Characterization of Bacteriophage T4 regA Protein-Nucleic Acid Interactions*

Kevin R. Webster and Eleanor K. Spicer
From the Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06511

The bacteriophage T4 regA protein is a translational repressor of a group of T4 early mRNAs. We have characterized the binding of regA protein to polynucleotides and to specific RNAs. Binding to nucleic acids was monitored by the quenching of the intrinsic tryptophan fluorescence of regA protein. regA protein exhibited differential affinities for the polynucleotides examined, with the order of affinity being poly(U) > poly(dT) > poly(dU) = poly(rG) > poly(rC) = poly(rA). The binding site size calculated for regA protein binding to poly(rU) was n = 9 ± 1 nucleotides. Cooperativity was observed in binding to multiple-site oligonucleotides, with a cooperativity parameter (w) value of 10-22. To study the specific interaction between regA protein and T4 gene 44 mRNA, the affinity of regA protein for synthetic gene 44 RNA fragments was measured. The association constant (K_a) for regA protein binding to gene 44 RNA fragments was 100-fold higher than for binding to nontarget RNA. Study of variant gene 44 RNA fragments indicated that the nucleotides required for specific binding are contained within a 12-nucleotide sequence spanning -12 to -1, relative to the AUG codon. The bases of five nucleotides (indicated in upper case type) are critical for specific regA protein interaction with the gene 44 recognition element, 5'-aaUGAGaauu-3'. These studies further showed that formation of a regA protein-RNA complex involves a maximum of 2-3 ionic interactions and is primarily an enthalpy-driven process.

The bacteriophage T4 regA gene encodes a polypeptide of 14.6 kilodaltons that is expressed during early and middle times of phage infection. regA protein regulates the expression of at least 12 T4 genes, while also regulating its own synthesis, at the level of translation (1). The genes regulated by regA protein exist on largely unlinked loci throughout the T4 genome. Thus, in contrast to most other known translational repressors, regA protein can recognize different species of mRNA. In addition, target genes demonstrate differential sensitivity to regA protein in vitro. Since the nucleotide sequences and regA protein sensitivities of the rIIB and gene 44 mRNAs are quite different, it seems likely that by defining the regA targets on each of these mRNAs, rules governing recognition of other regA-regulated targets will emerge.

MATERIALS AND METHODS

Reagents—RNA β-cyanoethyl phosphorodiamidites were purchased from Peninsula Laboratories Inc. Ribonucleotide triphosphates, ribonuclease V1, (dT)_10, poly(rI), poly(rA), poly(rC), poly(rU), poly(dT), and poly(dU) were purchased from Pharmacia LKB Bio-technology Inc. (dT)_10, (dT)_15, (dT)_20, and all oligonucleotides were synthesized by the Yale School of Medicine Protein and Nucleic Acid Chemistry Facility. Nuclease S1 was purchased from Boehringer Mannheim. T7 RNA polymerase (300,000 units/mg) was the generous gift of D. Mueller and J. E. Coleman (Yale University).

Strains and Plasmids—Escherichia coli AR120 (xal*, N+) and plasmid pAS1 (6) were obtained from A. Shatzman (Smith, Kline & French).

Purification of regA Protein—regA protein was purified from AR120 cells containing plasmid pAS1regA (5) following induction of transcription from the lambda Pl promoter by naldixic acid treatment (7), as described previously (8). Protein purification was essentially as described earlier (2), and 1 g of naldixic acid-induced cells yielded ~2 mg of pure regA protein. regA protein was stored at concentrations of 0.7-1 mg/ml in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl_2, 1 mM EDTA, 1 mM dithiothreitol, and 50% glycerol at ~70 °C.

In Vitro Transcription—Transcription reactions were carried out in 250 μl of 40 mM Tris-HCl (pH 8.1), 20 mM MgCl_2, 1 mM spermidine,


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5 mM dithiothreitol, 50 μg/ml bovine serum albumin, 0.01% Triton X-100, and 80 mM polyethylene glycol 8000 with 50 mM linearized gene 44-1ac2 plasmid templates (9). T7 RNA polymerase was added (10 units/μl final concentration), and the reaction was carried out at 37 °C for 4 h. Transcripts were purified on 6% polyacrylamide, 8 M urea gels. The mRNA bands were localized by UV shadowing, excised, and eluted overnight (at room temperature) in 2 ml of 0.5 M NH4Ac, 1 mM EDTA.

