The binding of C$_{10}$ RNA oligomers to wild type and mutant *Escherichia coli* transcription termination factor Rho provides a model for the enzyme-RNA interactions that lead to transcription termination. One surprising finding is that wild type Rho binds between five and six C$_{10}$ oligomers per hexamer with $K_D$ = 0.3 µM, and five to six additional C$_{10}$ molecules with $K_D$ = 7 µM. Previously, approximately half this number of oligomer-binding sites was reported (Wang, Y., and von Hippel, P. H. (1993) *J. Biol. Chem.* 268, 13947–13955); however, the E155K mutant form of Rho, thought at the time to be wild type, was used in that work. The present results with E155K Rho agree with the earlier work. C$_{10}$ binding with mutant forms of Rho that are altered in RNA interactions, bearing amino acid changes F62S, G99V, F232C, Trp-267Phe, and Thr-286Ala, indicate that the higher affinity binding sites constitute what has been termed the primary RNA site, and the lower affinity sites constitute the secondary sites. The binding data together with the crystal structures for wild type Rho (Skordalakes, E., and Berger, J. M. (2003) *Cell* 114, 135–146) support structurally distinct locations on Rho for the two classes of C$_{10}$ binding sites. The results are consistent with participation of residues 33 Å apart in secondary site RNA interactions. The data further indicate that not all RNA sites on Rho must be filled for full ATPase and transcription termination activity, and suggest a model in which RNA binding to the higher affinity sites leads to a protein conformation change that exposes the previously hidden lower affinity sites.

*Escherichia coli* transcription termination factor Rho is an ATP-fueled 5’ → 3’ RNA-DNA helicase (1). It acts by binding to an exposed region of nascent mRNA, moves directionally 5’ → 3’ along the RNA as it hydrolyzes ATP, and releases the message from paused RNA polymerase and the DNA template (for a review, see Ref. 2). A recently determined structure of the homohexameric Rho protein shows its subunits arrayed as an open lockwasher around a central hole (3). The mechanism of mRNA release from ternary transcription complexes by Rho is not certain but may employ the RNA-DNA helicase activity of Rho.

The interactions of Rho with RNA are extensive but remain incompletely understood. Treatment of Rho-poly(C) complexes with RNase showed that 60–80 bases of RNA are protected from digestion (4, 5); these results were confirmed by binding experiments using fluorescently labeled RNA (6). Longer RNA of 120 bases, however, is needed to maximally stimulate Rho ATPase activity (7), suggesting that additional RNA-binding sites exist.

Two classes of RNA-binding site on Rho, termed “primary” and “secondary,” were defined functionally, based on ATPase activation by poly(dC) and by RNA oligomers 6–10 bases long (8). Poly(dC) alone does not stimulate Rho ATPase activity, but it does reduce the RNA oligomer requirement for maximal ATPase activity by 5–10-fold (8, 9). The binding site for poly(dC) was termed the primary site and that for the RNA oligomer in the presence of poly(dC) was termed the secondary site.

Binding studies with RNA oligomers and a mutant form of Rho, E155K, that behaves like wild type (10) indicated two classes of sites on the homohexameric Rho protein, three with $K_D$ = 2.0 µM and three with 10-fold lower affinity (11, 12). The correspondence of these two classes of RNA sites with the proposed primary and secondary RNA-binding sites is not clear.

Although the results described above were interpreted as indicating two structurally distinct RNA-binding sites, other possibilities are that the secondary sites form subparts of the primary sites or that they are temporally distinct, acting as an alternative conformation of part of them (alternative protein conformations of three subunits, for example, could result in weaker binding to those subunits; see Ref. 13). Evidence against structurally distinct primary and secondary sites includes binding competition studies in which poly(dC) displaced poly(C) completely (6) and experiments in which poly(dC) prevented RNA oligomers from cross-linking to Rho (14). Seifried et al. (15), however, suggest that only the higher affinity sites were monitored in these experiments.

