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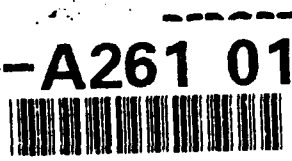

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13. ABSTRACT (*Maximum 200 words*)

The bacteriophage T4 regA protein (M_r=14,600) is a translational repressor of a group of T4 early mRNAs. To identify a domain of regA protein that is involved in nucleic acid binding, ultraviolet light was used to photochemically cross-link regA protein to [^{32}P]p(dT)$_{16}$. Two tryptic peptides cross-linked to [^{32}P]p(dT)$_{16}$ were isolated, and sequencing of the major cross-linked peptide yielded the sequence VISXKQKHEWK, corresponding to residues 103-113. Phenylalanine 106 was identified as the site of cross-linking, thus placing this residue at the interface of the regA protein:p(dT)$_{16}$ complex. The nucleic acid binding domain of regA protein was further examined by chemical cleavage of regA protein into six peptides using CNBr. Peptide CN6, which extends from residue 95 to 122, retains both the ability to be cross-linked to [^{32}P]p(dT)$_{16}$ and 70% of the non-specific binding energy of the intact protein. Site directed mutagenesis has been used to introduce substitutions at Phe 106. Studies to measure the contribution of Phe 106 to the free energy of RNA binding are in progress.

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MECHANISM OF ACTION OF BACTERIOPHAGE T4 TRANSLATIONAL REPRESSOR REGA PROTEIN

FINAL REPORT

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OCTOBER 9, 1992

U.S. ARMY RESEARCH OFFICE
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A. STATEMENT OF THE PROBLEM:

The bacteriophage T4 regA protein regulates the expression of at least twelve T4 genes while also regulating its own synthesis, at the level of translation (1). It has been shown that regA protein acts by binding to specific mRNAs and competing with the formation of initiation complexes by ribosomes (2). In our earlier studies we examined the region of one specific target gene (T4 gene 44) that is required for repression by regA protein (3). These studied indicated that regA protein acts by recognition of a specific RNA element, in which apparently both the sequence and structure are of importance (3,4). **What has not previously been examined is the domain structure of regA protein and the amino acid residue(s) involved in RNA binding.**

A number of RNA binding proteins have been shown to contain common structural motifs. For example, a family of eucaryotic RNA binding proteins share a common structural feature termed the RNP domain (or RNA recognition motif, RMM), which consists of approximately 80 amino acids including four well conserved phenylalanine residues (5-10). Within the 80 amino acid domain is an octapeptide sequence of basic and hydrophobic residues, including a conserved phenylalanine, that is postulated to play a role in RNA recognition. This domain is found in snRNP proteins, hnRNP proteins, poly A binding proteins and nucleolin (7).

A second type of RNA binding domain that has been identified is characterized by an arginine-rich motif which is common to a number of procaryotic and eucaryotic RNA binding proteins (11). These proteins include the bacteriophage N proteins, HIV tat and rev proteins, retroviral gag proteins and some ribosomal proteins (11). The arginine-rich domain has been implicated in the specific recognition of RNA stem-loop structures by the N protein during bacteriophage transcription antitermination events and in HIV tat protein-RNA interactions (11). In fact, a 14 residue synthetic peptide which contains the arginine rich sequences of tat protein has been shown to bind in a specific fashion to a target RNA stem-loop structure (the tar element)(12). Thus, it is now apparent that both the ability to bind nucleic acids and to exhibit specificity of binding, in some cases, may require only a small protein fragment.

RegA protein does not exhibit significant similarity with either of the RNA binding domains described above. The fact that regA protein recognizes a specific group of mRNAs (unlike RNP domain proteins) and that its target RNA element is single-stranded (unlike the elements recognized by N protein, tat and rev) suggests that a different structural motif may be involved in RNA recognition and binding. To localize the RNA binding domain of regA protein, we have used a variety of approaches, including photochemical cross-linking to nucleic acids, purification and characterization of regA protein fragments, and site-specific mutagenesis.