Chemical Synthesis and Purification of Oligoribonucleotides—Oligoribonucleotides were synthesized by the Yale University School of Medicine Protein and Nucleic Acid Chemistry Facility on an Applied Biosystems 380B oligonucleotide synthesizer. To deprotect the RNA, the dried oligonucleotide pellet from a 1 mol synthesis was resuspended in 5 ml of 1 M tetraethylammonium fluoride in tetrahydrofuran (40% stock) and incubated at room temperature for 4 h. The deprotection reaction was then lyophilized to a volume of approximately 1.5 ml followed by the addition of 1.5 ml of 0.1 M triethylammonium acetate (pH 6.8). The tetraethylammonium fluoride was then removed by reverse-phase ion-pairing chromatography on a C4 column (Vydac). Full-length deprotected oligoribonucleotide was then isolated by chromatography on a Nucleogen-DEAE 60-7 column (Macherey-Nagel), followed by desalting on a C4 column. The purified oligoribonucleotide was then stored either dried or suspended in diethylycarbonate-treated distilled H2O at -20 °C.

Fluorescence Spectroscopy—regA protein fluorescence was detected with an SLM model 8000 spectrofluorometer interfaced with an IBM PC-XT computer. Titrations were performed at a protein concentration of 0.2 μM in buffer A (10 mM Hepes (pH 7.2), 5 mM MgCl2, 1 mM EDTA, 1 mM β-mercaptoethanol) plus a specified concentration of NaCl unless otherwise noted. Data was acquired at the experimentally determined excitation maximum of 282 nm and emission maximum of 347 nm. The effects of photobleaching throughout the titration were corrected for by monitoring regA protein fluorescence in a control cuvette. Screening of incident light by poly- and oligonucleotides was corrected for by parallel titration of N-acetyl-L-tryptophanamide (Sigma). All data was acquired through “reverse” titration (the addition of lattice to ligand) (10). The regA protein binding site size (n) was determined for binding to poly(U) under stoichiometric binding conditions (10); thus, the apparent association constant (Kapp) was calculated using the equivalence point of the titration and a binding site size of n = 9 residue (assuming nonoverlapping sites) as follows:

\[
K_{\text{app}} = \frac{([Q]_0 - [Q]_m)}{([Q]_m - [Q]_m) + K_{\text{app}}} = \frac{[R]_0}{[R]_0 - [R]_m}
\]

where \( a = b - 1 \) and \( b = \text{number of binding sites} \) where \( F_0 \) is fluorescence of the protein, or (b) in cases where a plateau could not be obtained, the approach described by Bujalowski and Lohman (11) was used. This method relates the fraction of bound protein to \( [Q]_m \), which for regA protein binding to polynucleotides was found to be linearly related, allowing for extrapolation to determine \( [Q]_m \). The maximal percent quenching \( (%Q_{\text{max}}) \) for binding to polynucleotides and long RNA transcripts was determined by two methods: (a) the percent quenching of a stable plateau (invariant over protein concentration) was determined from 0.2 to 2.0 μM in the quenching of the intrinsic fluorescence of the protein, or (b) in cases where a plateau could not be obtained, the approach described by Bujalowski and Lohman (11) was used. This method relates the fraction of bound protein to \( [Q]_m \), which for regA protein binding to polynucleotides was found to be linearly related, allowing for extrapolation to determine \( [Q]_m \). The maximal percent quenching \( (%Q_{\text{max}}) \) for binding to polynucleotides was determined from a plateau in quenching or, in the case of weak binding, from a double-reciprocal analysis in which 1/\( \Delta F \) is plotted versus 1/[free oligo] (12, 13). The Y intercept of the plot yields 1/\( \Delta F_{\text{max}} \) for the particular titration. This relationship also allows the determination of \( K_m \):

\[
K_m = \left( m - \Delta F_{\text{max}} \right)^{-1}
\]

where \( m \) is slope.

For regA protein binding to poly- or oligonucleotides, the percent maximal quenching \( (%Q_{\text{max}}) \) was independent of both the concentration of NaCl and the temperature. For particular oligoribonucleotides (i.e. (rN)4, (gG)4-11, (gG)4-12, and (gG)4-14) which bound weakly in 150 mM NaCl at 25 °C, titrations were performed under conditions which allow tighter binding, i.e. low [NaCl] or low temperature, to obtain a more accurate measurement of \( %Q_{\text{max}} \).

A cooperativity parameter was estimated by the statistical analysis developed by Draper and von Hippel (14). This procedure yields estimates of \( n \) similar to those obtained for gene 32 protein binding to oligonucleotides by using poly (rA) in forward titrations (15).

\[
K_m = \left( S_{\text{eq}} \right)^{b/a} K_i
\]

where \( a = b - 1 \) and \( b = \text{number of binding sites} \). Binding to (dT)12 was used to measure the intrinsic affinity constant (\( K_i \)). The cooperativity parameter was calculated in two ways by assuming binding to be nonpolar in nature (\( S_2 = 2 \)) and by assuming polar binding (\( S_2 = 1 \)) (15).

