Thermodynamic and Enzymological Characterization of the Interaction between Transcription Termination Factor ρ and λ *cro* mRNA†

Ignacio Faus‡ and John P. Richardson*

Department of Chemistry, Indiana University, Bloomington, Indiana 47405

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ABSTRACT: Termination of transcription at tR_1, the ρ-dependent terminator between genes *cro* and cII of bacteriophage λ, is mediated by interactions between ρ protein and an RNA sequence element called *rut*. We show, using a filter retention assay technique, that ρ protein binds with about 10-fold lower affinity to variants of *cro* RNA lacking both parts of *rut* or to normal *cro* RNA having one or the other part of *rut* bound to a complementary DNA oligonucleotide than it binds to unmodified *cro* RNA. These same variant and modified forms are nearly devoid of the strong ρ ATPase cofactor activity of *cro* RNA. Estimates of binding energies of the ρ–*cro* RNA interaction under different conditions reveal that termination function correlates with about 12.6 kcal of binding energy, of which two-thirds is due to nonelectrostatic interactions. The *rut* segment is shown to contribute about 1 kcal, nearly all to nonelectrostatic interactions. KCl is found to be more effective than potassium glutamate as a competitive counterion, and a decrease in 1.4 kcal of binding energy due to counterion competition correlates with a loss of termination and ATPase activities. In sum, the results indicate that the *rut* sequence contributes substantially to the overall binding affinity, that ionic interactions are also important, and that mere binding of ρ to RNA is not sufficient for ρ ATPase activation.

In *Escherichia coli*, transcription termination at certain sites on DNA templates depends on the action of ρ protein (Roberts, 1969). Although the mechanism of ρ-dependent transcription termination is not fully understood, it involves interactions between DNA, RNA polymerase, the nascent RNA chain, nucleoside triphosphates, and ρ factor itself [reviewed by Platt (1986)]. Two sequence-dependent events appear to be important in the process of ρ-dependent transcription termination: pausing of RNA polymerase at the termination site and binding of ρ to the nascent RNA chain. It is generally accepted that transcriptional pausing precedes all termination events (Platt, 1981). However, not all polymerase pause sites are termination sites. Thus, a second sequence signal appears to be important in order to mediate ρ-dependent termination. This sequence signal is upstream from the termination site and encodes the segment of RNA that is presumably used to interact with ρ (Lau & Roberts, 1985; Chen et al., 1986; Chen & Richardson, 1987; Galloway & Platt, 1988). This upstream signal is known as the *rut* (*r*ho-*ut*ilization) site (Salstrom et al., 1979). The segment of RNA containing that sequence is also called *fer* (Platt, 1986) or *rat* (Bear & Peabody, 1988). To understand the mechanism of ρ-dependent transcription termination, it is therefore important to characterize the macromolecular interactions between ρ and an RNA molecule whose synthesis is terminated by ρ action.

Previous characterization of ρ–RNA interactions have been limited by the availability of pure, homogeneous RNA. Many of the early studies focused on the interaction between ρ and synthetic homopolymer RNA molecules, such as poly(C) (Galluppi & Richardson, 1980), and some partial characterization of the interaction between ρ and λ *cro* RNA, which contains the *rut* site of tR_1, have been reported (Bektesh & Richardson, 1980; Ceruzzi et al., 1985). These previous studies with λ *cro* RNA were done with very small amounts of RNA synthesized by transcription of purified restriction fragments with *E. coli* RNA polymerase. With the development of cloning vectors containing promoters for phage RNA polymerases (Green et al., 1983), it has become possible to synthesize much larger amounts of an RNA molecule of a defined sequence. We report here further characterization of the interaction between ρ and λ *cro* RNA, using homogeneous RNA that has been prepared by transcription of plasmid DNA with T7 RNA polymerase. Specifically, we analyze the extent to which various segments of *cro* RNA contribute to the stability of the ρ–*cro* RNA interaction, determine the relative contribution of ionic and nonionic forces to that interaction, correlate the influence of binding with the activation of ATP hydrolysis, and compare the effects of ionic conditions and temperature on both the binding and activation of ATP hydrolysis.

Materials and Methods

Biochemicals and Enzymes. Ampicillin, chloroamphenicol, rifampicin, and dithiothreitol were purchased from Sigma. RNase A and DNase I (code DPRF) were from Cooper Biomedical (now Worthington). Proteinase K was from Beckman and was self-digested by the method of Barbehenn et al. (1982). T4 DNA ligase, Klenow fragment of DNA polymerase I, and polynucleotide kinase were from Pharmacia. Calf intestinal phosphatase was from Boehringer Mannheim. RNasin was purchased from Promega Biotech. Restriction enzymes were purchased from various commercial sources. Unlabeled ribonucleoside triphosphates were from Boehringer Mannheim. [α-^{32}P]UTP (3200 Ci/nmol) and [α-^{32}P]ATP (650 Ci/nmol) were obtained from ICN Chemical and Radioisotope Division. Bovine serum albumin, from Sigma, was acetylated by the procedure of Gonzalez et al. (1977). ρ protein was prepared as described (Finger & Richardson,

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* Author to whom correspondence should be addressed.

