The RNA-Binding Domain of Transcription Termination Factor Rho: Isolation, Characterization, and Determination of Sequence Limits†

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ABSTRACT: The function of transcription termination factor rho from *Escherichia coli* is dependent upon its ability to bind RNA. To delineate the extent of the RNA-binding domain in the rho polypeptide, plasmid-borne copies of altered forms of the *rho* gene were expressed to yield truncated versions. These proteins were then isolated and assayed for their ability to bind an RNA oligonucleotide [oligo$(C)_8$] using an ultraviolet light-induced cross-linking assay. A fragment consisting of the first 116 amino acid residues, rho(1–116), bound oligo$(C)_8$ with nearly the same affinity and specificity as the intact protein. Smaller derivatives lacking 5, 13, or 22 residues from the N terminus or with 2 fewer residues at the C terminus bound RNA with reduced affinity, while derivatives lacking 27 N-terminal residues or having just the first 109 residues were unable to bind RNA. Derivatives lacking N-terminal residues were considerably less soluble than rho(1–116). The physical properties of rho(1–116) indicate that it possesses approximately 20% each of α-helix and β-sheet and is monomeric in solution. Thus, the results show that this fragment, which contains an RNP1 sequence motif, will be a good model for future physical–chemical studies of the protein–RNA interactions of rho.

Rho factor in *Escherichia coli* is needed to dissociate RNA transcripts from biosynthetic complexes in which RNA polymerase has reached a termination site on a DNA template (Platt & Richardson, 1992). RNA release is effected by actions of rho protein on the ternary transcription complex that are coupled to the hydrolysis of nucleoside triphosphates. An essential early step in the process is the binding of rho to the nascent RNA.

Rho protein functions as a hexamer of a single polypeptide chain with 419 amino acid residues (Finger & Richardson, 1982; Pinkham & Platt, 1983). Trypsin preferentially cleaves the subunits at two sites, one near residue 130 and the other at Lys_{283} (Bear et al., 1985; Dolan et al., 1990). Cleaved rho binds RNA in the segment that has the first 130 amino acids (Dolan et al., 1990). These results suggest that the amino-terminal segment of 130 residues comprises a distinct domain that might be able to bind RNA by itself and thus be studied as a separate subcomponent.

We report here the isolation and partial characterization of a 116-residue fragment of rho polypeptide that binds nearly as tightly to a test RNA ligand as the full-length protein. Smaller fragments lacking further residues from the C-terminus or lacking residues from the N-terminus either bound the test RNA very poorly or formed inactive aggregates. This 116 amino acid rho RNA-binding domain protein possesses properties which make it suitable for detailed molecular studies of fundamental RNA–protein interactions.

EXPERIMENTAL PROCEDURES

Biochemicals and Enzymes. Unlabeled ribonucleoside triphosphates and deoxynucleoside triphosphates were purchased from Boehringer-Mannheim Biochemicals and Pharmacia, respectively. [γ-^{32}P]ATP and [2,8-^{3}H]ATP were from ICN Chemical and Radioisotope Division, and [α-^{35}S]dATP was from Amersham. Restriction enzymes, T4 DNA ligase, DNA polymerases, Bal31 nuclease, and T4 polynucleotide kinase were from New England Biolabs; calf intestinal alkaline phosphatase was from Boehringer-Mannheim; proteinase K and Sequenase were from United States Biochemicals; myosin, polynucleotide phosphorylase, and bovine serum albumin were from Sigma; RNase A and DNase I were from Worthington Biomedical. Poly(C), poly(U), and poly(A) were from Miles Laboratories, and poly(dC) was from Pharmacia. The average length of these polymers was determined to be >100 nt[1] by analysis, after electrophoretic separation on 6% urea–polyacrylamide gels of samples of each labeled with ^{32}P at their 5′ ends by transfer from [γ-^{32}P]ATP catalyzed by polynucleotide kinase.

Oligoribocytidylates [oligo$(C)_n$] were prepared as described in Richardson (1982) and labeled at their 5′ ends with ^{32}P as described in Dolan et al. (1990). DNA oligonucleotides were synthesized on an Applied Biosystems Model 380A apparatus in the Institute for Molecular and Cellular Biology, Indiana University, Bloomington, IN.

Construction of pDMrho(1–116). The plasmid pCB111, which contains the *rho* gene of *E. coli* downstream from T7ϕ10 promoter and Shine–Dalgarno ribosome binding site (Richardson & Richardson, 1992a), was first digested successively with *Hpa*I and with alkaline phosphatase and then ligated to a 5′-phosphorylated self-annealed DNA oligonucleotide with the sequence 5′-GCATAAGCTTATGC. The resulting DNA was used to transform JM103, and the presence of pDMrho(1–116) was confirmed by sequence analysis. It encodes a truncated version of rho that has, as its last *rho*-encoded

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[1] Abbreviations: bp, base pair(s); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; HEPES, 4-(2-hydroxyethyl)piperazineethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; nt, nucleotide(s); PCI, phenol/chloroform/isoamyl alcohol (25:24:1); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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residue, Val_{116}, followed by an Ala encoded by the linker as the C-terminal residue.

Construction of Plasmids That Encode Rho Proteins Ending at Residues Prior to 116 (the C-Terminal Deletions). Plasmid pCB111 that had been digested with *Hpa*I was digested at 30 °C with Bal31 nuclease (0.1 unit/μg of DNA) in a solution containing 0.6 M NaCl, 0.05 M Tris-HCl (pH 7.5 at 30 °C), 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 50 μg/mL bovine serum albumin. Samples were removed at 1-min intervals, made 20 μM with EGTA, and extracted once with phenol and once with PCI. After repairing the ends with T4 DNA polymerase, the DNA was once again extracted with PCI. After ethanol precipitation, the Bal31-digested DNA was ligated to a 5′-phosphorylated self-annealed DNA oligonucleotide linker with the sequence 5′-CGCTTAATTAATTAAGCG. This oligonucleotide places chain-termination codons in all three phases downstream from the partially digested DNA. After adding 72 μL of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA), 8 μL of DMSO, and 1 μL of 0.2 M spermine to the 10-μL ligation solution, the sample was incubated on ice 30 min and centrifuged in a microfuge for 30 min. The precipitated DNA was rinsed with ethanol, dried, and resuspended in 150 μL of hybridization buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 5 mM EDTA). This solution was heated to 65 °C, and cooled slowly to 4 °C, and then kept cold until it was used to transform DH5αF′. Plasmids from transformed clones were first screened for the size of deletion by digestion with *Ase*I, which yields a DNA fragment that extends from the T7 promoter to the translation termination site in the linker DNA. The positions of the deletion end points were determined by DNA sequence analysis of the candidate plasmids using a primer with the sequence 5′-CCGTTCCGCGGACACTCC (nt 195–213 in *rho* with respect to the ATG encoding the initial Met). This procedure yielded the plasmids pDMrho(1–114), pDMrho(1–109), and pDMrho(1–103) encoding truncated forms of rho ending in the sequences Leu_{114}-Ser-COOH, Arg_{109}-Arg-Leu-Ile-Asn-COOH, and Pro_{103}-Ala-COOH, respectively. In each case the numbered residue is the last of the *rho*-encoded amino acids. The unnumbered residues were encoded by the linker.

