-RB69 *regA*, the *regA* gene was positioned downstream from the strong λ P$_L$ promoter and the λ *cII* gene AUG codon. As shown in Figure 1, in this construct the RB69 *regA* gene is preceded by 15 nt of upstream RB69 *gene 62* sequence. Translation of the P$_L$ transcript is expected to initiate at the *cII* gene AUG codon, then terminate at the *gene 62* termination codon, and finally, reinitiate at the *regA* AUG codon, through translational coupling.

Induction of *regA* expression from pAS1-RB69 *regA* by nalidixic acid treatment resulted in an increased level of *regA* expression, relative to that obtained from pEM141 (12). However, as shown in Figure 2, considerably higher levels of T4 RegA protein are produced from the same vector (pAS1-T4 *regA*). This suggests that some autorepression of RB69 *regA* translation occurs with this construct, since both the T4 and RB69 *regA* vectors use the same promoter and the same efficient translation initiation regions. In an effort to eliminate

A Translation initiation region (TIR) of the λ *cII*-RB69 *regA* fusion gene on pAS1-RB69 regA.

5'-UGUUAUCUA<u>AGGA</u>AAUACUUACAUauggauccggaauggu<u>aaa</u>*AUGAUU* -3'

B   pAS1-RB69 regA plasmids:

Figure 1. (**A**) Sequence of the TIR of RB69 *regA* on the pAS1-RB69 *regA* vector. The *regA* coding region is preceded by the λ *cII* gene ribosome binding site (underlined) and AUG start codon followed by a 15 nt ORF corresponding to the 3′-end of T4 *gene 62* (lower case). Translation initiated at the *cII* AUG will terminate at the *gene 62* stop codon (boxed), and then reinitiate at the *regA* AUG (italic). (**B**) Mutations introduced into the TIR of λ *cII*-RB69 *regA* to increase expression of *regA*. The ribosome binding site and start codons are in bold.
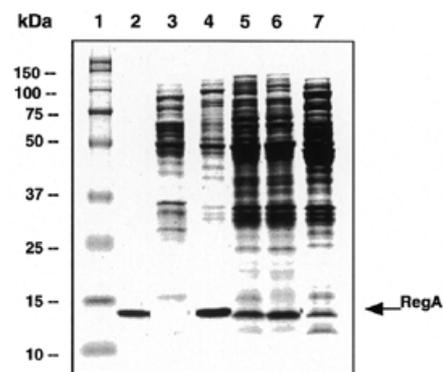

Figure 2. Expression of RB69 and T4 RegA proteins from pAS1 vectors. Total cell extracts from 5 µl of uninduced cell culture (lane 3), 5 µl of nalidixic acid-induced cell cultures expressing T4 RegA protein (lane 4), 10 µl of induced cell cultures expressing RB69 RegA protein from plasmids pG62A (lane 5) (see Fig. 1), pCIIB (lane 6) and WT pAS1-RB69 regA (lane 7), respectively, were applied to a 15% SDS–polyacrylamide gel. Lane 1, molecular weight markers; lane 2, purified T4 RegA protein.

autorepression of RB69 RegA protein, mutations were introduced into the TIR of RB69 *regA* on pAS1-RB69 *regA*. Because RB69 *regA* autorepression could occur through RegA protein occlusion of either the *cII* gene TIR or the *regA* TIR, mutations were introduced into both TIRs (Fig. 1B). In some mutants (e.g. G62A, G62B, CIIA and CIIB), cytosines were substituted for A or U, because RegA-sensitive mRNAs are generally deficient in cytosines. In mutant CIIB, 7 nt between the *cII* gene Shine–Dalgarno sequence and AUG codon were replaced by a 7 nt sequence from the TIR of T4 *gene 43*, which is insensitive to RegA repression. As shown in Figure 2, this mutation (CIIB; lane 6) resulted in a slightly higher level of expression of RB69 RegA than the WT (lane 7) or G62A mutant (lane 5) vectors. However, none of the pAS1-RB69 regA constructs expressed RegA to the level obtained for T4 RegA protein (Fig. 2, lane 4). This finding is consistent with the suggestion of Jozwik and Miller (12) that RB69 RegA

GENE	TRANSLATION INITIATION REGION
Gene44 (T4)	UG<u>**AAUGAGG--AAAUU**</u>--**AUG**AUUACUGUAAAUG
Gene44 (RB69)	AUA<u>AUGAGG</u>A<u>AAAUU</u>AC**AUG**AUUACUAUCAAUU
Gene45 (T4)	AAUUG-AAGGAAAUUAC**AUG**AAACUGUCUAAAG
Gene45 (RB69)	UCUUGAA<u>AGGAAAU</u>AA<u>A</u>**AUG**AAACUGUCUAAAG
regA (T4)	GCAUUGGAAUGGU-AAA**AUG**AUUGAAAUUACUC
regA (RB69)	GCAUUGGAAUGGUAAAA**AUG**AUUGAAAUUAAAU

Figure 3. Nucleotide sequence of the translation initiation regions of three RegA-sensitive mRNAs from T4 and RB69 phage. The binding site of T4 RegA protein on T4 *gene 44*, previously determined by RNase protection assays (3,6), is indicated by shading. The sequences of the oligonucleotides used in this study are indicated by underlining and the initiation codons are in bold type.