‡ Present address: Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Institut de Chimie Biologique, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cédex, France.

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1981). T7 RNA polymerase was either purchased from Promega Biotech or prepared as described by Tabor and Richardson (1985) from a strain of cells containing a cloned plasmid copy of the gene of T7 RNA polymerase under control of the *lac* UV5 promoter (Devanloo et al., 1984). DNA oligonucleotides were synthesized on an Applied Biosystems Model 380A apparatus and purified by gel electrophoresis in the Institute for Molecular and Cellular Biology, Indiana University, Bloomington, IN.

Plasmids. All plasmid-harboring *E. coli* strains were grown in LB–ampicillin medium and amplified with chloroamphenicol, as described (Maniatis et al., 1982). Plasmids were isolated by the alkaline hydrolysis method (Maniatis et al., 1982), with the following modifications, which were performed to remove RNA contaminants: After isolation, the plasmid preparation was incubated with RNase A (30 μg/mL) in the TE buffer for 1 h at room temperature. This was followed by a treatment with self-digested proteinase K (50 μg/mL) for 90 min at 37 °C in a solution containing 50 mM Tris-HCl[1] (pH 7.2), 0.1 M NaCl, 10 mM $MgCl_2$, and 5% SDS. The sample was extracted several times with phenol/chloroform, and the aqueous phase was saved. DNA was separated from oligoribonucleotides by centrifugation through 1 M NaCl (Maniatis et al., 1982). Plasmids were finally resuspended in TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA] and stored frozen at −20 °C.

Construction of Plasmids of the pIF2 Series. Plasmid pCYC2 (Chen & Richardson, 1987), which consists of a 1584 bp *Hind*III–*Eco*RI λ fragment inserted into pUC9, was digested with *Rsa*I and *Hin*cII, and a 522 bp fragment containing the entire *cro* gene was purified by gel electrophoresis. Plasmid pTZ18R, which contains a promoter for T7 RNA polymerase connected to a polylinker (Mead et al., 1986), was digested with *Eco*RI and *Hin*dIII, and the 2809 bp fragment was isolated, repaired by action of Klenow fragment of *E. coli* DNA polymerase I, and dephosphorylated with calf intestinal phosphatase. This vector was ligated with the 522 bp *Rsa*I–*Hin*cII fragment from pCYC2, and the ligation mixture was used to transform *E. coli* HB101 as described (Hanahan, 1983). Miniplasmid preparations were isolated from several of the ampicillin-resistant clones, and the DNAs were cut with *Hae*III. A clone with the insert in the right orientation was identified after examining the distribution of DNA fragments in a 6% polyacrylamide gel in TBE buffer (0.09 M Tris base, 0.09 M boric acid, and 2 mM EDTA, pH 8.4). It was named pIF2. pIF2dAR70 was prepared by replacement of the 255 bp wild-type *Bgl*II–*Nde*I fragment of pIF2 with the corresponding *Bgl*II–*Nde*I fragment from the mutant pCYC2dAR70 plasmid (Chen & Richardson, 1987). pIF2dAL131 was prepared by ligating a 381 bp *Rsa*I–*Hin*cII fragment from pCYC2dAL131 (Chen & Richardson, 1987) with pTZ18R vector that had been linearized with *Eco*RI and repaired by the action of the Klenow fragment of DNA polymerase.

Synthesis and Isolation of Unlabeled cro RNA. The synthesis of unlabeled RNA was carried out in a reaction mixture containing 40 mM Tris-HCl (pH 7.5), 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 100 units of RNasin, 450 units of T7 RNA polymerase, and 10–15 μg of pIF plasmid cleaved with the appropriate restriction enzyme. Samples were preincubated at 37 °C for 10 min, and transcription was initiated by the simultaneous addition of ATP, GTP, CTP, and UTP to 0.5 mM (final volume = 100 μL). Incubation was for 2 h at 37 °C. The reaction was stopped by the addition of EDTA to 30 mM and SDS to 0.2% (w/v). RNA was purified by extracting once with an equal volume of water-saturated phenol, once with a mixture of phenol/chloroform (1:1), and once with chloroform/isoamyl alcohol (24:1). After precipitation with ethanol and recovery by centrifugation, RNA was dissolved in 20 μL of TBE buffer with 7 M urea, 20% sucrose, and 0.025% each of bromophenol blue and xylene cyanol and separated by electrophoresis on a 6% polyacrylamide gel containing 8 M urea and TBE buffer. RNA, located in the gel by UV shadowing, was removed from slices of the gel matrix by using an IBI Model UEA electroeluter, precipitated with ethanol, resuspended in water, and desalted by passing the preparation through a Sephadex G-50 spin column (Maniatis et al., 1982). Purified RNA was stored frozen at −70 °C. A small amount (100 ng) of RNA was analyzed by gel electrophoresis to monitor for degradation. After electrophoresis, the gel was soaked in a solution of ethidium bromide (1 μg/mL), and the RNA products were visualized with a transilluminator.