One way to resolve the distinctness of primary and secondary RNA sites is to define their locations on Rho. The primary site is believed to be in the N-terminal domain of Rho, which, as an independent polypeptide, is still able to bind RNA (16). Structures for the monomeric N-terminal 130-amino acid Rho fragment (17, 18) show a cleft suitable for RNA binding, a function confirmed by a co-crystal structure of this Rho domain with C$_{9}$ oligomers (19). The co-crystal structures of full-length hexameric Rho with DNA or RNA oligomers (3) show oligomers bound only in the same N-terminal domain sites. The strongest evidence for assignment of these sites as primary involves residues Phe-62 and Phe-64 of the N-terminal RNA binding cleft (19); Phe-64 stacks with the base of a CMP residue, and Rho that is mutant at these phenylalanines binds long RNA poorly in nitrocellulose filter binding assays (20–22). Structures of Rho-oligomer complexes obtained thus far resolve only 2 bases.
of nucleic acid per N-terminal RNA-binding site; thus the understanding of how and where RNA binds to Rho in this region of the protein is not complete.

The location of secondary RNA-binding sites is ill-defined. Shigesada and co-workers (23), in an extensive study of Rho proteins mutant in the C-terminal 100 amino acids, found effects on both secondary and primary RNA interactions by mutations throughout this region. Widger and co-workers (24), studying the Rho inhibitory bicyclicin, concluded that bicyclicin interferes with RNA binding at the secondary RNA site, based on the commonly used criteria of a $K_m$ measurement using long RNA to show primary site interaction, and $C_m$ $K_m$ values to indicate secondary site behavior. Following homology modeling of Rho structure according to F$_1$-ATP synthase and site-directed mutagenesis work, they proposed that the secondary RNA site includes residues 318–325 and a two-part site lining the central hole of Rho that has multiple positively charged amino acids (25, 26). These predictions support a model, initially proposed by Richardson (27), in which RNA passes through the middle of the ring of Rho subunits.

Additional evidence for RNA passage through the center of the Rho hexamer was supplied by cross-linking studies (28) and by the finding that RNA protects Rho from $H_2O_2$/Fe-EDTA cleavage at sites predicted to be on the inner surface of the hexamer in the "Q" loop (residues 278–288, based on F$_1$ homology modeling) (28, 29). The crystal structure of hexameric Rho (3) confirms the existence of the Q loop. Mutagenesis studies replacing amino acids in the Q loop resulted in proteins defective in the secondary RNA-binding site, as measured by higher oligomer $K_m$ values and normal binding to long RNA (30). The results strongly support passage of RNA through the center of Rho and are consistent with a binding site there. However, as discussed by Wei and Richardson (30), an alternative explanation is that protein conformation changes caused by RNA binding elsewhere on Rho result in both the observed cleavage protection and altered oligomer $K_m$ values. In summary, whether the secondary RNA-binding sites of Rho are distinct from the primary sites is unresolved.

To obtain additional information concerning Rho-RNA interactions and, in particular, the relationship of primary and secondary sites to the higher and lower affinity sites demonstrated by Wang and von Hippel (12), we analyzed $C_{10}$ RNA oligomer binding to wild type Rho and to mutant Rho proteins previously characterized as altered in primary or secondary RNA-binding sites.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains—Escherichia coli AR120-A6(p39-ASE) is the source for wild type Rho (10, 31), and AR120(p39-AS) is the source for Rho E155K (32). The hosts for overproduction of Rho mutant proteins are specified below.** BSL107rho::kan is BSL2(DEL3) modified by a kan promoter in rho, for a source of Rho, it carries pPMrho (pPMrhoCam (21, 33)).

**Plasmids—For Rho F62S production, we mutated a plasmid, pRW72, that carries a wild type rho gene (33) in which rho is under the control of both its normal control region (including the rho attenuator) and an upstream T7 promoter. A 30-base mutant primer, 5'–GACGATCTCAGCGGAGCAGGATG–3', of both its normal control region (including the rho RNA site includes residues 318–325 and a two-part site lining the central hole of Rho that has multiple positively charged amino acids (25, 26). These predictions support a model, initially proposed by Richardson (27), in which RNA passes through the middle of the ring of Rho subunits.

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Equation 2 when two classes of binding site, 1 and 2, were assumed, and to (10)

formed as in Stitt and Xu (43). When cro KD Ultrafit (Biosoft; using robust weighting to exclude outliers) and Prism (10 TAGME buffer with various concentrations of labeled oligomer. A por-
tion (5'-32P-C10 binding was monitored by ultrafiltration in experiments

using the Microcon-30 ultrafiltration apparatus (Millipore). C10 radiolabeling was essentially as in Wang and von Hippel (12).