Figure 4. Gel mobility shift assay of RB69 RegA protein binding to RE RNAs. ^{32}P-5′-end-labeled 18mer RB69 *gene 44* RE RNA (5′-AUGAGGAAAAUU-ACAUGA-3′) (lanes 1–4), 16mer T4 *gene 44* RE RNA (5′-AAUGAGGAA-AUUAUG-3′) (lanes 5–7) and 16mer T4 *gene 44* $G_{-9}U$ RE RNA (5′-AAU-UAGGAAAUUAUG-3′) (lanes 8–10) were incubated with cell supernatants containing no RegA protein (lane 2) or 20 nM RB69 RegA protein (lanes 3, 4, 6, 7, 9 and 10). Samples were analyzed by electrophoresis on a native 6% polyacrylamide gel. Gels were dried and then analyzed on a PhosphorImager (see Materials and Methods).

protein generally represses translation more efficiently than T4 RegA protein.

Mapping of RB69 RegA protein binding sites on RB69 *gene 44* and *gene 45* RNAs

As noted above, T4 mRNAs vary in their sensitivities to RegA repression *in vitro* (1,3) and *in vivo* (8). Of the T4 genes examined to date, *gene 44* is the most sensitive, while the *regA* gene itself is the least sensitive to RegA repression *in vitro*. Although the T4 RegA protein binding sites on five T4 mRNAs have been mapped (1,3,14), the binding sites of RB69 RegA protein on RB69 mRNAs have not been examined. Comparison of the three RegA-sensitive RB69 genes that have been sequenced to date (11,24) reveals that the sequences of the TIRs are similar, but not identical in the two phage. As shown in Figure 3, relative to the phage T4 TIRs, the RB69 TIRs have three nucleotide insertions in *gene 44*, one nucleotide insertion and two base substitutions in *gene 45* and a single nucleotide insertion in *regA* mRNA. Like the T4 RegA-sensitive mRNAs, the RB69 mRNAs do not appear to contain potential secondary structures in the TIRs.

Given the similarities of the sequences of the RB69 TIRs, it is likely that RB69 RegA protein binds to the same regions on the homologous mRNAs as T4 RegA protein. To test this hypothesis, the ability of RB69 RegA protein to bind to a synthetic RNA corresponding to the TIR of RB69 *gene 44* was examined. For this assay, an 18mer RNA (5′-AUGAG-GAAAAUUACAUGA-3′) corresponding to nucleotides –14 to +4 (relative to the AUG) of RB69 *gene 44* mRNA (24) was synthesized. This sequence spans the 12 nt sequence previously found to be the binding site for T4 RegA protein on T4 *gene 44* mRNA (Fig. 3) (6). To detect binding, RNA gel mobility shift assays were performed using ^{32}P-labeled RB69 *gene 44* RE RNA (RB *gene 44* RE RNA) as a ligand. In these assays, induced cell supernatants containing RB69 RegA protein were used to eliminate the requirements for purification of WT or mutant RB69 and T4 RegA proteins (see below). We have previously demonstrated that RNA binding affinities of WT T4 RegA measured by similar gel shift assays are equivalent to those measured by equilibrium fluorescence quenching assays using purified RegA protein (19). As shown in Figure 4, addition of cell supernatants containing RB69 RegA protein to RB *gene 44* RE RNA produces a shift in the mobility of the RNA (lanes 3 and 4). Cell supernatant containing the parental
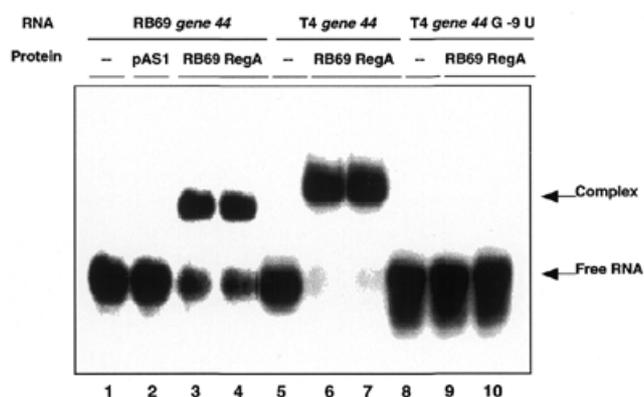
plasmid without regA (pAS1) did not produce a shift in RNA mobility (Fig. 4, lane 2). Also, a similar shift was observed when RB69 RegA was incubated with a 16mer RNA corresponding to the T4 *gene 44* RE (Fig. 4, lanes 6 and 7). This is consistent with the report of Allen and Miller (25) that RB69 RegA-His$_6$ binds a 23 nt RNA containing the RegA binding site of T4 *gene 44*. The specificity of RNA binding was tested by incubating RB69 RegA protein with T4 *gene 44* RE RNA containing a base substitution at nucleotide –9 relative to the AUG. This base substitution reduces the affinity of T4 RegA protein for *gene 44* RE RNA 100-fold (6) and eliminates the ability of T4 RegA to retard the electrophoretic mobility of the RNA. As shown in Figure 4 (lanes 9 and 10), RB69 RegA protein does not bind the $G_{-9}U$ variant of *gene 44* RE RNA, confirming that the mobility shift is specific.