Partial S1 and V1 Nuclease Digestions—RNA oligonucleotides were 5′-end labeled with 32P using T4 polynucleotide kinase and then purified on a 20% polyacrylamide, 8 M urea gel and eluted as described above. Enzymatic digestions were carried out in 10 μl of buffer A plus 150 mM NaCl, at 25 °C. Digestion with S1 nuclease was performed for 3 min at an enzyme concentration of 300 units/ml. Digestion with ribonuclease V1 was performed for 4 min at an enzyme concentration of 8.8 units/ml. All reactions contained 100,000 cpm (Cerenkov) of RNA oligonucleotide and 0.01 mg/ml yeast tRNA (digestion patterns were the same in the absence of tRNA). Reactions were terminated by the addition of 0.5 volume of gel loading buffer (56% formamide, 0.05% xylene cyanol and bromphenol blue) and freezing at -70 °C. Samples were then loaded onto a 20% polyacrylamide, 8 M urea gel and visualized by autoradiography.

RESULTS

Binding of regA Protein to Polynucleotides—To characterize the general properties of regA protein-nucleic acid interactions, the affinity of regA protein for a variety of polynucleotides was measured. Like many other nucleic acid-binding proteins, the intrinsic cryptophan fluorescence of regA protein is quenched upon binding to nucleic acids. This is illustrated in Fig. 1, in which the quenching of regA protein fluorescence is plotted as a function of increasing concentrations of poly(U). The titration with poly(U) shown in Fig. 1 was carried out under stoichiometric binding conditions (10); thus, at the equivalence point of the titration the molar ratio of nucleotide residues to regA protein is equal to the binding site size (n). As illustrated in Fig. 1, the binding site size (n) for regA binding to poly(U) was 9 ± 1. The site size for binding to poly(dT) was also found to be 9 ± 1 (data not shown).

The apparent association constants, \( K_{\text{app}} \), for regA protein binding to a variety of homopolynucleotides have been determined under conditions of low ionic strength, as summarized in Table I. A 200-fold range of affinities was observed in the binding of regA protein to the assorted homopolynucleotides. The preference of regA protein for RNA versus DNA is

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1 K. R. Webster and Y. Shamoo, manuscript in preparation.

2 The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Fig. 1. Titration of 2 μM regA protein with poly(U). Binding was carried out at 20 °C in buffer A plus 10 mM NaCl and was monitored by the change in regA protein fluorescence. The observed plateau in fluorescence quenching (%Q) was constant over a 10-fold range of initial regA protein concentration. Extrapolation of the binding site size (n) is indicated by an arrow.
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Table 1

<table>
<thead>
<tr>
<th>Polynucleotide</th>
<th>$K_{app}$ $M^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(rU)</td>
<td>$5 \times 10^5$</td>
</tr>
<tr>
<td>Poly(dT)</td>
<td>$8 \times 10^5$</td>
</tr>
<tr>
<td>Poly(dU)</td>
<td>$3 \times 10^6$</td>
</tr>
<tr>
<td>Poly(rG)</td>
<td>$3 \times 10^6$</td>
</tr>
<tr>
<td>Poly(rC)</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Poly(rA)</td>
<td>$1 \times 10^6$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oligonucleotide*</th>
<th>$K_{app}$ $M^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(dT)$_{10}$</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>(dT)$_{25}$</td>
<td>$2 \times 10^6$</td>
</tr>
<tr>
<td>(dT)$_{30}$</td>
<td>$2 \times 10^6$</td>
</tr>
<tr>
<td>(dT)$_{35}$</td>
<td>$8 \times 10^5$</td>
</tr>
<tr>
<td>(dT)$_{40}$</td>
<td>$1 \times 10^6$</td>
</tr>
</tbody>
</table>

*All oligonucleotides possessed a 5’-OH.

RNA targets, we have measured the affinity of regA protein for T4 gene 44 RNA transcripts and gene 44 RNA fragments. Previous deletion analysis and RNase protection studies of gene 44 mRNAs, utilizing gene 44-lacZ fusion genes, indicated that the regA protein binding site of gene 44 mRNA is located between bases -10 and +2, with respect to A of the gene 44 initiation codon (5). To measure the $K_{app}$ for regA protein binding to the gene 44 mRNA, transcripts containing variable amounts of gene 44 sequence (in addition to lacZ sequences) were synthesized in vitro using T7 RNA polymerase and gene 44-lacZ fusion genes. Titrations of regA protein with three gene 44 lacZ RNA transcripts compared with a T4 gene 32 (nontarget) RNA transcript are shown in Fig. 3A. regA protein binding to gene 44 target-containing RNA is at least 10-fold greater ($K_{app} \geq 1 \times 10^6 M^{-1}$) than binding to the nontarget gene 32 RNA ($K_{app} = 1 \times 10^5 M^{-1}$). regA protein also binds with approximately equal affinity to transcripts containing gene 44 sequences -11 to +9 and to a transcript containing