When low concentrations of C10 were used (0.1–10 μM), the specific activity was 10–100 cpm/nmol; for concentrations greater than 10 μM, it was 5 × 10–10 cpm/nmol. Confirming a high specificity of labeling, up to 96% of the radioactivity in labeled C10 preparations could be retained on Microcon filtration membranes because of binding to Rho, whereas no radioactivity was retained if Rho was omitted.

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using the Microcon-30 ultrafiltration apparatus (45). The concentra-
tions of total and free oligomers were measured, and the concentrations of bound C10 were determined by subtraction. In general, 50–100-μl mixtures of Rho (final concentration, 2 μM subunit) were made in TAGME buffer with various concentrations of labeled oligomer. A portion (10 μl) of the binding mixture was taken for determination of total radioactivity, and the remainder was placed above the membrane in the

Microcon. The device was centrifuged at 5000 × g for 30 s; the filtrate (10–20 μl) was discarded, and the same device still containing sample

above the membrane was placed in a fresh collection tube and was centrifuged again at 5000 × g for 3–4 min. A portion (10–20 μl) of the filtrate was subjected to liquid scintillation counting to determine the level of free ligand. Experimental points in which oligomer concentrations greater than 5 μM were used showed scatter in the data. Data were fit to Equation 1

\[ \text{bound} = \frac{c_{1}\text{cap}}{K_{D} + \text{free}} + \frac{c_{2}\text{cap}}{K_{D} + \text{free}} \]  

(Eq. 1)

when two classes of binding site, 1 and 2, were assumed, and to Equation 2

\[ \text{bound} = \frac{\text{cap}}{K_{D} + \text{free}} \]  

(Eq. 2)

when one class was assumed, using the nonlinear regression programs Ultrafit (Biosoft; using robust weighting to exclude outliers) and Prism 3 (GraphPad Software, Inc.; no weighting used). In these equations, “bound” is the number of sites/Rho monomer; “cap” is the number of sites/monomer with a particular KD value; “free” is the concentration (μM) of free ligand, and “KD” is the dissociation constant for ligand binding at a particular class of sites. In cases where RNA binding was weak, instead of varying the oligomer concentration, a constant amount of radiolabeled C10 (2 μM) was incubated with various Rho concentrations from 0.7 nM to 33.3 μM hexamer (10.6 μM for Rho F62S).

Structure Examination—The visualization programs RasMol (46), GRASP (46), and Swiss PdbViewer version 3.6b2 were employed with the wild type Rho coordinates from Skordalakes and Berger (3).

RESULTS

To obtain information relating the functionally defined primary and secondary RNA-binding sites of Rho to RNA oligomer higher and lower affinity binding sites, we decided to analyze C10 oligomer binding to Rho mutant forms classified previously as altered in primary or secondary RNA sites. C10 binding was also performed with wild type Rho; the results of these experiments were unexpected.

RNA Oligomer Binding to Wild Type and E155K Rho—The binding of C10 to wild type Rho (Fig. 1, filled symbols) was, as previously published (12), well fit by a model with two classes

of binding sites, in our hands with KD values of 0.3 and 7 μM (compare with 0.2 and 2 μM in Ref. 12). However, we found 5.4 and 4.8 oligomers per hexamer for the higher and lower affinity sites, respectively, nearly twice the number reported previously (Fig. 1, filled symbols; Table I). DNA sequencing of the plasmid used to produce Rho confirmed that it carried the wild type rho gene (data not shown).

Because the earlier published work (12) was carried out with Rho E155K, thought at the time to be wild type, we did binding