Synthesis of ^{32}P-Labeled RNA. A reaction mixture containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 1 mM spermidine, 5 mM dithiothreitol, 100 μg/mL acetylated bovine serum albumin, 1.8 pmol of pIF plasmid cleaved with the appropriate restriction enzyme, and 27 units of T7 RNA polymerase, in 66 μL, was incubated at 37 °C for 10 min prior to addition of ATP, GTP, and CTP to 0.67 mM and [α-^{32}P]UTP (3.2 μCi/nmol) to 100 μM. After incubation for 60 min at 37 °C, DNase I was added to 12 μg/mL and incubation continued for 10 min at 37 °C. The reaction was quenched by the addition of EDTA to 30 mM and SDS to 0.2% (w/v). After extraction of proteins as for the unlabled RNA samples, RNA was precipitated by addition of 0.1 volume of 5 M ammonium acetate and 2.5 volumes of ethanol. The RNA pellet was dried under vacuum and resuspended in water, and the ethanol precipitation step was repeated a second time, followed by a wash with 70% ethanol. RNA solutions were desalted by passing them through a Sephadex G-50 spin column. Some RNA preparations were also gel purified, as described for unlabeled RNA. However, this was usually unnecessary as the RNA was very pure after the synthesis reaction was completed, whereas gel-purified ^{32}P-labeled RNA samples were often partially degraded. The results obtained with undegraded, gel-purified RNA were the same as those obtained with undegraded RNA isolated by the standard procedure. The concentration of each RNA preparation was determined from the specific activity of the [α-^{32}P]UTP in the reaction mixture and the number of uridine residues in the particular transcript. RNA preparations were stored frozen in water at −70 °C. To ensure that no degradation had taken place during the synthesis and purification steps, all RNA samples were analyzed by gel electrophoresis, using autoradiography to visualize the labeled RNA.

ρ–RNA Binding Assays. Binding assays were performed in binding buffer [40 mM Tris-HCl (pH 8.0), 25 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM dithiothreitol] containing 250 μg of acetylated bovine serum albumin/mL, 0.1 nM [^{32}P]RNA, and ρ at concentrations that varied from 0.2 to 15.2 nM (expressed in terms of ρ hexamers). The mixture, in 100 μL, was incubated for 5 min at 37 °C, and then 80 μL was filtered through 13-mm Schleicher & Schuell BA85 nitrocellulose filters. The filters were washed twice with 250 μL of binding buffer. To quantitate the fraction of RNA bound, the filters were dried and assayed for radioactivity in

[1] Abbreviations: bp, base pair(s); EDTA, ethylenediaminetetraacetic acid; nt, nucleotide(s); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; w.t., wild type.