The ability of RB69 RegA protein to bind to RB69 and T4 *gene 44* RE RNAs and the observation that RB69 and T4 RegA proteins can both distinguish between WT and $G_{-9}U$ variant T4 *gene 44* RE RNAs suggests that RB69 and T4 RegA proteins bind to homologous sequences on their target mRNAs. To confirm this, the binding site of RB69 RegA protein on RB69 *gene 44* RE RNA was mapped by RNase protection assays. The 18mer RB69 *gene 44* RE RNA (described above) was digested with *E.coli* RNase I and pancreatic RNase A, in the presence or absence of cell supernatants containing T4 or RB69 RegA protein. As shown in Figure 5A, when RB69 *gene 44* RNA was digested with RNase I (which cleaves non-specifically), cleavages after nucleotides A_{-9} to A_{-4} were protected by both T4 (lanes 4 and 5) and RB69 (lanes 6 and 7) RegA proteins. When RNase digestion was performed in the presence of supernatants from cells lacking RB69 regA (AR120/pAS1), protection was not observed (Fig. 5A, lane 3). When the 18mer RB69 *gene 44* RNA was digested with RNase A (which preferentially cleaves on the 3′ side of U and C residues in pyrimidine–adenine sequences), cleavage after
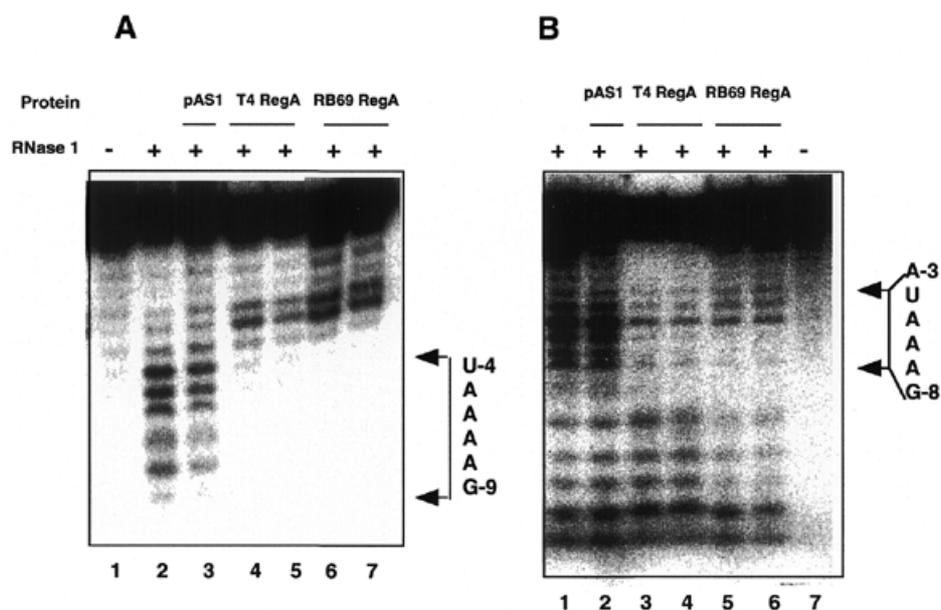
Figure 5. RNase footprint assays of RB69 and T4 RegA protein binding to RB69 *gene 44* and RB69 *gene 45* TIR RNAs. (**A**) RB69 *gene 44* RE RNA (5′-AUGAG-GAAAAUUAC**AUG**A-3′). Lane 1, RNA alone; lanes 2–7, RNA digested with RNase I. Lane 2, RNA digested in the absence of RegA protein; lane 3, RNA digested in the presence of pAS1 cell supernatant (which does not contain RegA protein); lanes 4 and 5, RNA plus cell supernatants containing 20 and 40 nM T4 RegA protein, respectively; lanes 6 and 7, RNA plus cell supernatants containing 20 and 40 nM RB69 RegA protein, respectively. (**B**) RB69 *gene 45* TIR RNA (5′-UGAA-AGGAAAUAAA**AUG**A-3′). Lanes 1−6, RNA digested with RNase I. Lane 1, RNA digested in absence of RegA protein; lane 2, RNA digested in the presence of pAS1 cell supernatant; lanes 3 and 4, RNA plus cell supernatants containing 20 and 40 nM T4 RegA protein, respectively; lanes 5 and 6, RNA plus cell supernatants containing 20 and 40 nM RB69 RegA protein, respectively; lane 7, RNA alone. Reactions in each panel contained 10 nM RNA and 0.01 U/ml of RNase I. RNA fragments were analyzed by electrophoresis on an 8 M urea/TBE gel and detected by PhosphorImager analysis. Nucleotides are numbered relative to the AUG start codon (in bold, above), so that U_{-4} is 4 nt upstream from the initiation A.

nucleotides U_{-3} and U_{-4} were strongly protected, and cleavage after C_{-1} was moderately protected by RB69 RegA protein (data not shown). Taken together these assays map the RB69 RegA binding site to the nucleotide sequence GAAAAUU immediately upstream the AUG start codon of RB69 *gene 44*.

To map the RB69 RegA binding site on *gene 45*, an 18mer RNA corresponding to nucleotides –14 to +4 (relative to the AUG start codon, 5′-UGAAAGGAAAUAAAAUGA-3′) was digested with RNase I. As shown in Figure 5B, addition of RB69 RegA protein (lanes 5 and 6) to RNase I digestion reactions led to protection of cleavages in the region of G_{-8} to U_{-4}, with weaker protection of nucleotide A_{-3}. A similar protection pattern was observed in the presence of T4 RegA protein (Fig. 5B, lanes 3 and 4), suggesting that the two proteins bind to the same general sites on RB69 *gene 45* RNA. Interestingly, the protected sequence in *gene 45*, GAAAUA, is similar to the purine-rich sequence protected in RB69 *gene 44* (GAAAAUU) and both sequences overlap the Shine–Dalgarno sequence of the respective mRNAs.