B. SUMMARY OF RESULTS:

Photochemical Cross-linking of RegA Protein to Nucleic Acid:

As a first step towards identifying residues of regA protein that are located at the interface of a protein:RNA complex, the ability of regA protein to be photocrosslinked to nucleic acid was examined. Complexes of regA protein and a radiolabeled target RNA termed g44-4 (5'-AAUGAGGAAAUU-3'), containing the gene 44 recognition element for regA protein (3), were exposed to increasing doses of UV light. Formation of covalently cross-linked complexes was then monitored by electrophoresis on SDS-polyacrylamide gels. Although cross-linking was observed, the efficiency of cross-linking (<5%) was not sufficient to enable isolation of a peptide cross-linked to g44-4 RNA (data not shown). It has previously been shown that thymine is the most photoreactive base, and that many single-stranded nucleic acid binding proteins can be photochemically cross-linked to oligo (dT) lattices (7,13-17). In addition, it has been shown that regA protein binds to oligo $(dT)_n$ and poly (dT) (3). Accordingly, the possibility of cross-linking

regA protein to $p(dT)_{16}$ was examined. As shown in Figure 1, regA protein is efficiently photochemically cross-linked to $[^{32}P]$-$p(dT)_{16}$. The maximal extent of cross-linking (~25% of the oligonucleotide) was obtained with a dose of 1.0×10^5 ergs/mm^2, which is comparable to the range of photon-flux used in cross-linking *E. coli* SSB, T4 gp32, A1 hnRNP protein and fd gp5 to nucleic acids (13).

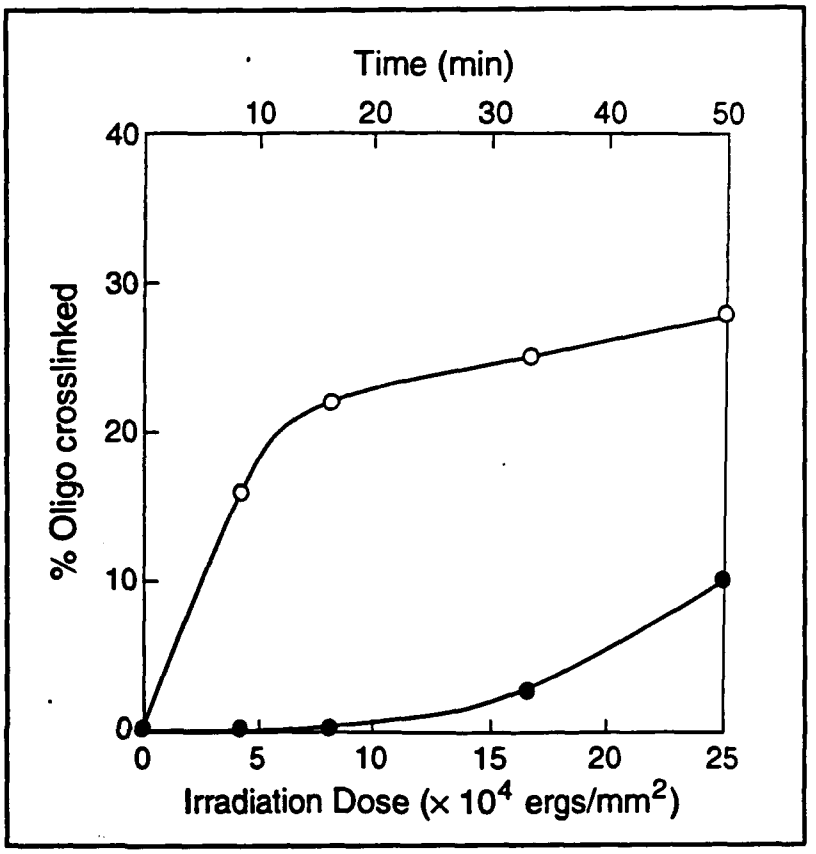

Figure 1: Dose-response curve for photochemical cross-linking of regA protein to $[^{32}P]p(dT)_{16}$. RegA protein (4 μM) was mixed with nucleic acid (2.8 μM oligonucleotide) in 25 μl buffer A (18). Samples were exposed to UV light at a dose of 5×10^3 erg/mm^2/min. for varying lengths of time, at 4°C. Percent oligonucleotide cross-linking was quantitated using a filter retention assay (as described in Ref. 18). O = $[^{32}P]p(dT)_{16}$ plus regA protein, ● = $[^{32}P]p(dT)_{16}$ plus an equimolar concentration of the g44-4 RNA in addition to regA protein.