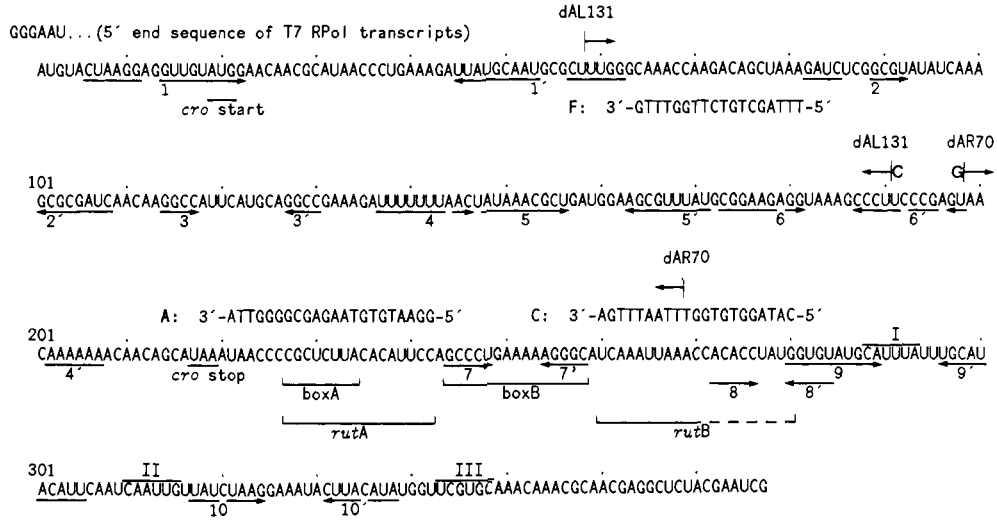

FIGURE 1: Sequence of *cro* RNA to residue 376 and sequences of oligodeoxynucleotides. Dots above the sequences indicate every tenth residue. The *cro* transcripts initiated by T7 RNA polymerase have the 5′ end sequence of GGGAAU- instead of AUG-. Complementary segments in the RNA are identified by numbered arrows (including interrupted arrows) under the sequence. The end points of the dAR and dAL deletions are indicated above the sequence. Landmarks are indicated, including the end-point nucleotides for transcripts terminated at sites I–III. The *rut*A and *rut*B regions have been postulated as being important for the interaction between ρ and the nascent *cro* RNA (Chen & Richardson, 1987).

a toluene-based scintillation mixture. Changes in this general protocol, when appropriate, are indicated in each figure legend. To analyze the bound RNA by gel electrophoresis, RNA was extracted with vigorous vortexing for 15 min in a siliconized glass tube from a filter into a mixture consisting of a solution containing 40 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 100 mM NaCl, 20 μg/mL tRNA, and 0.5 mL of buffer-saturated phenol. RNA was recovered from the aqueous phase by ethanol precipitation.

The filter retention assay measures the fraction of total RNA retained, which is

$$f_R = e\left(\frac{[\text{RNA}{-}\rho]}{[\text{RNA}] + [\text{RNA}{-}\rho]}\right)$$

where e is the retention efficiency. This equation can be used to substitute for the $[\text{RNA}{-}\rho]/[\text{RNA}]$ term of the association equilibrium constant equation to yield

$$K_a = \frac{f_R}{(e - f_R)}\frac{1}{[\rho]} \quad \text{or} \quad \frac{f_R}{[\rho]} = -K_a f_R + K_a e$$

The negative slope of a Scatchard plot ($f_R/[\rho]$ vs f_R) of the data from a binding set yields K_a, the apparent equilibrium constant. Values were calculated with a linear least-squares regression program. For this calculation, we assumed that ρ binds as a hexamer and that all the protein was ρ and was capable of binding. There is evidence that ρ binds to poly(C) as a hexamer (Finger & Richardson, 1982) and with a stoichiometry of 1 RNA molecule per hexamer (Galluppi & Richardson, 1980). In addition, the protein used was greater than 98% pure by various criteria and had been affinity purified by its RNA-binding property. Other aspects of the validity of the filter binding assay are discussed in a previous publication (Ceruzzi et al., 1985). Retention efficiencies (e) were determined from the extrapolated values of f_R at $f_R/[\rho] = 0$.

ATPase Assays. ρ ATPase activity was measured by the release of labeled ADP from [α-^{32}P]ATP, as follows: A reaction mixture (20 μL) containing 40 mM Tris-HCl (pH 7.9), 25 mM KCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA, 1 mM [α-^{32}P]ATP (10 300 cpm/nmol), 0.085 pmol of ρ as hexamer, and various amounts of unlabeled *cro* RNA was incubated for varying amounts of time at 37 °C. The reaction was quenched by addition of EDTA to 50 mM and ADP to 80 mM. Aliquots (2 μL) were applied to poly(ethylenimine)–cellulose strips (Brinkmann), which were then developed with 1 M formic acid–0.5 M $LiCl_2$. The strips were dried, and both the ADP and ATP spots were located under the UV lamp. The spots were cut and assayed for radioactivity in a toluene-based scintillation mixture.

Results

Binding of ρ Protein to Variant Forms of cro RNA. To facilitate our studies of the interaction between ρ and *cro* RNA, we prepared a series of plasmids that could be used as templates for transcription of the *cro* gene or mutant derivatives of the gene in vitro with T7 RNA polymerase. The sequence of *cro* RNA is shown in Figure 1. Figure 2 shows a map of the *Taq*I fragment from pIF2, diagrams of the various transcripts, and gel electrophoretic analysis of some representative transcripts. In these plasmids, the fourth base pair of the *cro* DNA was fused to the sixth base pair from the start point of the T7 promoter (ϕ_{10}). The resulting *cro* gene transcripts commence with the sequence 5′GGGAAC instead of 5′AUG (Figures 1 and 2). To test whether this difference affects the binding properties of the RNA, a small amount of λ *cro* RNA was synthesized from a *Taq*I-digested 1589 bp *Hin*dIII–*Eco*RI λ DNA fragment (isolated from pCYC2) by using *E. coli* RNA polymerase and compared to the T7 polymerase transcript of *Taq*I-digested pIF2 DNA. Binding was quantitated by the nitrocellulose membrane filter retention assay (Ceruzzi et al., 1985). The curve for the fraction of T7 polymerase transcript bound as a function of ρ concentration is shown in Figure 3 (w.t. RNA). Since ρ was in excess for all points on the curve, the data could be analyzed by the Scatchard procedure to determine the apparent association constant. The results (Table I) indicate that ρ binds to *cro* RNA synthesized by using T7 RNA polymerase from the pIF2 template with the same affinity as it binds to the transcripts synthesized with *E. coli* RNA polymerase. Therefore, we conclude that these nucleotide changes in the 5′ end of *cro* RNA do not affect its interaction with ρ. We thus used the T7 RNA polymerase transcript of *Taq*I-cleaved pIF2 (wild-type RNA) as the standard for further comparisons.