RNA binding affinities of T4 and RB69 RegA proteins

To compare the relative RNA binding affinities and specificities of T4 and RB69 RegA proteins, quantitative RNA gel mobility shift assays (19) were performed using T4 and RB69 RE RNAs as ligands. For these assays, the concentration of recombinant RegA protein in lysed cell supernatants was determined by quantitation of Sypro Orange stained SDS gels containing known amounts of purified RegA protein as a reference (see

Materials and Methods). Figure 6A shows a representative gel shift titration assay in which 2.5–20 nM RB69 (lanes 8–12) and T4 (lanes 3–7) RegA proteins were pre-incubated with 10 nM T4 *gene 44* RE RNA (5′-AAUGAGGAAAUUAUGA-3′). Products of the binding reaction were separated on a native 6% polyacrylamide gel, and the fraction of ^{32}P-labeled RNA bound by RegA protein was determined by PhosphorImager analysis (see Materials and Methods). These assays demonstrated that the affinity of RB69 and T4 RegA proteins for T4 *gene 44* RE RNA are 34×10^7 M^{-1} and 4.9×10^7 M^{-1}, respectively.

Because RB69 RegA protein has a lower pI value than T4 RegA protein (7.6 versus 8.8), complexes of *gene 44* RE RNA with RB69 RegA protein migrate faster than T4 RegA protein complexes in native polyacrylamide gels (Fig. 6A). This difference in migration rates enables competition assays to be performed with the two proteins. Figure 6B illustrates such a competition assay, in which varying ratios of T4 and RB69 RegA proteins were incubated with T4 *gene 44* RE RNA. At a molar ratio of ~7:1 T4 to RB69 RegA protein, approximately equal amounts of T4 RegA–RNA and RB69 RegA–RNA complexes were formed (Fig. 6B, lane 3). This is consistent with the 6.9-fold higher affinity of RB69 RegA protein relative to T4 RegA protein for T4 *gene 44* RNA measured by quantitative gel shift titration assays (Table 1).

To further explore the RNA binding specificity of the two RegA proteins, the affinities of T4 and RB69 RegA proteins for *gene 44*, *gene 45* and *regA* RE RNAs were measured. For these assays, 18–25 nt ligands were designed that span the
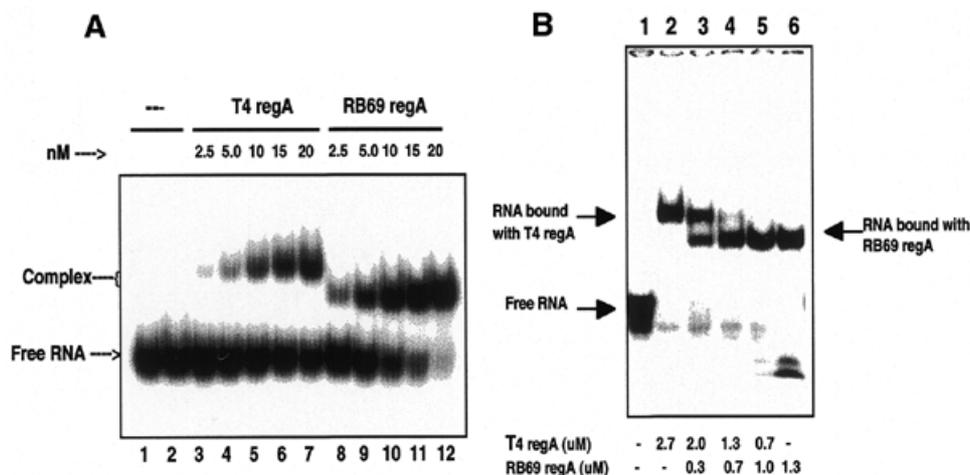
Figure 6. (**A**) Gel shift binding titration of T4 *gene 44* RE RNA with T4 and RB69 RegA proteins. Increasing concentrations of cell supernatants containing T4 or RB69 RegA proteins were incubated with 10 nM ^{32}P-T4 *gene 44* RE RNA. RB69 RegA–RNA complexes migrate faster than T4 RegA–RNA complexes, presumably due to RB69 RegA protein's lower pI value. T4 *gene 44* RE RNA: 5′-AAUGAGGAAAUUAUGA-3′. Samples were analyzed as in Figure 4. (**B**) Competition gel shift assay of T4 and RB69 RegA proteins for T4 *gene 44* RE RNA. ^{32}P-T4 *gene 44* RE RNA (50 nM) was incubated with cell supernatants containing T4 RegA protein (lane 2), RB69 RegA protein (lane 6) or mixtures of T4 and RB69 proteins (lanes 3–5). Lane 1 in (A and B) contains RNA incubated without cell supernatant. Concentrations of RegA proteins in cell lysates were determined by quantitation of Sypro Orange stained gels using a Molecular Dynamics Storm™ imager.