To determine if regA protein:$p(dT)_{16}$ cross-linking occurs through specific interactions and to determine whether the site of $[^{32}P]p(dT)_{16}$ binding is the same as the RNA binding site, a cross-linking reaction was performed in the presence of an equimolar concentration of unlabeled target g44-4 RNA, which is expected to have greater than 750-fold higher affinity ($K_a \sim 6 \times 10^8 M^{-1}$) for regA protein than $p(dT)_{16}$ (K_a = 2-8 $\times 10^5 M^{-1}$, based on the affinity of dT_{12} and dT_{24}) in 10 mM NaCl (3). The presence of the specific target site RNA virtually eliminated all cross-linking to $[^{32}P]p(dT)_{16}$ using cross-linking times below 20 minutes, as shown in Figure 1. This result confirms that, at the 20 min. radiation time used in this study, the $d(pT)_{16}$ photochemical cross-link is not random and occurs at the same site as g44-4 RNA binding within the protein.

Isolation of the Cross-linked RegA Tryptic Peptide and Identification of the Cross-linked Amino Acid Residue:

To identify the site of cross-linking between regA protein and $p(dT)_{16}$, a preparative reaction was performed with 315 nmoles of regA protein and 200 nmoles of $[^{32}P]p(dT)_{16}$. After photolysis, non-cross-linked $p(dT)_{16}$ was removed by TCA precipitation of the reacted complex. The TCA-precipitated protein and protein-DNA complex were then redissolved, denatured, carboxamidomethylated (to allow identification of cysteine during sequencing) and digested with trypsin. Cross-linked tryptic peptides were separated from non-cross-linked peptides by anion

exchange HPLC, as shown in Figure 2. By comparing the elution patterns in panels A ($p[dT]_{16}$ alone), B (regA protein tryptic digest alone) and C (tryptic digest of regA protein:$[dT]_{16}$