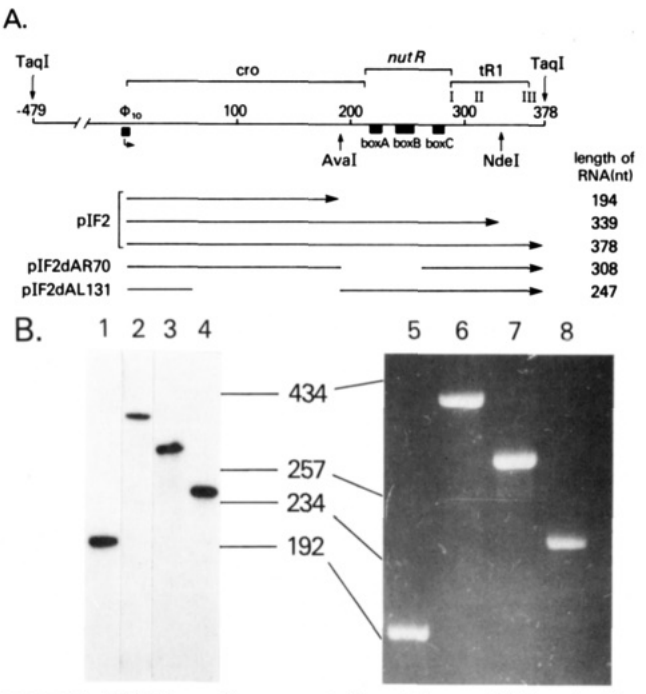

FIGURE 2: (A) Schematic representation of the *cro* RNA molecules synthesized by using T7 RNA polymerase and plasmids pIF2, pIF2dAR70, and pIF2dAL131 cleaved at different restriction sites. The length (in nucleotides) of each RNA molecule is shown at right. Other genetic landmarks are indicated, including sites I–III of tR_1 and the consensus sequence elements of *nut*R: boxA, boxB, and boxC. (B) Polyacrylamide gel electrophoresis analysis of transcripts used for binding and ATPase activation studies. Lanes 1–4 show an autoradiograph of ^{32}P-labeled RNA. Lanes 5–8 are from a photograph of an ethidium bromide stained gel of unlabeled RNA. (Lanes 1 and 5) pIF2/*Ava*I RNA; (lanes 2 and 6) pIF2/*Taq*I RNA; (lanes 3 and 7) pIF2dAR70/*Taq*I RNA; (lanes 4 and 8) pIF2dAL131/*Taq*I RNA. The numbers between the two gel panels indicate lengths in bp of marker DNA fragments (*Hae*III digest of pBR322 DNA).

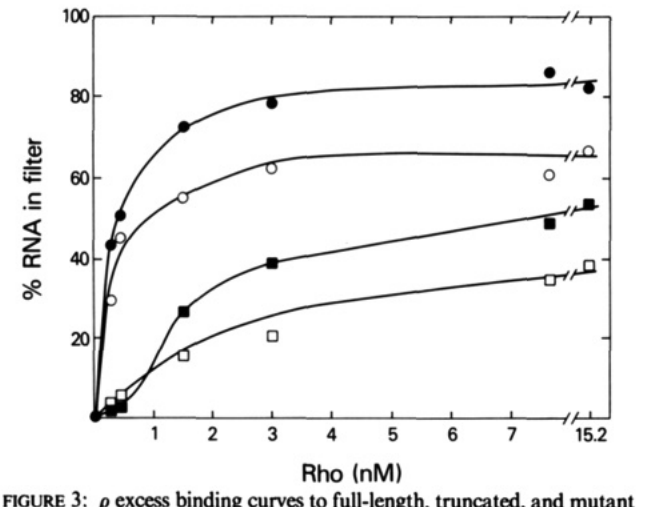

FIGURE 3: ρ excess binding curves to full-length, truncated, and mutant *cro* RNA molecules. Ten femtomoles of labeled *cro* RNA was incubated with ρ at the indicated concentrations at 37 °C for 5 min in 100 μL of binding buffer. The amount of RNA bound to ρ was determined as described under Materials and Methods. We verified by extraction and gel electrophoretic analysis that the RNA molecules retained in the filters were undegraded. (●) pIF2/*Taq*I (w.t.) RNA; (□) pIF2/*Ava*I (*Ava*I–w.t.) RNA; (■) pIF2dAR70/*Taq*I (dAR70) RNA; (○) pIF2dAL131/*Taq*I (dAL131) RNA.