Construction of Plasmids That Encode Rho Protein Fragments Lacking N-Terminal Residues and Ending at Residue 116 (the N-Terminal Deletions). To allow construction of deleted forms of the *rho* gene that could be expressed by T7 RNA polymerase, pDMrho(1–116) was altered by the following procedure. A DNA oligonucleotide with the sequence 5′-GGTCTAGAGTTTAACTTTAAGAAGGAGATATACCCATGGCATATGCC-3′ was annealed and converted to a double-stranded form by action of the Klenow fragment of *E. coli* DNA polymerase I (PolIK). After digestion with *Xba*I and *Nde*I, it was ligated to the large *Xba*I/*Nde*I fragment of pDMrho(1–116), to create a new plasmid called pDM2.

To prepare the variants with deletions in the part of the gene encoding the N-terminus of rho, the plasmid pDMrho(1–116) was digested with *Nde*I and purified by treatment with 0.2% SDS and 1 μg/mL proteinase K for 30 min at 37 °C prior to extraction twice with PCI and once with chloroform/isoamyl alcohol (24:1) and precipitation with ethanol.

Digestion with Bal31 was as described in preparing the C-terminal deletions except that 0.5 unit of Bal31 was used per microgram of DNA and samples were taken at 20-s intervals. After repairing the ends by action of T4 DNA polymerase, the DNA was digested with *Kpn*I and fragments with the length of 850–1000 bp were purified by gel electrophoresis on a 1% low-melting agarose gel. To extract the DNA, the gel segment was heated to 65 °C, mixed with 2 volumes of TE buffer, and quickly frozen at −70 °C. After thawing at 20 °C, the agarose was removed by centrifugation.

To construct plasmids with deletions that could be expressed, *Nco*I-digested pDM2 was repaired with PolIK and digested further with *Kpn*I. The 5.4 kbp fragment was then separated by electrophoresis through a 0.8% low-melting agarose gel, extracted from the gel, and ligated to the isolated 850–1000 bp fragments. Since the repaired *Nco*I-digested fragment ends with the Met initiation codon sequence placed downstream from the T7 promoter and a ribosome binding site, the plasmid, when fused in frame with the Bal31-digested fragments from pDM(1–116), could be used to express rho protein fragments. Sequence analysis of several candidates yielded the plasmids pDMrho(5–116), pDMrho(13–116), pDMrho(22–116), and pDMrho(28–116) in which the initiator Met is fused in frame with the sequence encoding residues 5, 13, 22, and 28 of rho, respectively.

Expression and Isolation of Rho(1–116). To achieve controlled synthesis of rho(1–116), pDMrho(1–116) was transferred to BL21DE3[pLysS], a strain with the T7 RNA polymerase gene under control of the *lac* repressor (Studier et al., 1990). After growth at 37 °C in a buffered, rich medium (Mott et al., 1985) to approximately 5×10^8 bacteria/mL (A_{600} = 1), rho expression was initiated by the addition of 1 mM IPTG. After 3 h the cells in 250 mL were harvested by centrifugation and resuspended in 10 mL of lysis buffer (50 mM Tris-HCl, pH 7.9, 2 mM EDTA, 1 mM dithioneitol, 1 mM 2-mercaptoethanol, 5% glycerol) containing 260 mM NaCl and 100 μg/mL lysozyme. After incubation at room temperature for 15 min, sodium deoxycholate and Triton X-100 were added to final concentrations of 0.25% and 0.1%, respectively, and the suspension was incubated on ice for 15 min. Next, $MgCl_2$ and DNase I were added to 25 mM and 5 μg/mL, respectively, and the mixture was incubated for 30 min. The mixture was then diluted with 1 volume of lysis buffer and centrifuged for 30 min at 13000*g*. Polymin P, pH 7.9, was added to the supernatant to a final concentration of 0.4%, and after 5 min at 0 °C, the precipitate was removed by centrifugation for 15 min. Proteins were precipitated from the supernatant by adding 0.5 g of $(NH_4)_2SO_4$ and 5 μL of 1 M KOH per mL, collected by centrifugation for 15 min at 20000*g*, resuspended in 2 mL of TEDG (50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol) with 0.2 M NaCl, and loaded on a 1.5 × 55 cm Sephadex G-50 column equilibrated with the same buffer. The column was washed with buffer, and the fractions containing the protein were pooled and dialyzed overnight into HEDG (50 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol) with 50 mM NaCl. The solution was passed over a Mono S column equilibrated with HEDG with 50 mM NaCl, and the flow-through fractions containing protein were pooled and dialyzed into TEDG with 50 mM NaCl. This material was passed over a Mono-Q column equilibrated with TEDG with 50 mM NaCl, and the flow-through fractions containing the protein were pooled and dialyzed into TEDG[50] (TED with 50% glycerol) with 50 mM KCl. This purified solution of rho(1–116) was stored at −20 °C at a concentration of 1.1 mg/mL. The yield of protein from 250 mL of culture was approximately 6 mg or 10% of the estimated amount of overproduced protein.

Expression and Isolation of the C- or N-Terminal Deletion Variants of Rho(1–116). To obtain partially purified proteins, the proteins were overexpressed in DH5αF′ using a system

which supplies the T7 RNA polymerase via an M13 phage derivative, M13[mGP1-2], carrying the gene for T7 RNA polymerase under control of the *lac* repressor (Tabor, 1987). The phage was introduced into the culture when A_{600} = 1 at a multiplicity of infection of 10, and IPTG was added to a concentration of 1 mM. The cells were grown for 3 h and then harvested and lysed as described for rho(1–116). Since the derivatives with C-terminal truncations were all soluble, the purification was identical to that for rho(1–116) through the ammonium sulfate precipitation step. Instead of using the Sephadex G-50, Mono-S, and Mono-Q columns, however, that fraction was passed through a 70-mL Sephadex G-75 column (90 × 1 cm) only. Fractions containing the deletion products, as identified by gel electrophoresis, were pooled and dialyzed *vs* TEDG^{50} with 100 mM NaCl. The purity of the various proteins was in the range of 70–80%.