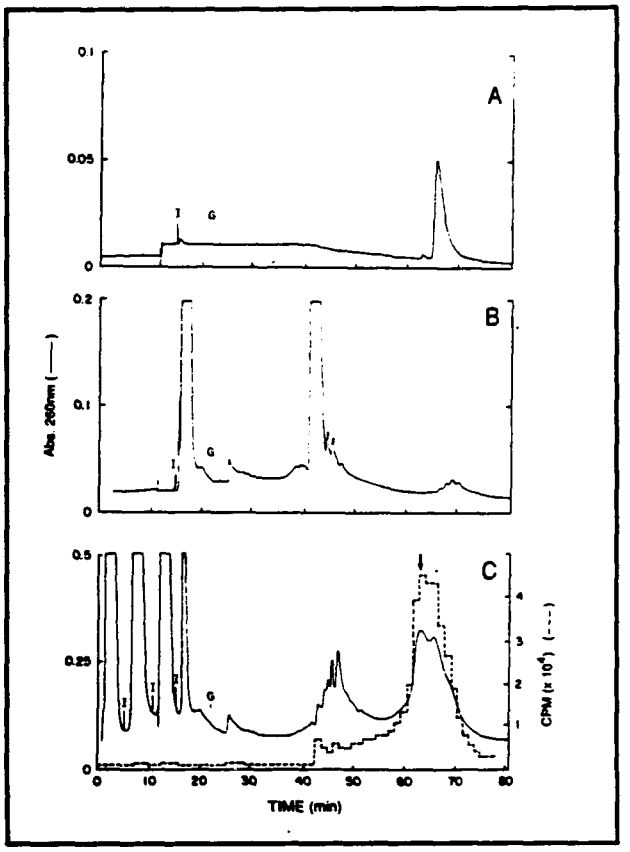

Figure 2: Anion-exchange HPLC purification of cross-linked regA tryptic peptides. Samples containing regA protein or regA protein mixed with ^{32}P-dT_{16} were irradiated with 1 X 10^5 ergs/mm^2/mm^2 of UV light, TCA-precipitated, carboxamidomethylated and then digested with trypsin (18). Samples were loaded on a Nucleogen DEAE 60-7 column equilibrated in buffer B (18). Peptides were eluted at 1 ml/min. with a 55 ml linear gradient of 0-1.5 M KCl in buffer B. I = injection point, G = start of gradient. Absorbance was monitored at 260 nm. A) [^{32}P]-$p(dT)_{16}$ alone. B) regA protein irradiated in the absence of dT_{16}. C) regA protein irradiated in the presence of [^{32}P]$p(dT)_{16}$. Symbols are: (—) OD_{260}; (---) cpm. The arrow in panel C indicates the position of the cross-linked tryptic peptides.

cross-linking reaction), one new radio-labeled peak eluting slightly earlier than the [^{32}P]$p(dT)_{16}$ was identified. Fractions from this peak, indicated by an arrow in Figure 2, were pooled and were found to contain approximately 20 percent of the initial TCA-precipitable ^{32}P-counts. The pooled sample was then desalted using a Waters SEP-PAK C_{18} cartridge and one third of the cross-linked peptide was subjected to amino acid analysis, which yielded a composition consistent with a yield of 2.3 nmoles of a 13 residue peptide.

Amino acid sequencing of the cross-linked peptide yielded a major sequence corresponding to residues 103-113 of regA protein (VISXKQKHEWK) and a minor sequence corresponding to residues 31-41 (VLYQSCHILQK) (ref. 18). The yield of the minor peptide in pool I, calculated from the molar yields at cycle 2, was 22% relative to the major peptide. The major peptide contained two tryptic cleavage sites which remained uncleaved, perhaps due to steric hinderance of trypsin cleavage by the cross-linked oligonucleotide. The absence of a high yield of the expected phenylthiohydantoin (PTH) derivative at cycle four indicates that this residue, which corresponds to phenylalanine 106, is the probable position of photochemical cross-linking in the major peptide. This result is consistent with the relatively low yield of phenylalanine in the amino acid composition. The site of cross-linking in the minor peptide is not as readily apparent; however, a potential site is at Cys 36.

RNA Binding Assays of regA Protein CNBr Fragments:

To identify the RNA binding region of regA protein by an independent approach, regA

protein was cleaved into fragments and individual fragments were tested for their ability to bind to RNA. For this purpose, regA protein was treated with CNBr which results in cleavage at methionine residues, producing six peptides, as illustrated in Figure 3. Using C-18 reverse-phase HPLC, four of the six CNBr peptides were purified as illustrated in Figure 4. The identities of the HPLC peaks were tentatively assigned by amino acid composition as: peak A, co-peptides CN3 and