**Fig. 2. Titrations of regA protein with single- and multiple-binding site oligo(dT).** Titrations were performed at 25 °C with 0.2 μM regA protein in buffer A plus 10 mM NaCl and were plotted using a monomer binding site size of 12 nucleotides. ○, d(T)$_{12}$; ●, d(T)$_{25}$; ▲, poly(dT).

**Fig. 3.** A, titration of regA protein with gene 44 lacZ run-off transcripts. Titrations were carried out with 0.2 μM regA protein in buffer A plus 150 mM NaCl, at 25 °C. Transcripts examined were gene 44-46-mer (which contains nucleotides -11 to +9 of gene 44 and 26 nucleotides of lacZ) (○), gene 44-65-mer (contains -11 to +9 of gene 44 and 45 nucleotides of lacZ) (▲), gene 44-88-mer (contains -37 to +26 of gene 44 and 25 nucleotides of lacZ) (△), and gene 32-151-mer (contains -105 to +21 of gene 32 and 25 nucleotides of lacZ) (●). B, titrations of regA protein with synthetic oligoribonucleotides. Titrations were carried out with 0.2 μM regA protein in buffer A plus 150 mM NaCl, at 25 °C. RNAs examined were gene 44/16-mer (corresponding to nucleotides -12 to +4 of gene 44) (○) and g32/23-mer (nucleotides -10 to +10 of gene 32 plus an additional three nucleotides) (●).
The value of $K_{app}$ calculated for binding to the gene 44 transcripts represents a minimal value for binding to the specific gene 44 target site, since the calculation averages the affinity of each binding site on the RNA (i.e. including both the specific and nonspecific binding sites). To eliminate the effects of nonspecific binding sites on measurement of apparent association constants and also to avoid potential structural differences in target site-containing RNAs of varying lengths, short fragments of gene 44 RNA were used as ligands to study the specific requirements for regA protein-RNA recognition.

A 16-base oligoribonucleotide, corresponding to bases -12 to +4 of gene 44, was chemically synthesized. This RNA fragment, designated g44/16-mer, contains the gene 44 mRNA region which was shown to be protected by regA protein from RNase digestion (nucleotides -10 to +2) (5). A comparison of regA protein binding to the gene 44/16-mer with binding to a gene 32/23-mer (which contains bases -10 to +10 of gene 32 mRNA) is shown in Fig. 3B. The $K_c$ for regA protein binding to the short gene 44 RNA target (1 x 10$^7$ M$^{-1}$) is 100-fold greater than the $K_c$ for nonspecific binding to the gene 32 oligonucleotide (1 x 10$^5$ M$^{-1}$). This degree of specificity is approximately 10-fold greater than that estimated for binding to the longer gene 44 RNA transcripts. Thus, the gene 44/16-mer appears to contain the necessary elements for specific binding by regA protein.

To further define the boundaries of the regA protein-gene 44 RNA interaction, the affinity of regA protein for RNA fragments shorter than the gene 44/16-mer was assayed. As shown in Fig. 4A, deletion of four nucleotides from the 5’ end of the g44/16-mer (oligonucleotide g44-3), which removed the gene 44 initiation codon, had no measurable effect on the affinity of regA protein ($K_c = 1 \times 10^7$ M$^{-1}$). The binding data for these RNAs is presented in a double-reciprocal plot in Fig. 4B, allowing the determination of $\Delta G_{m}^{\circ}$ for the weak-binding oligonucleotide (g44-3). Deletion of two additional nucleotides from either the 5’ end or the 3’ end of g44-4 produced a large decrease in the $K_c$ ($<5 \times 10^6$ M$^{-1}$), for either of the 10-mers). This large decrease in affinity observed for the smaller fragments indicates that the minimal regA protein recognition element comprises nucleotides -12 to -1 of gene 44 mRNA.

The contribution of individual bases within the 12-nucleotide gene 44 RNA fragment (g44-4) to the overall binding free energy was assessed by measuring the affinity of regA protein for g44-4 with single base substitutions. Base substitutions were selected so that no additional Shine-Dalgarno-like sequences or initiation codons were introduced into the RNA and the potential for formation of base pairs within the RNA was avoided. Fig. 5 shows five representative titration curves obtained for binding to synthetic variants of g44-4. The binding to a random RNA, oligo (rN)12, was measured for comparison. As evident from Fig. 5, regA protein exhibited a wide range of affinities for the different oligonucleotides.