### Table I

| Rho form (Ref.) | RNA | Original description | C$_{10}$ binding results$^a$ | $V_{max}$ ATPase activity$^d$ | Transcription termination in vitro |
|----------------|-----|----------------------|-----------------------------|-----------------------------|-----------------------------------|
|                |     |                      | n/subunit | $K_D$ | mRNA | $C_{10}$ | Helicase activity |
|                |     |                      | Class1 | Class2 | Class1 | Class2 |                |
| Wild type trp’ | 12 ± 5 pm (35) | µM 4 | 0.9 ± 0.3 | 0.8 ± 0.3 | 0.3 ± 0.2 | 7 ± 8 | 100 | 100 | 100 | >97% (21) | + |
| cro            | 0.2 nm (42, 21) | | | | | | | | | |
| E155K (10)     | 0.3 ± 0.6 | 0.6 ± 0.6 | 0.1 ± 0.4 | 0.2 ± 0.3 | 0.3 ± 0.2 | 7 ± 8 | 100 | 100 | 100 | >97% (10) | + |
| F62S (21, 22)  | >0.1 µM (21) (Chiefly 1° site) | 21 ± 12 | 0.7 ± 0.2 | 26 ± 14 | 125 | 5 | 100 | <13% (21) | Not available |
| G99V (35)      | trp’ 5 ± 2 pm (35) ~650 (35) | 0.8 ± 0.1 | 12 ± 3 | 157 | 5 | 0 | <5% (35) | –/+ (35) |
| F232C (48)     | trp’ 1.4 ± 0.2 nm (48) (2° site) | 0.6 | 0.2 ± 0.9 | 1.1 ± 2.9 | 0.3 ± 2.1 | 0.9 ± 1.7 | 112 | 100 | 20 | ~10% (48) | ~20% (48) |
| T286A (30)     | cro 0.2 nm (30) (Inactive) | 1.2 ± 0.1 | 0.6 ± 0.1 | 85 | Inactive | 0 | <5% (30) | Not available |
| K352E (35)     | trp’ 3 ± 2 pm (35) 100 ± 10 (35) | 0.9 ± 0.1 | 0.6 ± 0.2 | 68 | 5 | 0 | <5% (35) | –/+ (35) |

$^a$ Measured by nitrocellulose filter binding assay using the indicated mRNA.
$^b$ Measurement for C$_{10}$ in the presence of poly(dC).
$^c$ This work.
$^d$ ATPase activity was measured in the presence of the specified RNA ligand. 100% corresponds to 9–13 units at 37 °C when poly(C) was the RNA cofactor. 4–7 units for cro RNA, and 8–15 units for C$_{10}$ (in experiments where activity with poly(C) was 14–18 units).
$^e$ Model with one class of binding sites.
$^f$ Model with two classes of binding sites.

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experiments with E155K protein. The results were similar in potassium glutamate–(Fig. 1, open symbols; Table I) and KCl-containing buffers (data not shown). Although there is scatter in the data at higher C$_{10}$ concentrations, it is clear that E155K Rho has lower capacity for C$_{10}$ compared with wild type Rho. Two binding models fit the Rho E155K data (Fig. 1). (i) In a “two-site” model (Fig. 1A and B), as in the work of Wang and von Hippel (12), there are two classes of binding site, each present at approximately three per hexamer, with $K_D$ values of 0.1 and 2.3 µM. (ii) In a “one-site” model (Fig. 1C; Table I), there are 5–6 equivalent sites per hexamer, with $K_D = 0.7$ µM. The suitability of the two models is considered below (see “Discussion”).

RNA Oligomer Binding to Mutant Rho Proteins—The results of binding experiments with mutant Rho proteins altered in RNA interactions are shown in Figs. 2–6 and are summarized in Table I, as are some of the other characteristics of the proteins examined.

T286A and K352E—Two mutant forms of Rho that had been characterized as altered in secondary RNA-binding sites but normal in the primary site, T286A (30) and K352E (35), in our experiments each showed one class of C$_{10}$-binding site with a $K_D$ value that is of the higher affinity wild type Rho site (0.4, 0.6, and 0.3 µM, respectively), present at approximately one per Rho subunit (Figs. 2 and 3). These results are consistent with the initial characterization of these mutant proteins and suggest that the observed high affinity sites correspond to the primary site and that lower affinity sites on these mutant proteins are unavailable or too loose to measure.