To determine the relative contribution of different segments of *cro* RNA to the stability of its complex with ρ, we measured the binding affinity using a number of variant forms of *cro* RNA (Figure 2). A previous qualitative analysis of the ρ–*cro* RNA interaction showed that *cro* transcripts had to be elongated to almost full length before they could interact appreciably with ρ (Ceruzzi et al., 1985). It is also known that two segments near the 3′ end of *cro* RNA are required for ρ-dependent transcription termination at λ tR_1 (Chen et al., 1986; Chen & Richardson, 1987). These two segments, designated *rut*A and *rut*B, are encoded by the section between the unique *Ava*I and *Nde*I sites of pIF2 (Figures 1 and 2). It is therefore expected that an RNA molecule terminated at the *Ava*I of site of pIF2 would bind poorly to ρ, while an RNA that has been extended to the *Nde*I site would bind as well as one extended to the *Taq*I site. The data in Figure 3 and Table I show that these predictions are correct. When the *cro* RNA molecule was terminated at the *Ava*I site of pIF2, the affinity constant for the ρ–*cro* RNA interaction was 2.8×10^8 M^{-1}. When the RNA was extended to the *Nde*I site, the K_a equaled 34.8×10^8 M^{-1}, a value 12 times higher and very similar to that for the 378-nt "full-length" *cro* RNA molecule synthesized from *Taq*I-cleaved pIF2. These results suggest that a fragment of *cro* RNA encoded by the sequence between the unique *Ava*I and *Nde*I sites is very important for the primary (ATP-independent) interaction between ρ and *cro* RNA. This sequence includes the segment encoded by the *rut* sites as well as those present at the 3′ ends of ρ-terminated transcripts. Our further analysis showed that ρ also bound poorly to dAR70 RNA, a variant lacking *rut*A and part of *rut*B but containing the termination site sequences. To demonstrate that the presence of the *rut* sequences was more critical than the overall size of the transcript, we measured the binding to another variant RNA, dAL131, which had the *rut* sequences but lacked a 131-nt segment preceding the *rut* sequences, and found that it bound to ρ with nearly the same affinity as the wild-type *cro* RNA.

It is noteworthy that the binding of ρ to dAR70 *cro* RNA shows slight cooperativity (Figure 3). Although we do not fully understand the reason for such cooperativity, this effect might be related to the fact that the *rut*B region is probably not completely deleted in the dAR70 mutation, since ρ function is reduced 70% (but not 100%) when a mutant DNA template lacking these sequences is transcribed in vitro in the presence of ρ factor (Chen & Richardson, 1987). It is therefore possible that ρ makes use of the remaining *rut*B sequences by some cooperative interaction with a ρ molecule bound weakly to some other part of the RNA.

Table I: Affinity Constants for ρ–*cro* RNA Interactions[a]

RNA	$K_a \times 10^{-8}$ (M^{-1})
376-nt *cro* RNA (*E. coli* RNA polymerase transcript)	36.6 ± 10.2
pIF2/*Taq*I (full-length wild type)	32.9 ± 2.0
pIF2/*Nde*I	34.8 ± 11.8
pIF2/*Ava*I	2.8 ± 0.3
pIF2dAR70/*Taq*I	5.0 ± 0.1[b]
pIF2dAL131/*Taq*I	25.8 ± 6.3
pIF2/*Taq*I + oligonucleotide A	8.6 ± 0.5
pIF2/*Taq*I + oligonucleotide C	7.1 ± 3.5
pIF2/*Taq*I + oligonucleotides A and C	3.6 ± 0.5
pIF2/*Taq*I + oligonucleotide F	37.1 ± 4.9

[a] Values of K_a were determined from slopes of Scatchard plots of RNA-binding data from assays performed with 10 fmol of RNA and varying concentrations of ρ (in molar excess) in 100 μL of binding buffer. Complexes of RNA with oligonucleotides were prepared by incubating the 10 fmol of pIF2/*Taq*I (full-length wild-type) RNA with 300 fmol of the indicated oligonucleotide for 5 min at 37 °C. The sequences of the oligonucleotides are presented in Figure 1. Identification letters are those used in Chen et al. (1986). [b] Since the interaction between ρ and the pIF2dAR70/*Taq*I RNA shows slight cooperativity, the data points for ρ concentrations less than 1 nM were not included in the least-squares analysis. The remaining four points yielded an excellent straight line.