To isolate the derivatives lacking amino-terminal residues, their expression was induced with M13[mGP1-2] and IPTG, the cells were harvested and lysed as described above. However, instead of being in the supernatant after lysis, these proteins all were found to be predominantly in the pellet, presumably in the form of inclusion bodies. The cell lysate pellets were washed with ND buffer (TEDG containing 0.1 M NaCl and 0.01% sodium deoxycholate) and then solubilized in 1 mL of ND buffer containing 2 M guanidine hydrochloride per 5 mL of original culture for 1 h at 4 °C with constant rocking. The samples were centrifuged for 15 min to remove insoluble material and then diluted with 4 volumes of 2 M guanidine hydrochloride in ND buffer before dialysis for 15 h against ND buffer. The protein solutions were centrifuged again to remove material that precipitated during the dialysis and then, if needed, concentrated by applying dry Sephadex G-75 to the outside of the dialysis bag until the volume had been reduced sufficiently. Finally, they were dialyzed against ND buffer with 50% glycerol and stored at −20 °C.

Assays. Protein was determined with the Bio-Rad assay using bovine serum albumin as a standard. Polyacrylamide gel electrophoresis used for measuring the approximate size and amounts of the truncated forms of rho protein was in the sodium dodecyl sulfate–Tricine system as described by Schägger and von Jagow (1987). Low-molecular-weight standards were obtained from Sigma Chemical Co., and the amount of truncated protein in a partially purified preparation was estimated visually by comparison with known amounts of purified rho(1–116) run in neighboring lanes. Assays for the binding of oligo(C) to the proteins were performed by the direct UV cross-linking method described by Dolan et al. (1990). Details of the amounts of oligo(C) and rho that were used are presented in the figure legends. The binding of an RNA ligand [R, usually oligo(C)$_9$] to a site (P) on rho or a fragment of rho to form a complex (C) is described by the equation R + P ⇌ C. The equilibrium dissociation constant K_d for that reaction is

$$K_d = \frac{[R\text{–}C][P\text{–}C]}{[C]} \quad (1)$$

where [C] is the molar concentration of the complex and the values of [R] and [P] are the initial molar concentrations of the RNA and the sites for RNA on the protein. The amount of oligo(C) cross-linked to rho or rho(1–116), B, is related to [C] by a proportionality constant A, which includes the efficiency of cross-linking. Assuming the efficiency of cross-linking is constant for the range of [R] values used with a given rho, the equation $B = A[C]$ can be combined with the quadratic equation solution for C of eq 1 to yield eq 2, in which B is a function of [R]. Values of K_d and A were

$$B = (A/2)[(K_d + [P] + [R]) - ((K_d + [P] + [R])^2 - 4[R][P])^{0.5}] \quad (2)$$

determined from best fits of curves for plots of B as a function of [R] using the Grafit program of Leatherbarrow (1990).

ATPase activity was measured in 20-μL solutions containing the indicated amount of rho protein, 30 ng of poly(C), and 1 mM [^{3}H]ATP (10 000 cpm/nmol) in 0.04 M Tris-HCl, pH 7.9, 0.05 M KCl, 0.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.3 mg/mL acetylated bovine serum albumin. After 10 min at 37 °C the reaction was stopped with 4 μL of 25 mM EDTA and 25 mM ADP and the amount of [^{3}H]ADP release was determined as described in Richardson and Richardson (1992b).

CD Spectroscopy. Circular dichroism was carried out using a JASCO Model J20A CD spectrophotometer with a cell length of 1 cm over the range 250 to ~190 nm. The samples were dialyzed extensively *vs* 500 mL of 40 mM Tris-HCl, pH 7.9, 100 mM KCl, or 10 μM sodium phosphate buffer, pH 7.5, before analysis. The protein concentration was 2.5–5 μM. The percentages of α-helix, β-sheet, and turns were estimated using a computer program kindly supplied by J. T. Yang (Venyaminov et al., 1991).

RESULTS

Isolation of Rho(1–116). Our approach to the preparation of the RNA-binding domain of rho as a fragment was to have it expressed from an altered form of the *rho* gene. Since the fragment might be toxic for the host, we used a T7 expression system that allowed the conditional expression of the gene (Studier et al., 1990). The plasmid pCB111 has *rho* downstream from the promoter and Shine–Dalgarno sequence of T7φ10 (Richardson & Richardson, 1992a). By insertion of a DNA oligonucleotide containing an in-frame translational termination codon between the two *Hpa*I sites of the *rho* gene in pCB111, we created pDMrho(1–116), which is predicted to encode a fragment consisting of the first 116 residues of rho with an extra alanine residue at the C-terminus.

To achieve controlled expression of the fragment, pDM-rho(1–116) was transferred to *E. coli* BL21DE3[pLysS], a strain with the T7 RNA polymerase gene (T7φ1) under control of the *lac* repressor (Studier et al., 1990). After growth at 37 °C to approximately 5 × 10^8 cells/mL, *rho* expression was initiated by adding IPTG to 1 mM and the cells were cultured for an additional 3 h. A polypeptide with the expected gel electrophoretic mobility was induced by this procedure (Figure 1). A significant fraction of the protein was also in an extract prepared by detergent (sodium deoxycholate/Triton X-100) treatment of lysozyme-digested cells and could be purified by conventional column chromatography, as described in the Experimental Procedures section. The yield from 250 mL of induced culture was 6 mg of homogeneous rho(1–116) protein (Figure 1) or about 10% of the total initially present in the induced cells. Since the amount of protein obtained was adequate for our further analyses, no attempt was made to develop a higher yield purification scheme.