G99V—Rho G99V is a third mutant protein previously characterized as altered in RNA binding only at secondary sites (35, 47). Because of low RNA oligomer affinity for Rho G99V, in our experiments the protein concentration was varied (Figs. 4 and 5). Rho G99V showed one class of approximately five oligomer-binding sites per hexamer with $K_D = 12$ µM. Are these sites mutant high affinity sites or lower affinity sites? Because the wild type low affinity site has a 7 µM $K_D$, the observed interactions resemble those of wild type lower affinity sites. This could be fortuitous, however, because this interpretation then means that high affinity sites must bind RNA so poorly that we do not detect them, a situation that contradicts the original report of normal primary site interactions and 200-fold looser secondary site binding (35). We therefore think the 12 µM sites are weaker-than-normal high affinity sites, and that RNA binding by low affinity sites is too weak to detect (see “Discussion”).

F62S—Rho carrying F62S was shown previously to have at least 100-fold weaker affinity than wild type Rho for cro mRNA (21) and thus is a candidate for a Rho protein altered in its primary RNA site; no secondary site information has been published. We therefore measured the $K_m$ value of Rho F62S for C$_{10}$ in the presence of poly(dC), and we found it to be about 4-fold higher than that of wild type Rho, 21 ± 12 µM versus 4.6 ± 3.2 µM (n = 2; data not shown), and thus expected to see changes in both classes of oligomer-binding sites. Again in experiments in which the protein was used to titrate the RNA, F62S showed one class of approximately four C$_{10}$ sites per hexamer, $K_D = 26$ µM (Fig. 5). These results are similar to those for G99V, but because of the location of Phe-62 in the protein and the C$_{10}$-stimulated ATPase activity of Rho F62S (Table I), the observed sites are likely to be secondary sites (see “Discussion”).

F232C—Rho F232C was initially characterized as having 100-fold looser primary site binding and 5-fold tighter secondary site interactions (48). Our data (Fig. 6), in agreement with this characterization (Table I, two-site model, Fig. 6A), show a similar class of high affinity sites that are fewer in number, plus a class of low affinity sites that are tighter than those of wild type Rho. The data may alternatively be interpreted as a single, sub-micromolar class of sites (Table I, one-site model; Fig. 6C).
DISCUSSION

12 C_{10}-binding Sites per Rho Hexamer—The present studies of RNA C_{10} oligomer interactions with wild type Rho surprisingly suggest more oligomer-binding sites than were reported previously (12). The sites appear to fall into two affinity classes as published before, although the \( K_D \) values here, 0.3 and 7 \( \mu M \), are slightly different from those reported previously (Table I).1 The number of sites found is five of each type per hexamer (Table I), in agreement with the co-crystal structures of wild type Rho with RNA and DNA oligomers (3), in which five of the six identical subunits show occupancy at the same location in the N-terminal domain, which we assume is the higher affinity site. When the N-terminal 130 amino acids of Rho are produced as a discrete polypeptide, this domain by itself binds RNA (16). In addition to the N-terminal sites, recent work strongly suggests that there are RNA interaction sites in the Rho ATP binding domain, in a portion known as the Q loop which extends into the central hole of the Rho hexamer (see Introduction). These predicted sites do not show bound ligand in the available crystal structures, perhaps because their affinity for RNA is lower, or because Rho with RNA bound in these sites does not crystallize under the conditions used. As discussed below, the lower affinity C_{10}-binding sites probably involve the Q loop. In summary, each of the identical subunits of Rho probably has two RNA-binding sites with different affinities.

1 Although our discussion is in terms of two discrete classes of binding sites with different \( K_D \) values, the situation may be more complex, with site affinities changing as neighboring sites become occupied (49, 50). The complexity generated by a hexameric protein precludes our consideration of such situations.
but perhaps only five of the six subunits simultaneously bind oligomers.

Rho E155K Binds Oligomers Differently from Wild Type Rho—The previously published RNA oligomer binding studies that reported three high affinity and three low affinity sites per Rho hexamer (12) were carried out with a mutant form of Rho, E155K, that for many years was thought to be wild type. Close examination (10) found only minor differences in the behavior of Rho E155K and wild type Rho in a variety of activity assays. The present studies with Rho E155K confirm the 1993 work (12): we find that Rho E155K binds fewer C10 oligomers than wild type Rho. Models with either one or two classes of binding site fit the data (Fig. 1; Table I). As detailed below, however, the one-site model requires additional features and predicts behavior that was not found.