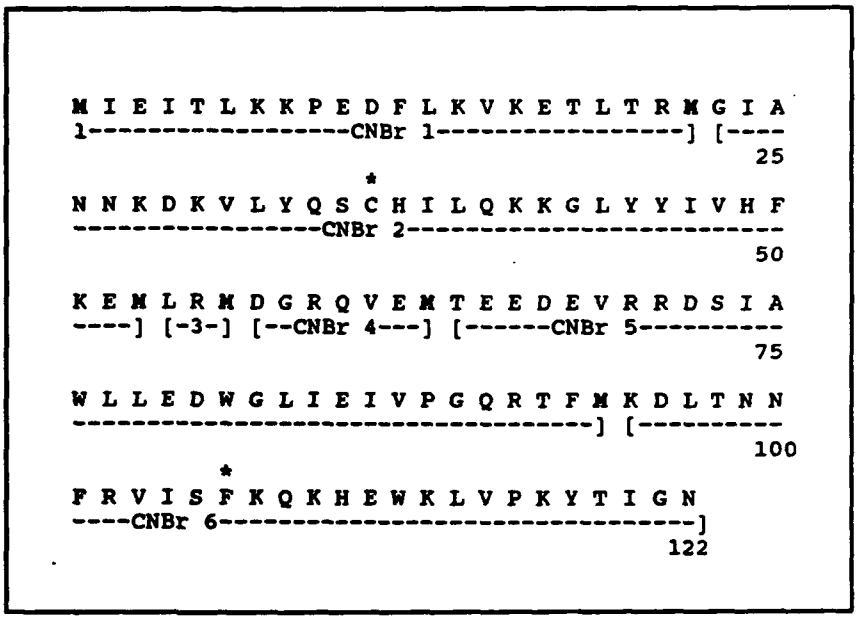

Figure 3: RegA protein amino acid sequence. Amino acids are represented by the one letter code. The * indicates the sites of photochemical cross-linking at Phe106 and Cys36. Underlining indicates the peptides generated by cleavage after Met, by CNBr treatment. Peptides are numbered CN1-6 from the N-terminus.

CN4 produced by partial cleavage; B, peptide CN6; C, peptide CN2; and D, peptide CN5. Amino-terminal sequencing revealed that pool B consisted of a mixture of peptides CN1 and CN6. Peptide CN6 was further purified to homogeneity, by C-18 chromatography.

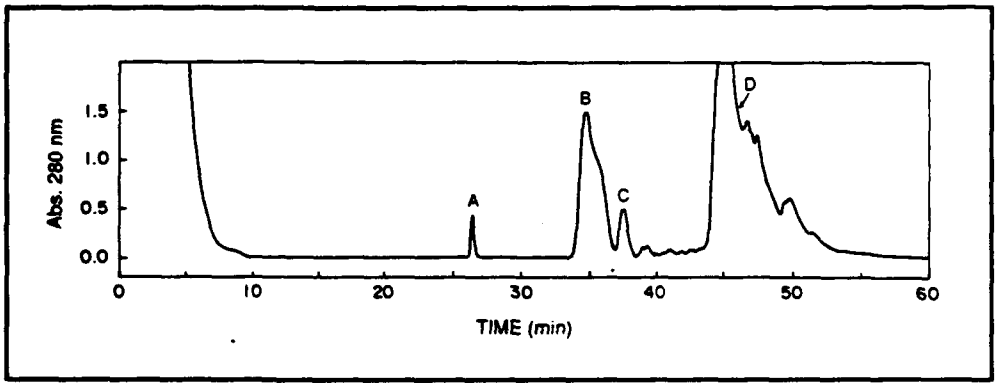

Figure 4: Reverse-phase HPLC purification of CNBr regA peptides. The sample was applied to a Vydac C-18 column and eluted with a gradient of 0-60% acetonitrile in 0.1% TFA (flow rate 1% per min.). The eluting peptides were monitored at 280 nm. Peaks A, B, C and D were pooled and the identity of the peptides determined by amino acid composition and amino-terminal sequencing.

In previous studies, the affinity of regA protein for RNA was measured by monitoring the quenching of the protein's intrinsic tryptophan fluorescence upon nucleic acid binding (3). Two of the six CNBr fragments of regA protein (CN5 and CN6) contain tryptophan residues, permitting use of a fluorescence quenching assay to detect RNA binding. Fluorescence binding assays

indicated that peptide CN6 retains the ability to bind to poly (U) as shown in Fig. 5. Binding studies of peptide CN5 were limited by the low yield of the peptide. However, at a concentration of 0.5 μM peptide CN5, no binding to poly (U) was detectable (data not shown). Peptide CN6 is 28 amino acids in length and consists of residues 95-122 of regA protein, which spans the major site of photochemical cross-linking (phenylalanine 106). The association constant, derived by fluorescence titration, for peptide CN6 binding to poly (U) in 10mM NaCl is 2 X 10^5 M^{-1}.