The maximum percent quenching of regA protein fluorescence produced by binding to g44-4 variants was similar to that produced by binding to the wild-type oligonucleotide g44-4. The single exception was g44-22, which yielded a significantly lower maximum percent quenching (Fig. 5). The association constants and %$Q_{max}$ determined for binding to the variant oligonucleotides are presented in Table II, and the binding affinities are schematically represented in Fig. 6. As is evident from Table II and Fig. 6, the affinity of regA protein varied with base substitutions across the entire length of the oligonucleotide. Five of the substitutions (at positions -10, -9,
TABLE II
RegA protein binding to gene 44 target variants

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>( K_{d} )</th>
<th>( K_{s} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>g44-16-mer</td>
<td>AUAUGGAAAUAUUG</td>
<td>55 ( (0.3 \pm 0.2) \times 10^{3} )</td>
<td>1 ( (0.2 \pm 0.2) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-4</td>
<td>AUAUGGAAAUUG</td>
<td>57 ( (0.1 \pm 0.1) \times 10^{3} )</td>
<td>7 ( (0.1 \pm 0.1) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-8</td>
<td>AUAUGGAAAUUG</td>
<td>64 ( (0.2 \pm 0.2) \times 10^{3} )</td>
<td>7 ( (0.2 \pm 0.2) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-9</td>
<td>AUAUGGAAAUUG</td>
<td>65 ( (1 \pm 1) \times 10^{3} )</td>
<td>8 ( (1 \pm 1) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-10</td>
<td>AUAUGGAAAUUG</td>
<td>60 ( (0.1 \pm 1) \times 10^{3} )</td>
<td>7 ( (0.1 \pm 1) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-11</td>
<td>AUAUGGAAAUUG</td>
<td>60 ( (0.1 \pm 1) \times 10^{3} )</td>
<td>1 ( (0.1 \pm 1) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-12</td>
<td>AUAUGGAAAUUG</td>
<td>60 ( (0.1 \pm 1) \times 10^{3} )</td>
<td>1 ( (0.1 \pm 1) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-13</td>
<td>AUAUGGAAAUUG</td>
<td>60 ( (0.1 \pm 1) \times 10^{3} )</td>
<td>1 ( (0.1 \pm 1) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-14</td>
<td>AUAUGGAAAUUG</td>
<td>63 ( (0.2 \pm 0.2) \times 10^{3} )</td>
<td>7 ( (0.2 \pm 0.2) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-15</td>
<td>AUAUGGAAAUUG</td>
<td>58 ( (0.1 \pm 0.1) \times 10^{3} )</td>
<td>7 ( (0.1 \pm 0.1) \times 10^{3} )</td>
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<tr>
<td>g44-16</td>
<td>AUAUGGAAAUUG</td>
<td>57 ( (1 \pm 1) \times 10^{3} )</td>
<td>7 ( (1 \pm 1) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-17</td>
<td>AUAUGGAAAUUG</td>
<td>57 ( (1 \pm 1) \times 10^{3} )</td>
<td>7 ( (1 \pm 1) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-18</td>
<td>AUAUGGAAAUUG</td>
<td>70 ( (1 \pm 1) \times 10^{3} )</td>
<td>7 ( (1 \pm 1) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-19</td>
<td>AUAUGGAAAUUG</td>
<td>70 ( (1 \pm 1) \times 10^{3} )</td>
<td>7 ( (1 \pm 1) \times 10^{3} )</td>
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<tr>
<td>g44-20</td>
<td>AUAUGGAAAUUG</td>
<td>70 ( (1 \pm 1) \times 10^{3} )</td>
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</tr>
<tr>
<td>g44-21</td>
<td>AUAUGGAAAUUG</td>
<td>70 ( (1 \pm 1) \times 10^{3} )</td>
<td>7 ( (1 \pm 1) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-22</td>
<td>AUAUGGAAAUUG</td>
<td>41 ( (0.1 \pm 1) \times 10^{3} )</td>
<td>7 ( (0.1 \pm 1) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-23</td>
<td>AUAUGGAAAUUG</td>
<td>62 ( (0.6 \pm 0.6) \times 10^{3} )</td>
<td>7 ( (0.6 \pm 0.6) \times 10^{3} )</td>
</tr>
<tr>
<td>g44-4DNA</td>
<td></td>
<td>$&lt;5 \times 10^{-6}$</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6. Summary of the effects of individual base substitutions on RegA protein affinity for the gene 44 recognition element. The y axis equals the \( K_{d} \) for RegA protein binding to the substituted oligoribonucleotide and is a logarithmic scale. The sequence of the gene 44 RegA protein target site is presented on the x axis. Each \( K_{d} \) corresponds to the affinity for an RNA 12-mer with a single mutation which is indicated on top of the respective bar. Positions at which more than one substitution was introduced are indicated by split bars, one corresponding to each mutation. The \( K_{d} \) for binding to the wild-type gene 44 target and a 12-mer of random sequence are represented by the dashed lines.