Rho(1–116) Binds a Short RNA Ligand. Because we expected that rho(1–116) might bind RNA as a monomer, we chose a test RNA ligand that would have an intrinsically high affinity and would be small enough to be unable to bind simultaneously to more than one subunit in native hexameric rho. In principle, such a ligand should bind as tightly to a monomeric RNA-binding domain as to a single complete

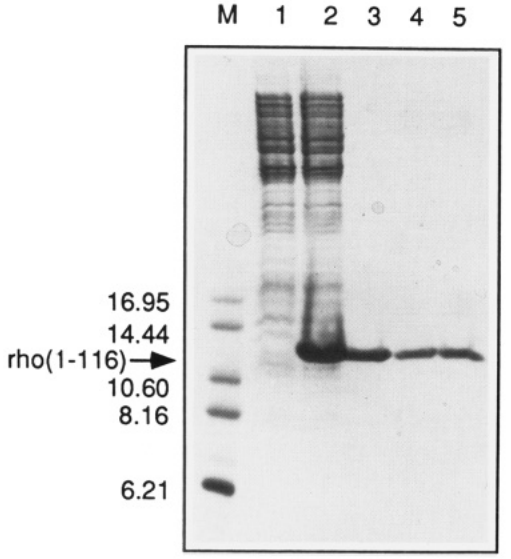

FIGURE 1: Induction and purification of rho(1–116). Samples of cell culture and of fractionated rho(1–116) were separated by electrophoresis on a 17% polyacrylamide/sodium dodecyl sulfate/Tricine-buffered system (Schägger & Von Jagow, 1987), and the proteins were stained with Coomassie Brilliant Blue. The samples in the indicated lanes were as follows: M, marker proteins; 1, 5×10^7 cells just prior to adding IPTG; 2, 5×10^7 cells 3 h after addition of IPTG; 3, Sephadex G-50 column fraction; 4, Mono-S column fraction; 5, Mono-Q column fraction. The yield was approximately 6 mg of protein in the Mono-Q fraction isolated from 250 mL of culture that contained approximately 40 mg of rho(1–116).

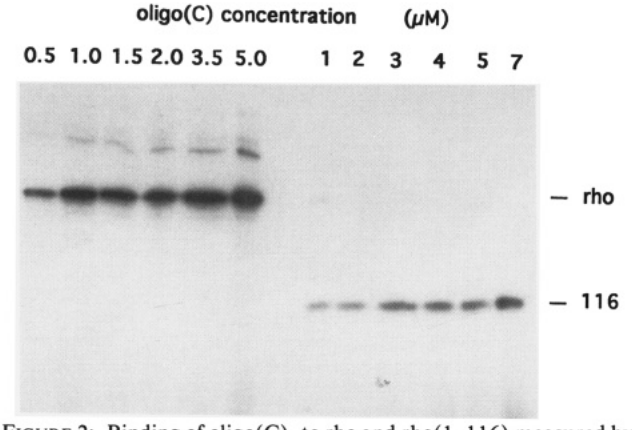

FIGURE 2: Binding of oligo(C)$_8$ to rho and rho(1–116) measured by UV cross-linking. Samples containing 1 μM (25 pmol, as subunits) of rho or rho(1–116) in 25 μL of 40 mM Tris-HCl, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 1 mM $NaHPO_4$, 0.1 mM dithiothreitol, and 0.1 mM EDTA were irradiated as described in Dolan et al. (1990) in the presence of the indicated concentrations of [^{32}P]p(Cp)$_7$C [oligo(C)$_8$]. The proteins with complexed oligonucleotides were isolated by acid preciptiation and separated by gel electrophoresis. The figure shows an autoradiograph of the gel. The amounts of radioactivity that comigrated with rho or with rho(1–116) were quantitated by phosphor-image autoradiography. The data were fit to the binding curve by the Grafit program (Leatherbarrow, 1990).

subunit in rho. The assay we used involves direct ultraviolet light-induced cross-linking of labeled oligo(C)$_8$ (Dolan et al., 1990). Figure 2 shows that after UV treatment of mixtures of oligo(C) with rho or rho(1–116) some of the oligonucleotide comigrated with the respective protein. The amounts linked were quantitated by phosphor-image autoradiography and the data used to determine the binding parameters by the curve-fitting procedure described in the Experimental Procedures. The dissociation constants (K_d) for the binding curves that fit the data best are 1.0 ± 0.6 μM and 8.3 ± 4.3 μM for rho and rho(1–116), respectively. Thus, rho(1–116) bound the RNA ligand with about 8-fold lower avidity than the complete rho protein. Similar binding titrations were also performed with oligo(C)$_9$, yielding K_d values of 0.35 ± 0.5 μM and 3.4 ± 0.4 μM with rho and rho(1–116), respectively (data not shown). These results confirm that the rho protein fragment binds the oligonucleotide less well than the intact protein.

Using an ultrafiltration technique to separate rho–oligo(C) complexes from free oligo(C), Wang and von Hippel (1993) found that oligo(C)$_{10}$ binds to about three sites per hexamer with $K_d = 0.22$ μM and another three sites with $K_d = 1.7$ μM. The data we obtained with the cross-linking procedure fit very well to a binding curve for a single type of site. Since the K_d value that we determined for the intact rho–oligo(C)$_9$ complex was, within experimental error, equivalent to the K_d value measured by Wang and von Hippel for the binding of oligo(C)$_{10}$ to the higher affinity site, we conclude that the cross-linking procedure was detecting primarily the interaction with the higher affinity site. One possible reason why binding to the lower affinity site was not detected as well could be because the efficiency of cross-linking is much lower for that site.

At saturation, the yields of cross-linking (the values of A in eq 2) were 0.11 and 0.06 pmol of oligo(C)$_8$ per 25 pmol (as subunits) of rho and rho(1–116), respectively. These yields depend on the efficiency, which is very low for direct ultraviolet linking [usually <2%; see Dolan et al. (1990)], and the number of available sites, which for native rho is one high-affinity site for every two subunits (Geiselmann et al., 1992b; Wang & von Hippel, 1993). The lower yields with the fragment may be reflecting structural differences of its site compared to the site(s) in intact rho and/or the presence of a significant fraction of misfolded (inactive) protein in the preparation.

RNA-Binding Properties of Smaller Segments of the Domain. After establishing that rho(1–116) bound a short RNA ligand reasonably well, we wished to determine the minimum segment of the rho polypeptide that can bind with similar affinity. We therefore devised methods for expressing and isolating shorter versions that were truncated at either the carboxyl or amino terminus. They were expressed from plasmids in which segments of the *rho* gene had been removed by progressive digestion with Bal31 nuclease. The detailed procedures for preparing the plasmids with the altered genes are described in the Experimental Procedures section.