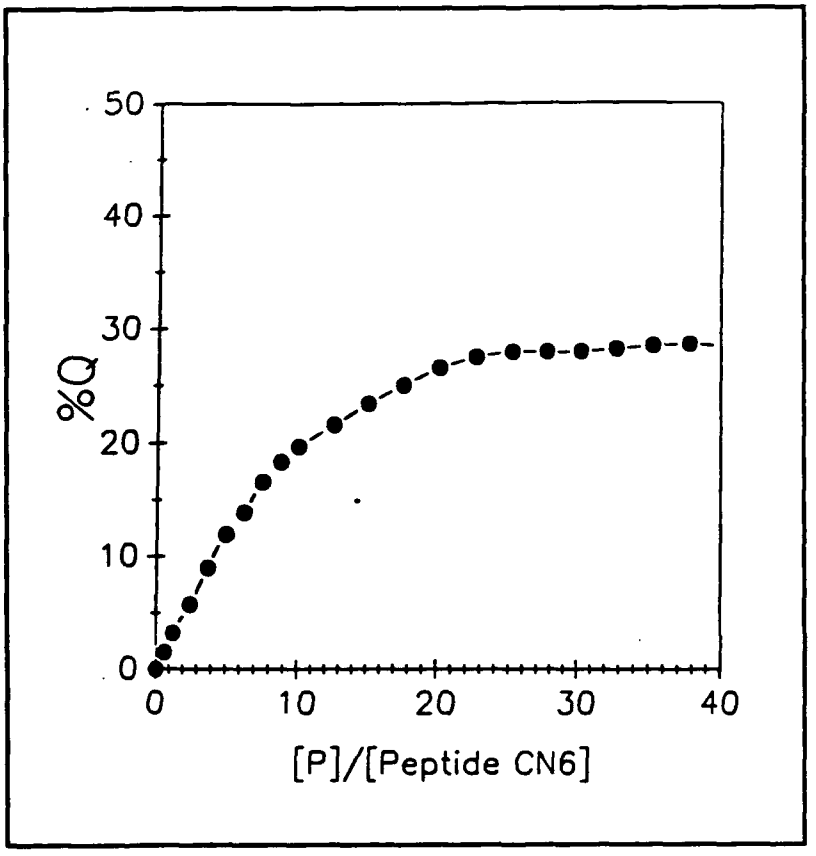

Figure 5: Titration of peptide CN6 with poly (U). Poly (U) was added to 10 μM peptide CN6 in buffer C (18) containing 10 mM NaCl, at 25 °C. Binding was monitored as a function of the quenching of the peptide's intrinsic fluorescence (λ_{em} = 347nm). The percent quenching (%Q) is plotted versus the molar ratio of uridine monophosphate to peptide.

This level of binding is approximately 250-fold less than the affinity of intact regA protein for poly (U) (K_1=5 X 10^7 M^{-1} in 10 mM NaCl) (3). The ionic contribution to the interaction of peptide CN6 with poly (U) was examined by measuring the dependence of logK_a on [Na^+] (19), which indicated that there is a maximum of one ionic interaction involved in CN6 binding to poly (U). The affinity of peptide CN6 for a specific target RNA [g44-4 (3)] was approximately 5X10^4 M^{-1} in 10 mM NaCl (data not shown) indicating that peptide CN6 does not retain the binding specificity of intact regA protein, which has a 12-fold higher (rather than a 4-fold lower) affinity for g44-4 RNA than for poly (U)(3).

The binding of CN1 and CN2 peptides was measured using a gel retardation assay, since neither peptide contains a tryptophan residue required for the fluorescence assay. Under conditions where both intact regA protein and peptide CN6 formed a complex with a 16 nucleotide RNA oligomer of gene 44, no binding to the RNA oligonucleotide was detected for either CN1 or CN2 (data not shown). Thus it appears that peptide CN2, which contains Cys36, the minor site of cross-linking, cannot bind nucleic acids as an independent domain, under the conditions tested.

In addition, the ability of peptides CN5 and CN6 to be photochemically cross-linked to [^{32}P]p(dT)$_{16}$ was examined. Peptide CN6 retained the ability to cross-link, whereas peptide CN5

was not covalently cross-linked to [^{32}P]p(dT)$_{16}$ (data not shown)(18). This is consistent with the binding properties measured by the fluorescence titrations described above. However a low extent of d(pT)$_{16}$ cross-linking to CN6 relative to the extent of regA protein crosslinking was observed, which probably resulted from several factors. The first is that whereas the regA protein:oligonucleotide ratio used in the cross-linking reaction was 1.5:1, the corresponding value for the CN6 peptide was only 0.5. In addition, the CN6 peptide lacks one of the two cross-linking sites (i.e., Cys 36) that we have identified in regA protein.