A One-site Model for C10 Binding to E155K Rho Is Unsatisfactory—If it is assumed that both classes of RNA site on Rho must be filled to stimulate rapid ATP hydrolysis, the wild type activity of Rho E155K can be explained according to the one-site oligomer binding model if two features are added. (i) Lower affinity RNA-binding sites on Rho are not normally accessible, but become exposed by a protein conformation change induced by filling high affinity sites (Scheme 1). RNA can then bind in these sites, permitting ATP hydrolysis. Evidence for protein conformation changes associated with RNA binding has been presented (51). (ii) Although in wild type Rho this proposed conformation change would occur when an RNA polymer, poly(dC), or RNA oligomers fill the high affinity sites, because of the E155K mutation, only long polynucleotides readily induce the proposed necessary conformation change in Rho E155K. Thus, Rho E155K could show normal ATPase activity with long polynucleotides but display only higher affinity binding sites in C10 binding experiments (the lower affinity sites are sequestered). A prediction of this model is that the ATPase activity of Rho E155K stimulated by C10 alone should be poorer than that of wild type Rho, because its lower affinity sites are less accessible. To test this prediction, the $K_m$ values for C10 in ATP hydrolysis (with no poly(dC) present) were determined for wild type Rho (14 ± 6 μM) and Rho E155K (18 ± 14 μM) (data not shown). The values are equal, within experimental error, with Rho E155K $V_{max}$ activity about 60% that of wild type (10 ± 3.4 and 17.2 ± 2.8 units, respectively). C10 thus must be able to bind in both classes of site on Rho E155K very similarly to the way it binds to wild type Rho, and therefore the one-site model, suggesting that the secondary sites are less accessible on Rho E155K, is rejected.

How can Rho E155K bind fewer C10 molecules than wild type? One possibility is that the observed stoichiometry results from an abnormal situation. von Hippel and co-workers (11, 38) reported that Rho E155K forms dodecamers in the presence of RNA oligomers 9–20 residues long. It is possible that some oligomer-binding sites become unavailable when dodecamers form. It is not currently known whether wild type Rho also dodecamerizes in the presence of short oligomers; if it does not, this could explain the observed difference in oligomer binding. An alternative explanation is as follows. Rho is thought to act
as a trimer of dimers (43, 45, 52–54), with subunits of identical amino acid sequence alternating between two conformations. A mutation could cause the dimers to overlap so that RNA-binding sites on every other subunit are obscured. Could mutation to a lysine at Glu-155 cause such a conformation change? The position of Glu-155 in the x-ray structures of wild type Rho (3) does not at present help in understanding the Rho E155K phenotype. Glu-155 is at or near the C-terminal end of a 28-residue portion of polypeptide (amino acids 121–157) that first connects N- and C-terminal Rho domains, then from residues 130 to 139 is near an adjacent subunit, and finally extends across the outer surface of the C-terminal domain to α-helix 6 (Fig. 7A). The side chain of Glu-155 extends from the protein surface, and its carboxylate oxygens are 8–9 Å from N1 of bound AMPPNP (3), too distant for interaction. The Glu-155 side chain is not involved in any H-bonds and is not close to residues from adjacent subunits. Structural information about Rho E155K would be very useful in understanding its behavior, and complete understanding of its phenotype requires further work.

The lower C10 binding stoichiometry of E155K compared with wild type Rho together with the wild type behavior of E155K suggest that not all RNA sites may need to be occupied for full enzyme activity. This property could be useful for a protein that must normally function with mRNA molecules that have different sequences and secondary structures, and that must bind and release RNA to travel along it. If all RNA-binding sites do not need to be filled for Rho to function, it then becomes clear how E155K Rho, with fewer sites, could behave so similarly to wild type and predicts that occupancy of a higher proportion of the RNA-binding sites of E155K may be required for full activity.

C10 Binding to Mutant Forms of Rho—Our C10 binding studies with mutant forms of Rho, characterized previously as defective in primary or secondary RNA sites, show that the experiments whose results were used to classify the Rho mutant proteins were only partly successful. To evaluate primary site interactions, binding studies using long RNA molecules were performed. However, because of the multiple RNA-binding sites on Rho, proteins altered in some of these interactions could continue to show high affinity binding to long RNAs, as is the case, for example, with Rho E155K. To assess secondary site interactions, Km measurements for C10 (in the presence of poly(dC)) have often been used. Interpretation of Km measurements as reflecting binding affinity must also be done cautiously, because kcat also contributes to Km (Km = KD + kcat/km).