Three peptides that had fewer *rho*-encoded residues than rho(1–116) at their carboxyl termini, rho(1–103), rho(1–109), and rho(1–114), were readily isolated from soluble extracts of cells in which they had been expressed. Because of the nature of the fusion to a chain-termination linker used for preparing the deletions, these peptides had one or more non-rho residues at their carboxyl termini. According to the DNA sequence analysis of the altered genes, rho(1–114) ends with the sequence -Leu$_{114}$-Ser; rho(1–109) with -Arg$_{109}$-Arg-Leu-Ile-Asn; and rho(1–103) with -Pro$_{103}$-Ala. Because of the extra residues on some of the derivatives, only rho(1-103) migrates significantly faster than rho(1–116) when the isolated peptides were analyzed by polyacrylamide gel electrophoresis (Figure 3, right panel). The estimates of the size of the isolated fragments based on their gel electrophoretic mobility are all very close to the predicted sizes (Table 1), indicating that they have not been extensively modified after expression.

Four fragments were prepared that had fewer residues than rho(1–116) at their amino termini. The names used designate the rho residues present besides the N-terminal methionine. Thus, rho(5–116) has the N-terminal Met fused to residues

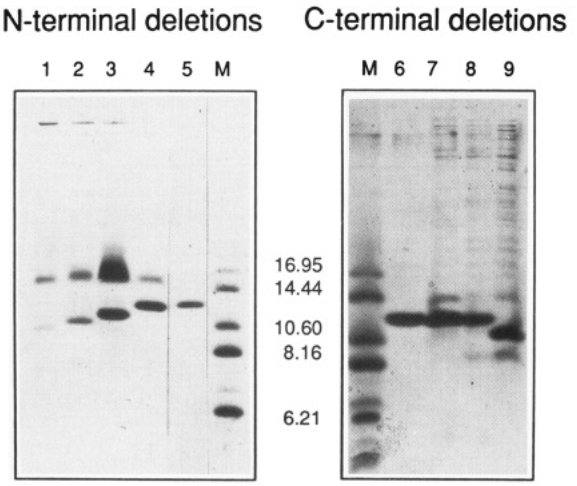

FIGURE 3: Gel electrophoretic analysis of isolated rho protein fragments. Samples were separated by gel electrophoresis and stained with Coomassie Brilliant Blue. Lanes M, protein size standards; lane 1, rho(28–116); lane 2, rho(22–116); lane 3, rho(13–116); lane 4, rho(5–116); lane 5, rho(1–116); lane 6, rho(1–116); lane 7, rho(1–114); lane 8, rho(1–109); lane 9, rho(1–103).

Table 1: Comparison of Predicted Size of the Rho Fragments with the Size Estimated from Electrophoretic Mobility

protein	total no. of residues[a]	predicted size from sequence[a]	estimated size from SDS–PAGE[b]
rho(1–116)	117	13 015	12 600
rho(1–114)	115	12 786	12 500
rho(1–109)	113	12 606	12 500
rho(1–103)	104	11 485	11 300
rho(5–116)	114	12 687	12 500
rho(13–116)	105	11 819	11 400
rho(22–116)	96	10 819	10 900
rho(28–116)	90	10 222	10 200

[a] These values take into account the residues added due to sequences introduced as a result of the construction of the derivative. [b] These values were estimated from the relative mobility with respect to the size standards in Figure 3.

5–116. Unlike the C-terminal deletions, these peptides were not present in soluble form in the extracts and thus had to be isolated from pellets of lysed cells by solubilization with guanidine hydrochloride. As a control, some rho(1–116) was also prepared by solubilization from the cell debris [rho(1–116_{CD})]. This procedure yielded peptides that were pure except for one major contaminant that had the same mobility as the lysozyme used to prepare the extracts (Figure 3, left panel). The sizes of the isolated peptides again agreed with those predicted from the sequence (Table 1).

Each of these isolated proteins was assayed for its ability to become cross-linked to oligo$(C)_9$ by the standard UV light treatment either without or with various polynucleotides present. The results from a representative experimental set are shown in Figure 4, and the averages of data from three experimental sets are presented in Table 2. No cross-linking was observed with either rho(1–109) or rho(28–116). Although all the other peptides yielded some cross-linked material, they could be distinguished from rho(1–116) by the ability of various polynucleotides to reduce the cross-linking. The three N-terminally truncated derivatives that could still bind showed partial selectivity in that the cross-linking of oligo$(C)_9$ was more sensitive to the presence of poly(C) than to the other polymers. In contrast, the two C-terminally truncated derivatives with partial binding showed two patterns of nonselective binding: the low amount of oligo$(C)_9$ bound

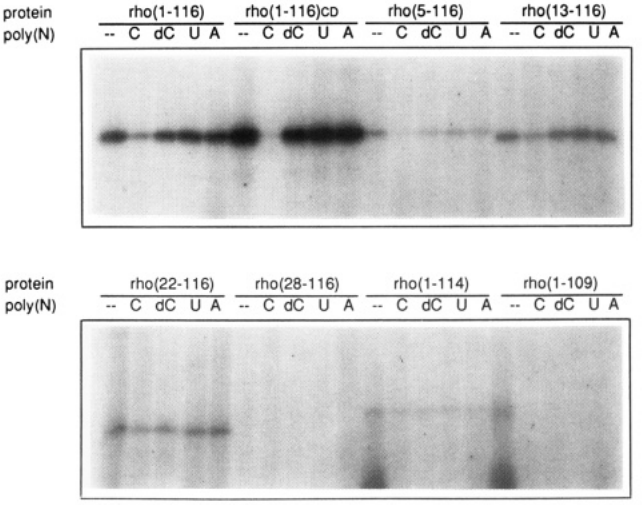

FIGURE 4: UV light-promoted cross-linking of oligo$(C)_9$ to truncated forms of rho protein. [^{32}P]Oligo$(C)_9$ at 8 μM (1.8 nmol of residues) was first mixed in 25 μL of binding buffer with 1.6 μM of the indicated peptide fragments, and 9 nmol (residue amount) of the indicated polynucleotides was then irradiated with UV light. The samples were separated by gel electrophoresis. The figure shows autoradiographs of the section of gels containing the peptide fragments. The lane designations are as follows: - -, no added polynucleotide; C, poly(C); dC, poly(dC); U, poly(U); and A, poly(A).