-8, -7, and -5) resulted in a greater than 10-fold decrease in the affinity of RegA protein for the oligonucleotide compared with g44-4. A second group of substitutions, at positions -11, -4, and -3, caused a smaller but still significant (>2-6-fold) decrease in the \( K_{d} \). In contrast, a third group of substitutions (at bases -12, -6, -2, and -1) produced either less than a 2-fold decrease or, in two instances, an increase in the \( K_{d} \).

To determine if the contribution of the individual nucleotides to the free energy of binding is additive, the affinity for an RNA with a double substitution, g44-20 (A→G, A→U), was measured. The free energy for binding to this variant equaled -7.36 kcal mol$^{-1}$, 23% less than observed for binding to g44-4 (-9.54 kcal mol$^{-1}$). This difference is approximately equal to the sum of the decreases observed for binding to RNAs with single base substitutions, g44-18 and g44-19 (20 and 4.6%, respectively). Thus, the contributions of these two nucleotides to the binding free energy are additive.

It is notable that substitutions at position G99 lead to a 200-fold decrease in affinity, effectively reducing binding to the level of nonspecific interactions. RegA protein also discriminated effectively between RNA and DNA, as is evident from the association constants for binding to g44-4 and the corresponding deoxyoligonucleotide g44-4DNA, which differed by a factor of 200 (Table II).

**Structure Mapping by Nuclease Digestion**—The large decreases in RegA protein affinity observed for gene 44 fragments with substitutions at residue G99 (g44-11, g44-12, and g44-13) suggest that a change in the structure of the oligoribonucleotide had occurred as a consequence of the single base substitution. Structural predictions generated using the method of Zuker and Stiegler (16) failed to identify any potential, stable secondary structures for the wild-type sequence (g44-4) or the G99 variants. To examine the effect of substitutions introduced at position -9 on the overall structure of the oligonucleotide, comparative S1 nuclease and ribonuclease V1 digestions were carried out, as shown in Fig. 7. S1 nuclease is known to cleave both single-stranded DNA and RNA in a non-sequence-dependent manner, whereas base-paired nucleic acids are resistant to S1 digestion (17). Ribonuclease V1 recognizes phosphodiester backbone structures of nucleotides participating in Watson-Crick base-pairing and base-stacking interactions (18–20). Comparison of the S1 digestion of g44-4 and the mutant G99 oligonucleotides demonstrates distinct differences in the cleavage patterns of these oligonucleotides, as indicated by arrows in Fig. 7. Oligonucleotide g44-4 is cleaved by S1 at each position except after nucleotide -9, whereas g44-11 and g44-12 variants are efficiently cleaved at nucleotide -9. In addition, cleavage at position -7 does not occur in the g44-11 and g44-12 variants but is observed in the (wild-type) g44-4 oligonucleotide.

Similar differences in cleavage patterns were observed with ribonuclease V1. As shown in Fig. 7, ribonuclease V1 cleaves at sites extending from -11 to -4 of the oligonucleotides. Since the same sites are cleaved by S1 nuclease, it is likely that V1 cleavages are occurring at nucleotides participating in base-stacking interactions rather than at nucleotides in a stable hairpin-loop structure. The differences in oligonucleotide...
tide cleavages by V1 illustrated in Fig. 7 suggest that base-
stacking interactions within the g44-11 and g44-12 variants 
different from those occurring within the (wild-type) g44-
4 oligonucleotide. This change in RNA structure may be 
responsible for the considerable decreases observed in regA 
protein affinity. Oligonucleotides g44-15 and g44-16 were also 
subjected to enzymatic cleavage, and in both cases S1 and V1 
cleavage did not occur at position -9, as observed for g44-4 
(shown in Fig. 7). In addition, oligo g44-15 was cleaved by S1 
and S1 at position -7, however, g44-16 was only cleaved by 
S1 at position -7. Oligos g44-14 and g44-18 were also cleaved 
at position -7 but not at -9 by S1 and V1 (data not shown). 
Thus, each of the variants tested behaved similarly to g44-4 
with respect to cleavage at position -9, except for variants 
with G+ substitutions. 