T286A and K352E—We find that of five mutant forms of Rho previously classified as defective in primary or secondary site RNA interactions, T286A and K352E proteins behave completely consistently with their initial classifications as normal in primary site binding and defective in secondary sites. Thr-286, evolutionarily conserved as Ser or Thr among nine Rho sequences from five bacterial phyla (56), is part of the Q loop on the inside surface of Rho hexamers that has been strongly implicated as part of the secondary RNA-binding site (30). The Q loop projects into the central cavity of Rho, constricting it. The side chain of Thr-286 is exposed to the inside cavity, where it could contact RNA (Fig. 7, B and C). Lys-352, evolutionarily conserved as Lys or Arg (56), also lies on the surface of Rho with its side chain extending from the protein and is at least 33 Å from Thr-286 (either the Thr-286 on the same subunit or on adjacent subunits of the hexamer), near the base of the protein as depicted in Fig. 7, at the opposite end from the N-terminal RNA binding domain (Fig. 7, A and B).

The locations of Thr-286 and Lys-352 in the protein structure clearly show that the secondary RNA site is not a subset or altered conformation of the primary site. The identical behaviors of these two mutant proteins together with the fact that their side chains at the mutation sites are exposed suggest that both amino acid residues may interact directly with RNA in the secondary binding sites. This conclusion supports, in part, a previous proposal that the secondary site extends over a large area (25, 26). The 33-Å distance between residues 286 and 352 is such that a molecule of C10, which is 29 Å long in helical form in solution (57) and likely more extended when bound to protein, could interact simultaneously with both side chains. Although the available Rho structures do not show a distinct groove or series of positively charged residues between Thr-286 and Lys-352 that could accommodate an RNA chain, such features are not required for RNA binding (58). An alternative explanation for the similar protein phenotypes, that glutamate at residue 352 indirectly affects the affinity of secondary sites for RNA, is a possibility which seems unlikely based on the currently available structural information.

One question concerning oligomer binding to the lower affinity Rho sites is whether five C10 oligomers could simultaneously bind, given that the Q loop, the location of Thr-286, constricts the diameter of the central hole of the hexamer to 20 Å (3). One or two RNA molecules, each with dimensions of ~9 Å from the phosphodiester backbone through the base, and ~5 Å for the O2-C6H distance (59, 60), would easily fit through the center of the hexamer, but it is not evident that five molecules could bind. Modeling C10 with the current Rho structures is problematic because the conformation of C10 and the location of the secondary binding sites are both ill defined; however, the following points may be considered. (i) Rotation of the bases in C10 about their N1–C1′ glycosyl bonds could reduce the molecular “width” on the base side of the single strand, allowing more molecules to bind. Limits on such rotation (59, 61) could be modulated by interactions with the protein. (ii) The Rho hexamer is a spiral, with a total displacement along an axis through the central hole of 45 Å (3). This displacement could allow one C10 oligomer to bind above another on one or more subunits, depending on the C10 configuration. (iii) When RNA oligomers bind in the lower affinity sites, the protein conformation may change. One or more of these features could permit the binding of five oligomers per hexamer in association with the Q loop. It does, however, seem possible that when a single long polynucleotide is bound to Rho, only one of the lower affinity sites may be occupied at a time.

Rho G99V shows only one class of lower affinity C10-binding sites, but its initial characterization was of normal primary sites and extremely defective secondary sites. Gly-99, a con-
served residue (56), is in the β4-strand of the N-terminal RNA binding domain but is 15–20 Å from the RNA-binding site (Fig. 7, A and D). It is a surface residue. When we used Swiss PDB Viewer to substitute valine for Gly-99, the program predicted clashes between the valine side chain and backbone carbonyl groups of neighboring residues Lys-115 and Ser-98. Resolution of these clashes might indirectly change the primary RNA-binding site, resulting in the observed looser (12 μM $K_D$) binding. What about the lower affinity sites? If they were unaffected, a total of 10 sites of −10 μM affinity would be expected, five primary and five secondary, but only five sites were found (Fig. 5; Table I). The explanation proposed in Scheme 1 might apply here; in Rho G99V the primary sites could show a weaker value, 12 μM $K_D$, and the hypothetical conformation change that makes secondary RNA sites available does not occur, rendering the secondary sites inaccessible. Consistent with this model, C10 alone does not stimulate the ATPase activity of Rho G99V (Table I). A difficulty with this model, however, is that a 12 μM $K_D$ value for the higher affinity sites of Rho G99V seems inconsistent with its originally reported wild type affinity for long RNA molecules. The observed oligomer-binding sites are not likely to be secondary sites, even though the measured $K_m$ value invalidates such a conclusion. The interpretation that is most consistent with available data is that the observed C10-binding sites in Rho G99V are primary, high affinity sites, secondary sites may become available only when long RNA binds, and thus binding to both classes of sites has been affected.