Table 2: Summary of the Cross-Linking of Oligo$(C)_9$ to Rho Fragments

rho fragment	fmol of oligo$(C)_9$ cross-linked per pmol fragment (residual % bound with competitor present)[a]				
	none	poly(C)	poly(dC)	poly(U)	poly(A)
rho(1–116)	1.70	0.54 (32)	0.88 (51)	1.58 (93)	1.28 (75)
rho(1–114)	0.19	0.17 (88)	0.16 (85)	0.16 (86)	0.16 (82)
rho(1–109)	0.15	<0.05	<0.05	<0.05	<0.05
$rho(1–116)_{CD}$	1.98	0.62 (32)	1.57 (79)	2.23 (113)	1.58 (80)
rho(5–116)	0.41	0.20 (48)	0.24 (59)	0.36 (88)	0.34 (81)
rho(13–116)	0.55	0.35 (64)	0.46 (84)	0.46 (84)	0.44 (81)
rho(22–116)	0.47	0.29 (61)	0.35 (74)	0.44 (93)	0.40 (84)
rho(28–116)	<0.05	<0.05	<0.05	<0.05	<0.05

[a] The values in the table are the averages of three separate determinations and are *not* corrected for the fraction of protein that is active. The number in parentheses is the percent (%) cross-linked in the presence of the competing polymer relative to that without polymer. The standard deviations of these values were about ±15 percentile units.

to rho(1–109) was completely abolished by the presence of any of the four polynucleotides while nearly all the low amount of binding by rho(1–114) was resistant to all the competitors including poly(C). Although the one or more non-*rho*-encoded amino acid residues at the C-termini of the peptides that have fewer *rho*-encoded residues than rho(1–116) could be influencing the binding properties, the finding that rho(1–114), which has only one non-*rho*-encoded residue, is so much less effective than rho(1–116) at binding oligo$(C)_9$ suggests that residue 116 is at or very close to the C-terminal boundary of a functional RNA-binding domain.

Since the derivatives with N-terminal truncations had to be solubilized from the cell debris after lysis and renatured in vitro, we performed binding saturation studies with two of them and with $rho(1–116)_{CD}$ to determine differences in the extent of saturation and in the binding affinity. Although $rho(1–116)_{CD}$ was at least as active as rho(1–116) isolated by the conventional procedure (see Table 2), rho(5–116) bound considerably less oligo$(C)_{10}$ at saturation than an equivalent amount of $rho(1–116)_{CD}$ (Figure 5), which suggests that it contained a large fraction of protein that was inactive. However, the shape of its binding curve indicates that the

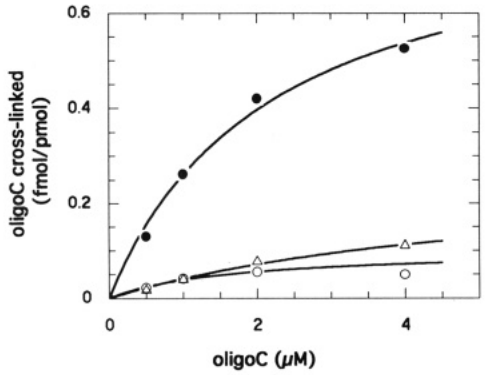

FIGURE 5: Oligo$(C)_{10}$ concentration dependence of cross-linking to rho(1–116), rho(5–116), and rho(22–116). [^{32}P]Oligo$(C)_{10}$ was mixed at the indicated concentration (whole molecule, not residue) with 50 pmol (1 μM) of rho(1–116) (●), 50 pmol (1 μM) of rho(5–116) (○), or 16 pmol (0.32 μM) of rho(22–116) (△) in 50 μL of binding buffer. The amount cross-linked was determined by phosphor-image quantitation of the label that comigrated with the protein fragments on gel electrophoresis. The data presented were normalized for the amounts of protein used.

affinity of oligo$(C)_{10}$ for the fraction that was active was not very different from the affinity for rho(1–116)$_{CD}$. In contrast, the binding curve for rho(22–116) rose more slowly with increasing concentration of oligo$(C)_{10}$, suggesting that the active fraction of the renatured rho(22–116) polypeptide had a substantially reduced binding affinity. Because of the problems with the solubility and folding, none of the derivatives with N-terminal truncations were deemed suitable for further structural and functional studies.

Physical Properties of Rho(1–116). On the basis of its predicted amino acid sequence, the polypeptides encoded by rho(1–116) should have M_r = 13 015. When the isolated protein was analyzed by electrophoresis in a polyacrylamide gel in the presence of sodium dodecyl sulfate, it migrated at the position of a protein with M_r = 12 600 (Figure 1). The closeness of this estimate to the predicted value indicates that the protein had not suffered extensive degradation before it was isolated. However, the measurement procedure is not precise enough to rule out the possibility that some residues may have been removed.

To determine whether rho(1–116) is monomeric or multimeric under nondenaturing conditions, its relative size was determined by gel-exclusion chromatography. When chromatographed in Sephadex G-75 along with several marker proteins, it eluted just behind myoglobin. As shown on the curve of log(M_r) *vs* elution volume (Figure 6), rho(1–116) migrated at the position expected for a globular protein with M_r = 13 800, which is very close to that of the denatured polypeptide determined by electrophoresis. Since any interaction that caused the subunits to partially associate or a folding that created a nonglobular structure would both lead to greater exclusion of the protein from the gel matrix and consequently yield a higher estimate of its relative mass, this result indicates that rho(1–116) is a largely monomeric, globular protein.

As an independent test of whether this segment of rho lacks domains or motifs that are responsible for the assembly of the hexamer, we treated rho(1–116) and mixtures of native rho and rho(1–116) with the lysine residue-reactive cross-linking reagent dimethyl suberimidate. Under conditions in which native rho by itself readily reacts to form dimers, trimers, tetramers, pentamers, and hexamers [Figure 7, lane 6; see also Finger and Richardson (1982)], no multimeric forms of rho(1–116) (lane 2) or heteromeric rho(1–116)-rho complexes

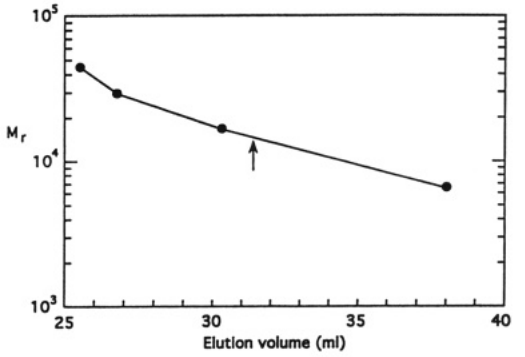

FIGURE 6: Estimation of M_{app} of rho(1–116) by gel-exclusion chromatography. A mixture containing 1.0 mg of aprotinin (M_r = 6500), 0.7 mg of sperm whale myoglobin (M_r = 16 900), 1.0 mg of bovine carbonic anhydrase (M_r = 29 000), 1.4 mg of chicken egg albumin (M_r = 45 000), and 2.5 mg of rho(1–116) in 1.4 mL of buffer containing 0.2 M NaCl in TEDG was chromatographed on a 65 × 1 cm column of Sephadex G-75 superfine equilibrated in that same buffer. Samples of fractions were analyzed by gel electrophoresis to determine the elution volume for the various proteins. The plot is of M_r for the standards on a logarithmic scale as a function of elution volume. The arrow shows the elution volume of rho(1–116).