Site-specific Mutagenesis of regA Protein

To determine if Phe 106, the major site of photo-crosslinking to nucleic acid, plays a significant role in regA protein binding to RNA, site-specific mutagenesis was used to introduce substitutions at residue 106. Oligonucleotide-directed mutagenesis was carried out using a degenerate oligonucleotide (complementary to: 5' C CGA GTT ATT TCT T/GNT AAA CAA AAA CAT GAA TGG 3') and a previously constructed phage M13 derivative carrying the wild type regA gene. Three clones with nucleotide changes coding for substitutions of Val, Tyr and Cys at Phe 106 were obtained. Restriction fragments carrying the regA gene from the three mutant clones were subcloned into the inducible expression vector pAS$_1$. The mutant proteins were produced at high levels by induction of expression from the pAS$_1$ vector, as illustrated for the F106Y and F106V mutants in Figure 6. All three proteins have recently been purified using a previously described method involving chromatography on DEAE-cellulose and poly(U)-agarose (20). Studies are now being initiated to examine the effects of the amino acid substitutions on regA

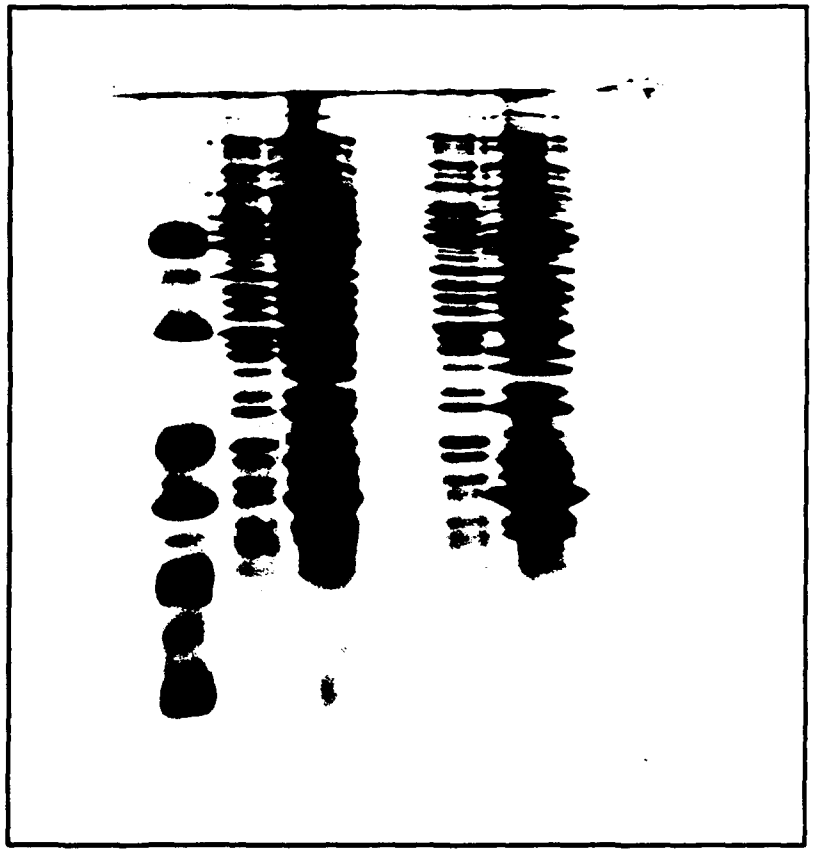

Figure 6: SDS-polyacrylamide gel analysis of induction of mutant regA proteins expression. Total cell extracts from AR120 cells containing pAS$_1$-regA expression plasmids were applied to a 15% polyacrylamide-SDS gel. Lanes: 1) M.W. standards; 2) uninduced cells containing pAS$_1$-regA F106Y; 3) nalidixic acid-induced cells containing pAS$_1$-regA F106Y; 4) uninduced cells containing pAS$_1$-regA F106V; 5) nalidixic acid-induced cells containing pAS$_1$-regA F106V.

protein's nucleic acid binding properties. The activities of the three mutant proteins will be assayed by photo-crosslinking, fluorescence quenching assays, and *in vitro* translational repression assays,

in order to fully assess the contribution of Phe 106 to the free energy of binding to nucleic acids.