Table 1. Affinities of T4 and RB69 RegA proteins for RE RNAs

RNA name and sequence	T4 RegA [K_{app}[a] ± SD (10^7 M^{-1})]	RB69 RegA [K_{app}[a] ± SD (10^7 M^{-1})]
T4 *gene 44* RE; 5′-AAUGAGGAAAUUAUG-3′	4.9 ± 1.1	34.0 ± 7.0
RB69 *gene 44* RE; 5′-AUGAGGAAAAUUACAUGA-3′	2.5 ± 0.4	9.5 ± 1.0
T4 *gene 45* RE; 5′-UUGAAGGAAAUUACAUGA-3′	3.2 ± 0.9	2.0 ± 0.7
RB69 *gene 45* RE; 5′-UGAAAGGAAAUAAAAUGA-3′	19.9 ± 4.0	1.8 ± 0.4
T4 *regA* RE; 5′-CAUUGGAAUGGUAAAAUGAUUGAA-3′	0.83 ± 0.13	0.16 ± 0.05
RB69 *regA* RE; 5′-CAUUGGAAUGGUAAAAAUGAUUGAA-3′	1.3 ± 0.3	0.6 ± 0.2

[a]K_{app} was determined by quantitative RNA mobility gel shift assays (see Materials and Methods). Binding was performed in 10 mM Tris–HCl pH 7.5, 50 mM NaCl and 1 mM EDTA. Values are the mean of two to four experiments.

RegA protected regions of the TIRs of RB69 mRNAs (described above) and T4 mRNAs (14). The binding affinities of T4 and RB69 RegA proteins for RB69 *gene 44* RE RNA (see above), T4 *gene 45* RE RNA (5′-UUGAAGGAAAU-UACAUGA-3′), RB69 *gene 45* RE (see above), T4 *regA* RE RNA (5′-CAUUGGAAUGGUAAAAUGAUUGAA-3′) and RB69 *regA* RE RNA (5′-CAUUGGAAUGGUAAAAAUGA-UUGAA-3′) were measured by RNA mobility gel shift assays. As illustrated in Figures 6 and 7, these assays revealed that both T4 and RB69 RegA proteins have a preference for their cognate *gene 44* RE RNAs over their cognate *gene 45* RE RNAs (for example, Fig. 7A and B, compare lanes 7–10). Interestingly, RB69 RegA protein has 5- and 7-fold higher affinities than T4 RegA protein for RB69 and T4 *gene 44* RE RNAs, respectively (Table 1). However, T4 RegA protein exhibits a higher affinity than RB69 RegA for *gene 45* RE RNAs (Table 1). Comparison of the affinities of T4 and RB69 RegA proteins for *regA* RE RNAs showed that both proteins have the lowest affinity for T4 *regA* RE RNA (Table 1). In terms of overall RNA binding specificity, T4 RegA exhibited the following hierarchy of affinities: RB69 *gene 45* RE > T4 *gene 44* RE > T4 *gene 45* RE > RB69 *gene 44* RE > RB69 *regA* RE > T4 *regA* RE (Table 2). For RB69 RegA the order of affinities was: T4 *gene 44* RE > RB69 *gene 44* RE > T4 *gene 45* RE > RB69 *gene 45* RE > RB69 *regA* RE > T4 *regA* RE.

Homology modeling of RB69 RegA protein structure

Using structure-guided mutagenesis, the RNA binding domain of T4 RegA protein recently was localized to a helix–loop groove motif in the protein (19). Three residues within helix A (Lys14, Thr18 and Arg21) and one residue on the loop following Helix C (Trp81) were found to play essential roles in RNA binding. Given the differences in the RNA binding affinities of the two proteins, we were interested to see where divergent amino acid residues are located in RB69 RegA protein. We expected that residues that are folded into the core of the protein would be fully conserved, to maintain the overall structure of the protein. Divergent residues would be expected
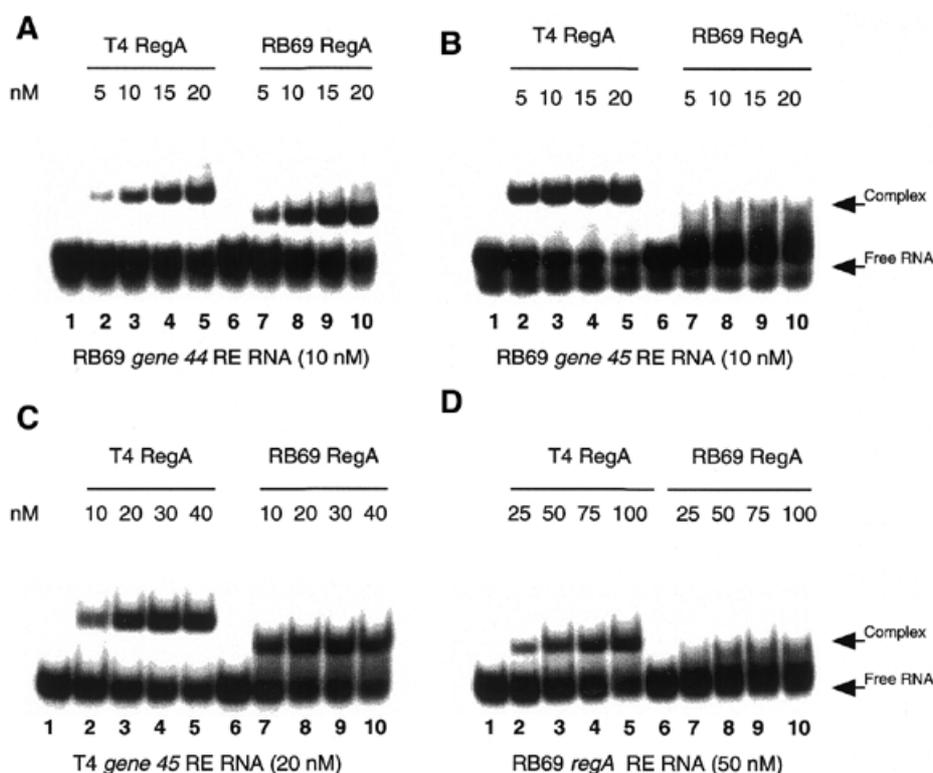
Figure 7. Comparative gel shift assays of T4 and RB69 RegA proteins binding to RB69 *gene 44* (**A**), RB69 *gene 45* (**B**), T4 *gene 45* (**C**) and RB69 *regA* (**D**) RE RNAs. Lanes 1 and 6 in all panels contain RNA incubated without cell supernatants. Lanes 2–5 represent RNA incubated with cell supernatants containing T4 RegA at the indicated concentrations. Lanes 7–10 represent samples containing RB69 RegA at the indicated concentrations. Reactions in (A) and (B) contain 10 nM RB69 *gene 44* and *gene 45* RE RNAs, respectively; samples in (C) contain 20 nM T4 *gene 45* RE RNA; samples in (D) contain 50 nM RB69 *regA* RE RNA. Binding reactions were analyzed by electrophoresis on native 6% polyacrylamide gels.