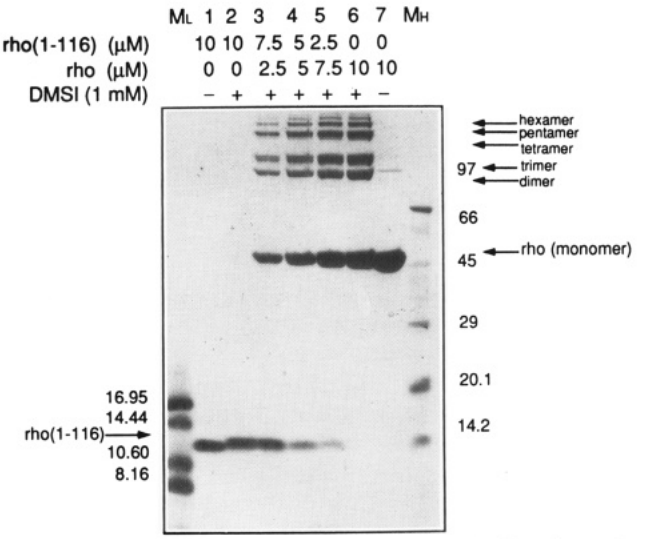

FIGURE 7: Use of dimethyl suberimidate to probe for oligomeric structure of rho(1–116). Samples of native rho and rho(1–116) at the indicated concentrations were mixed together in 30 μL of a solution containing 0.1 M NaCl, 10 mM $MgCl_2$, 0.2 mM dithiothreitol, 0.1 mM EDTA, 0.1 M triethanolamine hydrochloride buffer, pH 8.0, and 1.5% glycerol. After 20 min at 24 °C, 10 μL of freshly prepared 4 mM dimethyl suberimidate in the same buffer solution was added. After another 30 min at 24 °C, 2 μL of 1.5 M ethanolamine hydrochloride, pH 8, was added to react with the remaining reagent. After 20 min at 24 °C, 10 μL of 5× electrophoresis sample buffer was added, and the samples were heated to 70 °C for 5 min and separated by electrophoresis on a 10% polyacrylamide/SDS gel. The gel was stained with Coomassie Brilliant Blue.

(lanes 3–5) were detected. The fact that the dimethyl suberimidate-treated rho(1–116) migrated slightly less fast than the untreated sample (compare lane 2 with lane 1) indicates that rho(1–116) reacts with the reagent. However, since cross-linking depends on the presence of reactive lysines on adjacent subunits that are within 11 Å of each other, the lack of cross-linking does not rule out some interaction of rho(1–116) with itself. At least, the results indicate that rho(1–116) is unable to make the contacts that are responsible for the extensive cross-linking of native rho with the reagent. This conclusion was further supported by the fact that rho(1–116) also did not interfere with the cross-linking of the intact rho; the relative yields of the multimeric forms did not decrease as the ratio of rho(1–116) to rho increased (lanes 6 to 3). The

Table 3: Rho(1–116) Lacks ATPase Activity and Does Not Interfere with ATPase of Native Rho

proteins assayed[a]	ATP hydrolyzed[b] (nmol)
rho(0.6 pmol)	7.2
rho(1–116) (3 pmol)	<0.02
rho(0.6 pmol) and rho(1–116) (3 pmol)	7.3

[a] The indicated amounts of proteins (as subunits) were pre-equilibrated in 10 μL of reaction buffer for 20 min at 0 °C prior to assay for ATPase with poly(C) as described in the Experimental Procedures section. The amount of poly(C) was 5-told above that needed to saturate activity with 0.6 pmol of native rho. [b] Amount of ADP produced after 10 min at 37 °C.

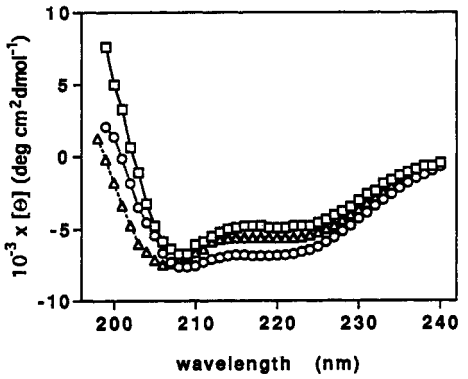

FIGURE 8: Circular dichroism spectra of rho, rho(1–116), and rho(1–109). CD spectra were measured in a 1-cm path cell with a JASCO Model J20A spectropolarimeter. The samples containing 5 μM rho (○), 5 μM rho(1–116) (□), or 2.5 μM rho(1–109) (△) were prepared for analysis by dialysis into 10 mM sodium phosphate buffer, pH 7.5.

subunits of hexameric, native rho are known to exchange between molecules (Richardson & Ruteshouser, 1986). Since they equilibrate in less than 20 min at 0 °C, we felt that the 20-min equilibration of the mixture prior to treatment with reagent was adequate to allow normal subunit exchange. Thus, we conclude that rho(1–116) does not contain the domains that are responsible for the subunit–subunit interactions that are characteristic of the full-length rho polypeptide.

Another approach that was taken to uncover a possible interaction of rho(1–116) with native rho was to determine whether it interfered with poly(C)-activated ATPase of rho. As expected, this truncated form of rho lacking the ATP-binding domain had no detectable ATPase activity (Table 3); the presence of 3 pmol of rho(1–116) caused less than 0.02 nmol of ATP (below the limit of detection) to be hydrolyzed in 10 min. In contrast, 0.6 pmol of rho (as hexamer) catalyzed the hydrolysis of 7.2 nmol of ATP in 10 min. When rho(1–116) was mixed with 0.6 pmol of native rho, the rate of ATP hydrolysis was essentially the same as with native rho alone, indicating that it does not cause an interference. These results constitute further evidence that rho(1–116) does not interact with normal rho and demonstrate that the RNA-binding segment of rho by itself lacks the ability to hydrolyze ATP.