Phe-62, evolutionarily conserved as Phe or Tyr (56), is a

**Fig. 7. Locations of various amino acids in wild type Rho (based on Ref. 3).** A, subunits C and D of the Rho ABCDEF hexamer (ribbons), viewed from outside the hexamer. Rho ligands are shown in ball and stick format; two residues of RNA are in the primary RNA site of each subunit, and one molecule of AMPPNP is bound per subunit. Selected amino acids are viewed in space-filling format (see text), colored similarly in the two subunits, and labeled. The polypeptide segment that connects N- and C-terminal domains is shown in black (backbone only); Glu-155 is at its end. This segment includes α-helix 5 (residues 131–135) and is otherwise either unstructured or it may have two short, three-residue stretches of β-strand structure (amino acids 136–138 and 143–145). Two segments of it are disordered (amino acids 127–128 and 148–151). B, the C subunit of Rho, viewed from the central hole of the hexamer. Two residues of RNA are shown (stick format) in the primary RNA site. Selected amino acid side chains (see text) are labeled and rendered as space-filling. C, a Rho hexamer (α-carbon trace) viewed from above. Thr-286 is shown in black (space filling). D, portion of the N-terminal domain of Rho (ribbons) with two residues of RNA (sticks) bound in the primary RNA site. Side chains of the labeled amino acids (see text) are also shown as sticks. E, portion of C/D subunit interface of Rho, centered around Phe-232 of subunit D. Side chains of Phe-232 and of residues within 4 Å of Phe-232 are shown as sticks. For orientation, a ribbon diagram that includes 3–4 residues on each side of the stick residues is superimposed. α-Helices and β-strands have been labeled as in Skordalakes and Berger (9).
surface residue in the primary RNA binding cleft, where it is part of a hydrophobic shelf for a ribose of the RNA ligand (Fig. 7, A, B, and D) (19); its alteration to serine could compromise the primary binding sites. It is difficult to understand how alterations in Phe-62 could result in altered secondary site interactions. If Scheme I is correct, that accessibility of secondary RNA sites depends on filling primary sites, then the observed 26 μM oligomer sites in F62S could be the altered primary sites, and the secondary sites again do not become accessible when oligomers fill the primary sites. ATPase measurements in the presence of C10 only, however, show good activity (Table I), contradicting this conclusion. Alternatively, the mutation could favor the required hypothetical protein conformation change in addition to preventing oligomer binding in the primary sites, so the observed binding is to secondary sites, where binding is looser because the required protein conformation change is not quite correct. Because of its position in the protein structure, the F62S change is likely to affect the conformation change that we have proposed that makes secondary sites available. ATP binding by Rho (52, 64, 65), studies of the assembly of the hexamer (50), and pre-steady-state kinetics results (43, 45, 63, 66) all suggest that the protein behaves as a trimer of dimers in many circumstances; a functional result not evident from the recent crystal structures (3). It could be that a trimeric configuration is adopted only when long polynucleotides are bound or in the absence of RNA ligands.

In conclusion, C10 oligomer binding studies with wild type and various mutant forms of Rho suggest that the higher affinity binding sites found by Wang and von Hippel (12) are the primary sites found by Richardson (8), and the lower affinity sites, which are in a distinct location, are the secondary sites. Surprisingly, wild type Rho binds a total of 10–12 C10 oligomers per hexamer, nearly twice the capacity of the otherwise normally behaved Rho E155K protein. A model to explain the behaviors of several mutant Rho forms proposes that RNA binding in the primary site normally leads to a conformation change that permits RNA access to the secondary sites.

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