to be on the surface of the protein, except in the helix–loop groove, which would also be mainly conserved. To determine the potential location of divergent residues, homology-based computer modeling of RB69 RegA protein was performed, using the program SYBYL (Tripos). Interestingly, as shown in Figure 8, the divergent residues in RB69 RegA protein cluster in two separate areas on the surface of the protein, rather than being dispersed across the surface. As illustrated in Figure 8, residues within the helix–loop groove are fully conserved in RB69 RegA protein.

To confirm that the conserved helix–loop groove motif in RB69 RegA serves as the RNA binding domain, a Trp81Ala mutation was introduced in RB69 *regA*. This mutation in T4 RegA protein has been shown to eliminate RNA binding, without significantly disrupting RegA protein conformation (19). As shown in Figure 9, introduction of the Trp81Ala mutation (lanes 3, 6 and 9) eliminated the ability of RB69 RegA to bind any of the three RE RNAs tested.

DISCUSSION

RNA gel mobility shift assays have demonstrated that RB69 RegA protein binds to an 18mer RNA corresponding to the translation initiation region of RB69 *gene 44* and to a 16mer RNA containing the translation initiation region of T4 *gene 44*. Interestingly, RB69 RegA protein, like T4 RegA protein, does

not bind a T4 *gene 44* RNA variant with a $G_{-9}U$ mutation. In addition, RNase protection assays have shown that T4 and RB69 RegA proteins protect the same binding sites on two RB69 RE RNAs. For RB69 *gene 44* RE RNA, the protected site is GAAAAUU (Fig. 5A) which spans nucleotides –9 to –3, relative to the start codon, AUG. For RB69 *gene 45* RE RNA, the protected site is GAAAUA (Fig. 5B), covering the region –8 to –3 relative to the start codon. Interestingly, the protected sequences for both RB69 *gene 44* and *gene 45* mRNAs overlap the Shine–Dalgarno sequence of the mRNAs. Previous studies (6) showed that in T4 *gene 44* mRNA the T4 RegA binding site spans nucleotides –10 to +2, which includes the Shine–Dalgarno region and an AU-rich element.