The circular dichroism spectrum of rho(1–116) and rho(1–109) were determined in the range of 198–240 nm to gain insights into the extent of α-helix and β-sheet secondary structure in the protein (Figure 8). The CD spectrum of native rho was determined previously by Finger and Richardson (1981) but was repeated here for comparison and to allow analysis with more recent structure prediction programs (Venyaminov et al., 1991). It is consistent with contents of 25% α-helix, 20–25% β-sheet, and 10% turns, values which are in good agreement with the previously reported analysis. With rho(1–116) the corresponding values were 20% each of α-helix and β-sheet and 30% as turns. Thus, approximately 23 amino acids are involved in α-helix and 23 in β-sheet. The spectrum of rho(1–109) indicates that it is similar to rho(1–116). Since it has similar amounts of ordered secondary structures, its inability to bind well to oligo(C) can be attributed to the absence of specific residues.

DISCUSSION

The first 116 residues of the rho polypeptide comprise a stable, compact globular domain that, by itself, binds short RNA ligands with an affinity and specificity that represents the interaction of RNA to a single binding site in native rho protein. These 116 residues constitute a minimum RNA-binding domain that will serve as a useful model for further structure and function studies of rho as a transcription termination factor.

As part of its normal function, native rho readily forms a hexameric structure (Oda & Takanami 1972; Finger & Richardson 1982; Geiselmann et al. 1992a). Thus, a rho monomer has surfaces that allow it to bind to other subunits. The physical properties of rho(1–116) indicate that these surfaces are not within the first 116 residues. Not only was rho(1–116) monomeric in solution, it also did not interfere with the multimerization of native rho. Thus, the surfaces that are responsible for hexamer formation are in some other segment of the rho subunit.

A characteristic property of hexameric rho is that it binds short RNA ligands, such as oligo(C)$_9$, with a stoichiometry of three per hexamer in high-affinity sites (Geiselmann et al., 1992b) and another three per hexamer in lower affinity sites (Wang & von Hippel, 1993). One mechanism that could allow this type of binding is to have the sites at the interfaces between the subunits, with the high-affinity sites at one type of interface and the low-affinity site at another type of interface. However, our finding that monomeric rho(1–116) binds RNA ligands by itself indicates that a low-affinity site, at least, can be completely within a single polypeptide. This result leads us to favor a mechanism in which the limitation of one ligand bound in a high-affinity site per two subunits in native rho is a consequence of "half-of-the-sites" reactivity that is controlled by allosteric interactions between subunits. The RNA-binding domain in a normal subunit is likely to have surfaces that contact the surfaces of other domains within the same subunit, particularly the ATP-binding domain (Dolan 1990). Also, even though rho(1–116) did not self-associate as an isolated protein, it could still have a surface that allows contact between two RNA-binding domains in adjacent subunits of native rho. These postulated other interactions could allosterically cause the RNA-binding domain of one subunit in a hexamer to become a low-affinity site by the binding of a ligand to the domain in an adjacent subunit. The difference in affinity between the strong and weak binding sties for oligo(C)$_{10}$ in the rho hexamer is about a factor of 10 (Wang & von Hippel, 1993). Our finding that the affinity of oligo(C)$_9$ for rho(1–116) is also about 10-fold lower than its affinity for the strong binding sites in native, hexameric rho could be an indication that the binding site for oligo(C)$_9$ in rho(1–116) is in the conformation that is characteristic of the weak sites in the hexamer.

The finding that the 116-residue amino-terminal fragment of the rho polypeptide binds RNA confirms and extends the conclusions from two previous experiments that had partially localized rho's RNA-binding domain. Dombroski and Platt (1988) showed that an isolated 151-residue amino-terminal fragment could bind to poly(C), and Dolan et al. (1990) found that oligo(C) bound to the 130-residue amino-terminal

fragment in rho that had been digested briefly with trypsin. Our results have thus narrowed the carboxyl-terminal boundary of the RNA-binding domain to residue 116. This boundary corresponds very well to the end of a segment with many conserved amino acid residues in the proteins encoded by homologues of the *rho* gene from other bacteria (Opperman & Richardson, 1994). The proteins encoded by these *rho* gene homologues contain several blocks of strongly conserved residues. Although most are in the ATP-binding domain, there is a block of conserved residues that runs from residue 30 to residue 120 in *E. coli* rho.

The fact that derivatives lacking residues from the amino terminus were nearly totally insoluble after synthesis suggests that the first few residues are essential for the proper folding of the domain. However, in its soluble form, the truncated protein that started at residue 22 retained some weak RNA-binding activity, whereas the one that started at residue 28 was completely devoid of RNA-binding activity. This boundary is also reflected in the phylogenetic analysis. The proteins encoded by the *rho* gene homologues are more diverged prior to residue 30 in *E. coli* rho than in residues after residue 30 (Opperman & Richardson, 1994). We propose therefore that the segment starting at residue 22 and ending near residue 116 is the "core" of the RNA-binding domain. This proposal is further supported by the finding that the site of photo-cross-linking of *trpt'* RNA to rho is at a point between residues 45 and 100 (Brennan & Platt, 1991). However, the finding that a mutational change of *E. coli* rho from Leu to Phe at residue 3 increases the termination activity of rho and broadens its base specificity in its primary RNA interaction (Mori et al., 1989) indicates that the amino-terminal residues are important for the normal functioning.

The RNA-binding domain of rho contains a sequence motif that is found in other RNA-binding proteins, particularly in proteins that are subunits of both small nuclear and heterogeneous nuclear ribonucleoprotein particles (RNPs) (Dreyfuss et al., 1988; Query et al., 1989). This sequence motif is known as RNP1 and is represented by the sequence DGFGFLR (residues 60–66) in *E. coli* rho. The residues in this sequence are known to be very important for RNA binding, as derivatives with different residues at any one of these positions have defects in binding RNA and some with certain changes have greatly reduced RNA binding (Martinez, 1994).

The structure of two RNA-binding domain polypeptides, each having an RNP1 motif, have been determined. One, from the snRNP A protein, was determined by both X-ray crystallographic (Nagai et al., 1990) and nuclear magnetic resonance procedures (Hoffman et al., 1991). The other, from the hnRNP C protein, was determined by nuclear magnetic resonance procedures (Wittekind et al., 1992). The two proteins have very similar structures. They are both compact and globular, and each has structure with a four-stranded antiparallel β-sheet that is supported from behind by two α-helices. The preliminary physical evidence indicates that rho(1–116) could have a similar structure. It is compact and globular, and the CD analysis indicated that it has a similar number of residues in β-sheet and α-helix secondary structure as do these two RNP proteins. We conclude that even though rho(1–116) lacks a discernible RNP2 motif that is found in these other RNA-binding proteins (Kenan et al., 1991), it is likely to have a very similar structural organization. With the ability to make substantial amounts of rho(1–116), we envision using this protein for further structural and functional characterizations.

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