Table II: Correlations of Binding Affinity of the *ρ–cro* RNA Interaction in Termination Function[a]

RNA	conditions	$K_a \times 10^{-8}$ (M^{-1})	$\Delta G^{\circ\prime}_{total}$ (kcal/mol)	ΔG_n (kcal/mol)	ΔG_i (kcal/mol)	termination
wild-type *cro*	0.05 M KCl	8.0	−12.6	−8.5	−4.1	+++
dAR70	0.05 M KCl	1.3	−11.5	−7.5	−4.0	+/−
dAL131	0.05 M KCl	10.0	−12.8	−10.8	−2.0	++
wild-type *cro*	0.15 M KCl	0.8	−11.2	−8.5	−2.7	−
wild-type *cro*	0.05 M KGlu	14.1	−12.9			+++
wild-type *cro*	0.15 M KGlu	5.6	−12.4			+++

[a] The values for K_a and free energies are from the data presented in Figure 4. The relative termination activations are qualitative representatives of data from Chen and Richardson (1987) and from unpublished results.

Another approach for determining which segments of *cro* RNA are important for *ρ* action involves the use of DNA oligonucleotides to block access of *ρ* to specific segments of RNA. It was found (Chen et al., 1987) that oligonucleotides complementary to the two *rut* segments strongly interfered with *ρ* action at tR_1, whereas oligonucleotides complementary to other segments did not, and that those complementary to the tR_1 sequence did not affect *ρ* action at other *ρ*-dependent terminators. Presumably, the inhibitory oligonucleotides were blocking segments of RNA used to make strong contacts with *ρ*. This assumption, that the initial binding is blocked by these oligonucleotides, can be tested directly by measuring the effect of the oligonucleotides in the *ρ–cro* RNA binding assay.

As shown in Table I, when *cro* RNA was preincubated with a 30-fold molar excess of either oligonucleotide A or C prior to the addition of *ρ* factor, the binding affinity between *ρ* and *cro* RNA was lowered. When both A and C were preincubated with *cro* RNA, the binding affinity was lowered even further. In contrast, oligonucleotide F, added also at a 30-fold molar excess over RNA, had no effect on the *ρ–cro* RNA interaction. Tests with different levels of the inhibitory oligonucleotides A and C indicated that a 4-fold molar excess was enough to saturate their effects, while half-maximal inhibition was achieved with a 2-fold molar excess (data not shown). These results further support the notion that two single-stranded regions in the untranslated tail of *cro* RNA that are blocked by oligonucleotides A and C, the *rut* sites, are important for allowing a tight interaction between *ρ* and *cro* RNA.

Role of Ionic and Nonelectrostatic Contacts in the ρ–cro RNA Interaction. Both ionic and nonelectrostatic contacts are important in the overall interaction between proteins and nucleic acids. Since *ρ* is a relatively basic protein (Blumenthal et al., 1976), ionic contacts probably play a role in the overall binding reaction. However, since nonelectrostatic contacts appear to be important for the specificity of protein–nucleic acid interactions (Mougel et al., 1987), we also expected these types of contacts to contribute to the *ρ–cro* RNA interaction.

In order to determine the contribution of ionic and nonelectrostatic contacts to the *ρ–cro* RNA interaction, binding constants for the interaction were measured at different KCl concentrations. Previous results have indicated that the retention of *ρ*–poly(C) complexes by nitrocellulose filters is not affected by ionic strength in the range from 0.025 to 2.0 M KCl (Galluppi & Richardson, 1980). Thus, we assume that the filter binding separation procedure is suitable for measurements with other RNA molecules. The results are presented in Figure 4 as plots of ln K_a vs ln [K^+]. Record et al. (1976) have developed a quantitative analysis of the salt dependence of K_a based on ion displacement. By use of their model, the nonelectrostatic contributions to the interaction between *ρ* and an RNA, ΔG_n, can be estimated from the extrapolated value of ln K_a at 1 M KCl (ln $K_{a,ex}$). A correction is made for the number of lysine-like ion pairs N. Thus, $\Delta G_n = -RT \ln K_{a,ex} + N\Delta G_{Lys}$. We will use the values of ΔG_{Lys} of 0.2 kcal/mol determined by Record et al. (1976) for 1 M NaCl. The number of ion pairs, N, is calculated from the slope of the line in Figure 4, by using the equation:

$$d \ln K_a / d \ln [K^+] = -N\Psi$$

where Ψ is the fraction of monovalent cations released per phosphate bound and has a value that ranges between 0.68 and 0.78 for single-stranded RNA (Record et al., 1976). Although the value of Ψ for the *ρ–cro* RNA interaction is unknown, it is generally accepted that *ρ* binds to relatively unstructured regions in RNA molecules (Adhya et al., 1979; Richardson & Macy, 1981; Chen et al., 1986). Therefore, in our calculations we have used $\Psi = 0.73$, the average of the upper and lower limits, to yield $N = 3$ for wild-type *cro* RNA. By this analysis, we estimate that ΔG_n for the interaction between *ρ* protein and *cro* RNA is −8.1 kcal/mol. Hence, under the standard reaction conditions for monitoring *ρ*-dependent termination in vitro, which is with 50 mM KCl, nonelectrostatic interactions appear to contribute about two-thirds of the total binding energy of −12.6 kcal/mol (Table II).