These studies revealed that in spite of their similar RNA binding domains, T4 and RB69 RegA proteins exhibit measurable differences in their RNA binding specificities. Although the two RegA proteins exhibit the same hierarchy of affinities for their cognate RNAs (i.e. *gene 44* > *gene 45* > *regA*), the two repressors exhibit different overall RNA binding specificities (Figs 6 and 7; Table 2). It was previously proposed that RB69 RegA is a generally stronger repressor of protein synthesis than T4 RegA protein (12). However, *in vivo* studies of relative mRNA repression sensitivities are complicated by the fact that RegA repression involves competition between 30S ribosomal subunits and RegA protein for the translation initiation regions of mRNAs. Thus, the apparent affinity of the two RegA
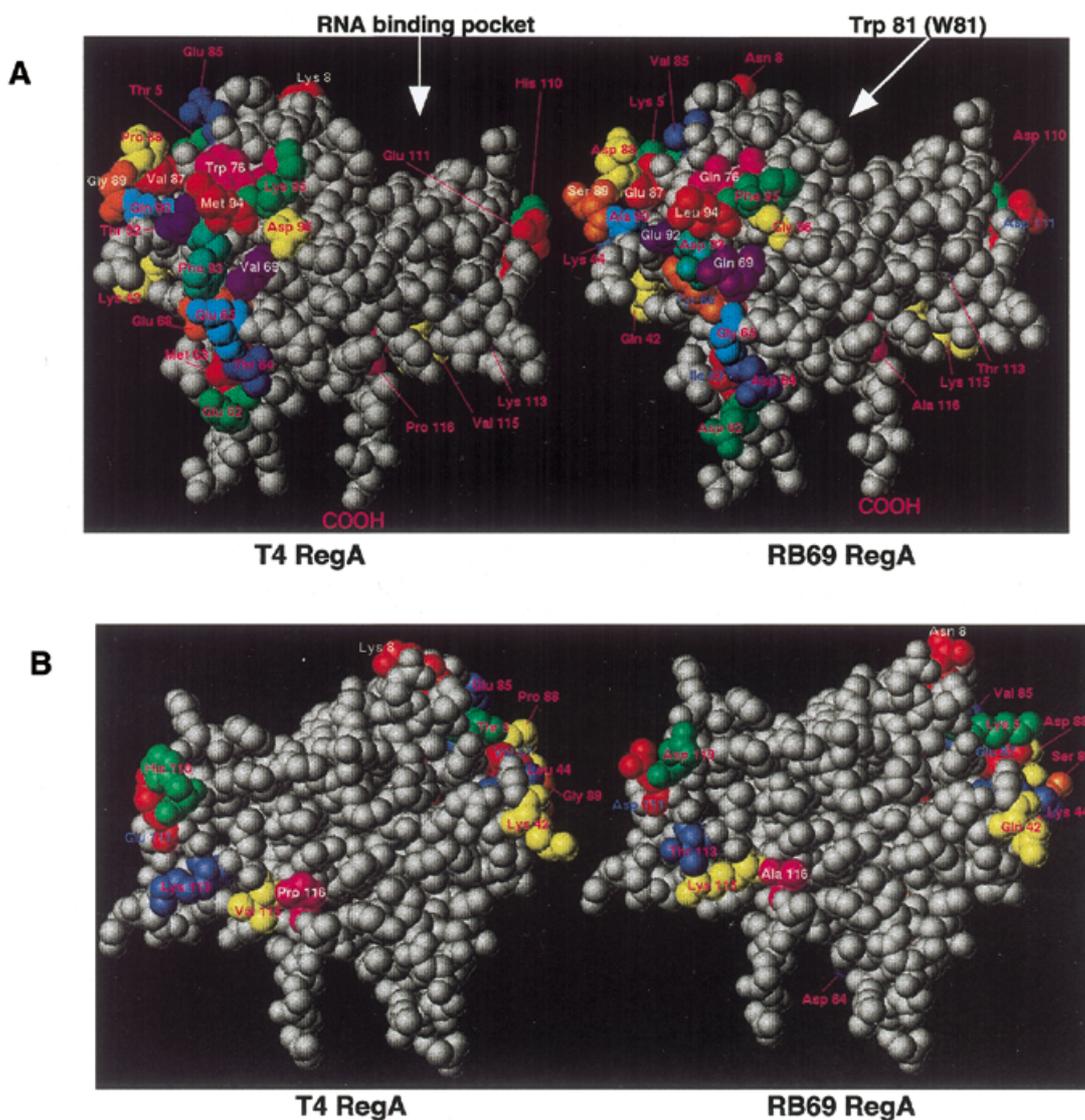
Figure 8. Space-filling models of RB69 and T4 RegA proteins, showing conserved and divergent amino acids. Divergent residues are colored, conserved residues are white. (**A**) front view, (**B**) back view, rotated 180°. Note that residues in the RNA binding groove are fully conserved in the two proteins. Arrows indicate the RNA binding pocket and the location of residue Trp81.

proteins is influenced by the relative concentrations of ribosomes, RegA protein and target mRNAs *in vivo*. To eliminate the effects of possible differences in substrate–ligand concentrations on RegA protein interactions with different RNAs, we have examined the affinity of RegA protein for purified target RNAs, using known concentrations of protein and RNA. These simplified assays have revealed that with respect to the six genes examined here, RB69 RegA protein exhibits a higher affinity than T4 RegA protein for two RNAs (Table 1). However, for the four other RNAs, T4 RegA protein exhibited the higher affinity (Table 1). The overall highest affinity was exhibited by RB69 RegA protein for T4 *gene 44* RE RNA. Interestingly, RB69 RegA protein also exhibited a larger range between highest (T4 *gene 44* RE) and lowest (T4 *regA* RE) affinities for the RNAs examined here (Table 1). This suggests

that *in vivo* the range of RB69 RegA protein concentrations required to repress target mRNAs may be broader.

To better understand the nucleotide sequence preferences of the two proteins, we have examined the relationship between nucleotide variations in the target RNAs and binding affinities of both proteins. Table 2 shows an alignment of the sequences of the core recognition region (i.e. the RNase protected or analogous region) plus 1–3 nt on either side, including nucleotide –9 in T4 *gene 44* (position 1 in Table 2), where base substitution has a large effect on RegA affinity (Fig. 4). The aligned REs were narrowed to 11 nt, corresponding to the site size determined for T4 RegA protein binding to T4 *gene 44* RNA, to focus on the potentially most important nucleotides for protein recognition.

Table 2. Sequence variations in the core recognition region of RNA ligands and hierarchy of RNA affinities for T4 and RB69 regA proteins

T4 regA protein	#1	#11	K_{app} (10^7 M^{-1})
RB *G45*	AAGG<u>GAAAU</u>AAA		19.9
T4 *G44*	GAGG<u>GAAAU</u>**U**A**U**		4.9
T4 *G45*	AAGGAAAUUAC		3.2
RB *G44*	GAGG<u>AAA</u>**A**U**U**A		2.5
RB *regA*	A**U**GG**U**A**A**AAA**U**		1.3
T4 *regA*	A**U**GG**U**A**A**AA**UG**		0.8
RB69 regA protein	#1	#11	K_{app} (10^7 M^{-1})
T4 G44	GAGG<u>GAAAU</u>UAU		34.0
RB *G44*	GAGG<u>GAAA</u>**A**U**U**A		9.5
T4 *G45*	**A**AGGAAAUUA**C**		2.0
RB *G45*	**A**AGG<u>GAAAU</u>**A**AA		1.8
RB *regA*	**AUGGUAAAA**AU		0.6
T4 *regA*	**AUGGUAAAAUG**		0.16