CONCLUSIONS:

To identify the nucleic acid binding domain of regA protein, it was photo-cross-linked to $[^{32}P]p(dT)_{16}$ and a major cross-linked peptide spanning residues 103-113 of regA protein (VISXKQKHEWK) was identified by gas-phase sequencing. The lack of a PTH-derivative at cycle 4 of the sequencing indicated that the site of cross-linking was phenylalanine 106. A second peptide corresponding to residues 31-41 was cross-linked with lower efficiency. The site of cross-linking to the minor peptide was tentatively assigned to cysteine 36. The fact that only two sites of cross-linking were observed and that cross-linking to $p(dT)_{16}$ was eliminated (using cross-linking times below 20 min.) by the addition of an equimolar amount of a specific RNA target (g44-4), makes it unlikely that cross-linking occurred through nonspecific adduct formation and, in fact, the latter has never been shown to occur with photochemical cross-linking (13). It is important to point out, however, that while photochemical cross-linking can be readily used to identify residues at the interface of protein:nucleic acid studies, it does not yield information regarding the contribution of these residues to the overall free energy of binding. Hence, *in vitro* mutagenesis studies are needed to better evaluate the level of involvement of phenylalanine 106 and cysteine 36 in regA protein binding. These studies are in progress now.

The RNA binding domain of regA protein was further examined by chemical cleavage with CNBr. Four CNBr peptides were purified by reverse-phase chromatography and tested for RNA binding. In 10 mM NaCl, peptide CN6 bound to poly (U) with an affinity of 2×10^5 M^{-1} and UV exposure induced covalent cross-linkage between CN6 and $p(dT)_{16}$. Based on salt sensitivity studies, which indicated that binding involves no more than one ionic interaction, it seems unlikely that the binding of peptide CN6 is driven by ionic interactions even though the peptide has a net charge of +4. This agrees with earlier studies indicating that intact regA protein binding is primarily enthalpy driven (3).

The results presented here suggest that the region of regA protein corresponding to CN6 has many of the necessary elements for non-specific regA protein-nucleic acid interactions but lacks determinants for specific interactions. Thus, even though peptide CN6 represents less that 25% of the primary sequence of regA protein, it nonetheless retains nearly 70% of the free energy of binding to poly (U). In contrast, peptide CN6 retains only about 50% of the free energy of binding to a specific target. Since peptide CN6 shows no specificity towards the g44-4 target, specificity of regA protein binding must involve the participation of additional residues elsewhere in the protein. Likely candidates would be residues in the NH_2-terminal third of regA protein, including residues in the second cross-linked peptide (residues 31-41). This is consistant with the observation that *regA protein with a Leu for Val substitution at position 15 was approximately 3-5 fold less active in in vitro* translational repression assays (21,22). In addition, Jozwik and Miller (23) have identified NH_2-terminal mutants of regA protein with altered mRNA repression specificity.

Future experiments will examine the contribution of Phe 106 to nucleic acid binding by measuring the effects of specific amino acid substitutions at residue 106 on RNA binding affinities. The role of the COOH-terminal region of regA protein will be further examined by partial proteolysis and additional site specific mutagenesis studies.

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1. Abstracts:

a. Sattar, A.K.M., O'Malley, S.M., Williams, K.R., Konigsberg, W.H. and Spicer, E.K. (1992). "Structure-Function Studies of RegA Protein: Probing Domains of Nucleic Acid Binding." *Translational Control*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY p.

b. O'Malley, S.M., Sattar, A.K.M. and Spicer, E.K. "Structure-Function Studies of RegA Protein: Probing Domains of Nucleic Acid Binding." (1993). *Biophysics Society Meeting*, Washington, DC.

2. Journal Articles:

a. Webster, K.R., Keill, S. Konigsberg, W., Williams, K.R. and Spicer, E.K. (1993). "Identification of Amino Acid Residues at the Interface of a RegA Protein-Nucleic Acid Complex." *J. Biological Chemistry*, in press.

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A.K.M. Sattar	6/1/92 to 6/30/92 Postdoctoral Associate

E. REPORT OF SCIENTIFIC INVENTIONS:

None.

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