Effect of Ionic Strength on RNA Binding—To assess the 
contribution of ionic interactions to regA protein-RNA com-
plex formation, the affinity of regA protein for four represen-
tative lattices (poly(U), g44 88 mer, oligo g44 4, and 
oligo(N12)) was measured as a function of NaCl concen-
tration. The effect of [Na+] on binding affinity is shown in Fig. 
8, in which log $K_0$ is plotted versus log [NaCl] (21). For all 
four RNAs, a linear relationship was observed over the range 
of NaCl concentrations examined. As is evident from the 
slope of the log $K_0$ versus log[NaCl] plots, binding of regA 
protein to the specific target RNAs (g44-88-mer and g44-4) 
varied with NaCl concentration to about the same degree as 
binding to the nonspecific RNAs. According to Record et al. 
(21), the dependence of $K_0$ on [Na+] can be described by the 
following equation:

$$-\log K_0(\text{log}[NaCl]) = m' \Psi$$ (6)

where $\Psi$ represents a monovalent counterion binding param-
eter for the specific oligo- or polynucleotide, and $m'$ is the 
number of ionic interactions between the protein and RNA. 
By estimating approximate values of $\Psi$ for each of the RNAs 
on the basis of their base composition and the reported values 
for homopolynucleotides (21) and determining the slope of 
the log $K_0$ versus log[NaCl] plots, the maximum number of 
ionic interactions involved in regA protein-RNA binding ($m'$) 
was calculated to be between two and three. The observation 
that poly(U) and g44-88-mer exhibit similar sensitivity to salt 
concentration as g44-4 and (N12) suggests that cooperativity, 
$\omega$, is not sensitive to salt concentration.

Temperature Dependence of RNA Binding—To calculate the 
enthalpy changes that occur upon complex formation, the 
affinity of regA protein for oligonucleotide g44-4 was deter-
mimed at 15, 25, 30, and 37 °C. As shown in Table III, regA 
protein affinity decreased as the temperature was raised. 
Using the van't Hoff relationship, the enthalpy of reaction 
was calculated to be $\Delta H = -17.5$ kcal mol$^{-1}$, indicating that 
regA protein binding to g44-4 is exothermic. The free energy 
and entropy of binding were also calculated and are presented 
in Table III. These thermodynamic parameters indicate that 
regA binding is primarily enthalpy driven, in agreement with 
the observation that ionic interactions (which contribute to 
the entropy of reaction through release of ions upon binding 
(21)) do not play a major role in regA protein-RNA interac-

discrete

tions.

We have examined the parameters that influence the bind-
ing of T4 regA protein to both specific and nonspecific nucleic 
acid lattices. regA protein exhibits both base and sugar spe-
ificity in binding to homopolynucleotides, with a 200-fold 
preference for polyribonucleotides compared with polynucleo-
ribonucleotides. The order of affinity for the polynucleotides 
examined, poly(rU) > poly(dT) > poly(dU) > poly(rG) > 
poly(rC) = poly(rA), may be due to preference for both the 
type of base and for the conformation of the polynucleotide. 
This hierarchy may reflect the lower energy required for the 
protein to interact with freely rotating bases (in poly(dT) and 
poly(U)) versus stacked bases (in poly(A) and poly(G)), which 
has also been suggested for IE gp5 binding to polynucleotides 
(22).

Comparison of the intrinsic affinity of regA protein for 
binding to a single binding site and the apparent affinity for 
binding to a multiple-site lattice indicates that regA protein 
binding is cooperative, with $\omega = 10^{2}$. This degree of coop-
ervativity is similar to that measured for E. coli rho protein

\begin{table}
\centering
\caption{Thermodynamic parameters of regA protein binding to oligonucleotide g44-4}
\begin{tabular}{|c|c|c|c|c|}
\hline
$K$ & $K_0$ & $\Delta G$ & $\Delta H$ & $\Delta S$ \\
\hline
M$^{-1}$ & kcal mol$^{-1}$ & kcal mol$^{-1}$ & cal mol$^{-1}$ & \\
\hline
288 & 2.2 x 10$^7$ & -9.7 & -17.5 & -27.1 \\
298 & 1.0 x 10$^7$ & -9.5 & -17.5 & -26.8 \\
303 & 5.6 x 10$^6$ & -9.4 & -17.5 & -26.7 \\
310 & 2.5 x 10$^6$ & -9.1 & -17.5 & -27.1 \\
\hline
\end{tabular}
\end{table}
RegA Protein-Nucleic Acid Interactions

(23) and heterogeneous nuclear ribonucleoprotein protein A1
binding to poly(trA)3 and is 2 orders of magnitude lower than
that measured for T4 gp32 binding to polynucleotides (24).