Nucleotides in bold indicate base substitutions relative to preferred, high affinity RNA.
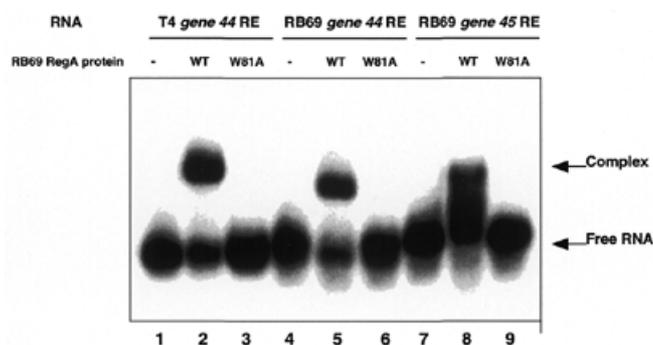Underlining indicates RNase protected nucleotides

Figure 9. RNA gel shift assay of WT and mutant Trp81Ala RB69 RegA proteins. Lanes 1–3 and 4–6, 10 nM T4 and RB69 *gene 44* RE RNAs, respectively; lanes 7–9, 50 nM RB69 *gene 45* RE RNA. Lanes 2, 3, 5 and 6, 10 nM WT or W81A RB69 RegA proteins was used. Lanes 8 and 9, 50 nM RB69 RegA proteins was used. Samples were analyzed as in Figure 4.

Examination of the hierarchy of affinities of T4 RegA protein for the six RNAs, shown in Table 2, reveals that the RNAs with the second and third highest affinities have two base substitutions (bold in Table 2) relative to the highest affinity RNA. These two base substitutions produce 4–6-fold decreases in the affinity constant. Interestingly, at position 11 in the aligned sequences, an A→U substitution (present in T4 G44) appears to be preferred over an A→C substitution (in T4 G45), which is consistent with the low C content of all RegA-regulated mRNAs. Comparison of the affinities of T4 RegA protein for T4 G44 and T4 G45 RNAs suggests that an A→G substitution at position 1 does not reduce affinity significantly, although previous studies have shown that a G→U substitution at this position in T4 G44 has a large affect (6). A third nucleotide

substitution (present in RB G44) is associated with a further reduction in affinity, while the fifth and sixth lowest affinity RNAs (RB *regA* and T4 *regA*) have four and five base substitutions, respectively. Thus, decreases in the affinity of T4 RegA protein for the six RNAs correlate with progressive nucleotide substitutions within the proposed 11 nt REs. Taken together these studies suggest that the preferred sequence for T4 RegA is (A/G)AGGAAAUAAA and that individual base substitutions at six positions within this sequence produce cumulative decreases in affinity (Table 2).

A similar comparison of the effects of base substitutions on RNA binding affinities for RB69 RegA protein suggests that some bases within the RE affect affinity more than others. For example, the second highest affinity RNA (RB G44) has three base substitutions relative to the highest affinity target (T4 G44) while the third highest affinity RNA has only two base substitutions (Table 2). This suggests that RB69 RegA affinity is more sensitive to the base at position 1 than to bases at positions 8 and 10. However, additional base substitutions relative to the T4 G45 target (with third highest affinity) produces progressive decreases in RB69 RegA protein affinity. These studies indicate that the preferred sequence for RB69 RegA protein is GAGGAAAUUAU. It should be noted that all six RNAs contain the sequence NNGGNAANNNN, so the relative importance of the bases at positions 3, 4, 6 and 7 was not evaluated by these studies. However, the conservation of these bases within the six RegA-regulated mRNAs suggests they may be important for RegA protein recognition.

It has been known for some time that RegA-regulated mRNAs vary in their sensitivity to RegA repression *in vivo* (8) and *in vitro* (1,5). However, until now, it has not been clear how specific nucleotide variations between closely related RNA sequences affect RegA protein affinity. The studies reported here help to clarify the mechanism of RNA discrimination by quantifying the effects of base substitutions on RegA protein affinity. This is important because it is the differential affinity for target RNAs that allows the RegA regulatory system to exhibit a gradient of effects on gene expression, as a function of increasing RegA protein concentration during the early and middle stages of phage infection. Presumably, these differential RNA affinities enable RegA protein to turn off expression of target genes in a stepwise fashion and thus optimize the temporal expression of inter-related enzymatic activities during the viral life cycle (4).

From the point of view of understanding protein–RNA recognition, perhaps the most intriguing observation from these studies is that, although the amino acids in the proposed RNA binding domain of T4 RegA (19) are fully conserved in RB69 RegA (Fig. 8), the two repressors exhibit notable differences in RNA binding specificities (Table 2). This observation suggests that some of the divergent residues in the two RegA proteins (Fig. 8) may contribute to differences in RNA binding specificities. However, recent studies of the effects of amino acid substitutions of divergent residues do not support a role for divergent residues in RNA binding (26). Alternatively, studies of the RNA binding specificity of the MS2 coat protein by Johansson *et al.* (27) suggests that there may be subtle differences in the geometry of amino acid side chains in the binding pocket of the two RegA proteins (that are not apparent in the homology modeling), which enable closer fitting of certain RNAs in the groove of each protein. Ultimately, the

solution of the structures of specific RegA–RNA complexes is needed in order to determine which of these possibilities is correct and to fully understand the mechanism of multiple RNA recognition and discrimination by RegA protein.

ACKNOWLEDGEMENTS

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