Measurement of regA protein-nucleic acid affinity under
different ionic conditions has shown that protein-RNA complex
formation (specific and nonspecific) is only weakly influ-
enced by the [Na+]*. There are a maximum of two to three
ionic interactions involved in binding regardless of the lattice
tested. This result is consistent with a van’t Hoff analysis of
complex formation, which indicates that formation of the
regA protein-RNA complex is primarily an enthalpy-driven
process, indicating a small ionic contribution. This is ana-
logous to what was found for T4 gp32 binding to poly(trA) (25)
and is in contrast to E. coli S4 r-protein binding to α-mRNA,
which is an entropy-driven reaction (26). The fact that all
of the different lattices tested behaved in a similar fashion
suggests that the general mechanism of regA protein binding
is similar.

By using both in vitro synthesized T7 transcripts and chemically synthesized oligoribonucleotides, a detailed analy-
sis of the regA protein target site within the gene 44 mRNA has been performed. regA protein affinity for specific mRNAs
is approximately 10-fold higher than that observed for E. coli ribosomal protein S4 binding to α-mRNA, Kₐ = 3 × 10⁶ M⁻¹
in 250 mM NaCl at 0°C) (26) and similar to R17 coat protein
binding to the replicase initiator region (Kₐ = 3 × 10⁶ M⁻¹ in
80 mM KCl, at 2°C) (27). This affinity is 100-fold greater
than observed for regA protein binding to nonspecific RNAs.

Studies of gene 44 RNA fragments, we have found that
a 12-base oligoribonucleotide extending from position −12 to −1 of gene 44 (5′-AAUGAGGAAAU-3′) is sufficient for
specific binding by regA protein. Deletion of additional nucle-
otides from either the 5′ or 3′ end of the 12-mer results in a
significant decrease in Kₐ, indicating that the boundaries of
the recognition element are clearly defined. This target se-
quence is centered upon the gene 44 Shine-Dalgarno region,
consistent with earlier work which located the regA protein
binding site to between residues −10 and +2 (5).

Studies of gene 44 RNA variants have demonstrated that
the bases of five nucleotides (indicated in upper case type)
are critical for specific regA protein interaction with the 12-
nucleotide recognition element. This same phenomenon may also occur on
specific nucleation site in combination with cooperative interac-
tions, may shed new light on the overall mechanism of regA
protein recognition of the 12-residue oligoribonucleotide.

Having determined the relative importance of specific bases
within the recognition element of gene 44 mRNA, we reex-
amined the regA target on rIB mRNA for possible sequence
similarities with the gene 44 recognition element. Genetic
studies of the rIB gene have shown that mutations which
diminish regA repression of rIB expression lie between nu-
cleotides −1 and +8 relative to the AUG codon (4). It has also
been demonstrated that regA protein protects 18 nucleotides
of rIB mRNA (extending from −5 to +13) from RNase
digestion (3). In comparing the 12-nucleotide gene 44 target
sequence with the sequence surrounding the rIB initiation
codon, optimal alignment is obtained with the sequences from
−13 to −2 and −1 to +11, as shown in Fig. 9. However, within
each potential target site there are differences which, in light
of the gene 44 binding study reported here, would reduce the
affinity of regA protein for the RNA targets to a level approx-
imately equivalent to the affinity for nonspecific RNA. The
presence of two potential binding sites of weak affinity and a
large region of RNAse protection, coupled with the observation
of cooperative regA protein binding to homopolynucleotides,
leads us to hypothesize that two or more regA proteins may
bind to the rIB mRNA. This could allow for a cooperative
interaction which would amplify the intrinsic affinity for this
mRNA to a level sufficient for specific interaction. Transla-
tional regulation by gene 32 protein is thought to occur by a
similar mechanism, whereby a 2-3-fold preference for a spe-
cific nucleation site in combination with cooperative interac-
tions leads to amplification of the intrinsic affinity of the
protein for its own mRNA, thus achieving a higher level of
specificity (28). This same phenomenon may also occur on
gene 44 mRNA, where presumably initial binding occurs at
the 12-nucleotide recognition element.

We have shown that regA protein may be sensitive to both
the structure and the sequence of its recognition element. The contribution of mRNA topology to regA protein recognition
and binding offers an explanation for the apparent lack of a
strong consensus target sequence among the different target
mRNAs. This fact, in addition to the observation that regA
protein can participate in cooperative protein-protein inter-
actions, may shed new light on the overall mechanism of regA
protein function. Further experiments are being carried out
to assess the contributions of both mRNA structure and
coooperative protein interactions to regA protein repression of
the gene 44 and rIB mRNAs.

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