To test whether the *rut* sequence contributes primarily to the nonelectrostatic component of the binding interaction, we performed a similar set of affinity measurements for the

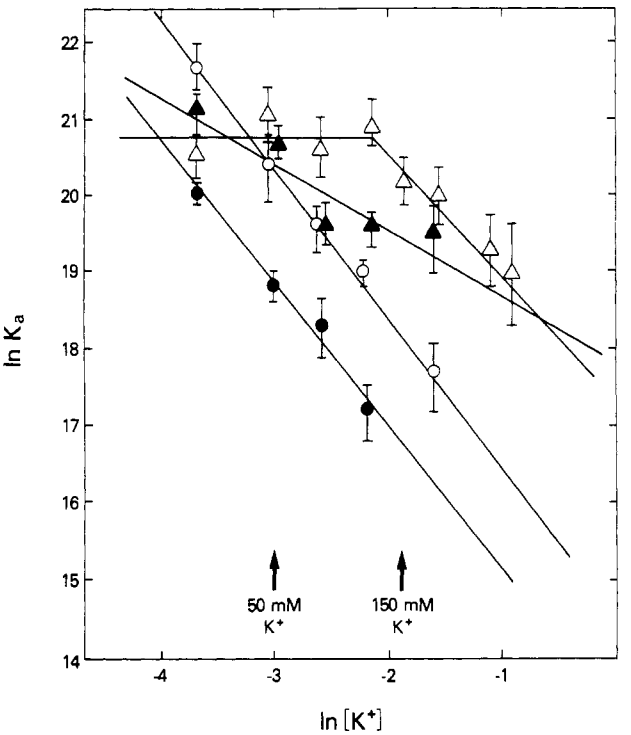

FIGURE 4: Dependence of equilibrium binding constants (K_a) of *ρ–cro* RNA complexes on K^+ ion concentration. Values of K_a were determined at each KCl and potassium glutamate concentration from Scatchard plots of RNA retained as a function of *ρ* concentration. (○) pIF2/*Taq*I (wild-type) RNA in KCl; (●) pIF2dAR70/*Taq*I RNA in KCl; (▲) pIF2dAL131/*Taq*I RNA in KCl; (△) pIF2/*Taq*I (wild-type) RNA in potassium glutamate.

variant RNA lacking the *rut* sequence (dAR70) as well as another shortened variant containing the *rut* sequence but lacking another upstream segment (dAL131) (Figure 4). With the dAR70 RNA, the electrostatic component again involved three ion pairs, but the nonelectrostatic component was reduced by 1 kcal. In contrast, binding to dAL131 RNA involved only one ion pair and a nonelectrostatic component that was 2 kcal higher than that for the complete *cro* RNA (Table II).

Effect of Potassium Glutamate on the ρ–cro RNA Interaction. Since it has been reported that protein–DNA interactions are stabilized at high K^+ concentrations when glutamate is replaced by chloride as the counterion (Leirmo et al., 1987), we also investigated the properties of ρ–*cro* RNA interactions in potassium glutamate. As shown in Figure 4, the equilibrium constant for the ρ and *cro* RNA interaction was independent of the potassium glutamate concentration in the range between 25 and 100 mM. Above 100 mM potassium glutamate, K_a decreased with increasing salt concentration following the same relationship as for KCl; the slope of that point of the ln K_a vs ln $[K^+]$ plot suggests that the number of ion pairs formed in the interaction was the same in the presence of potassium glutamate as in the presence of KCl. However, this interpretation is based on the assumption that the protein binds no anions (Record et al., 1976), but clearly the difference in the inhibition of binding with potassium glutamate and KCl must be an outcome of the relative ability of ρ to bind the two anions. The results suggest that chloride ions bind more tightly to ρ than glutamate ions and are thus less readily displaced by RNA.

Temperature Dependence of the ρ–cro RNA Interaction. Since many protein–nucleic acid interactions are stabilized by large entropic contributions (Record & Mossing, 1986), we also calculated the entropic and enthalpic contribution to the free energy of the ρ–*cro* RNA interaction. Complete binding curves were obtained under standard binding conditions in the range from 4 to 37 °C, and ln K_a was plotted vs $1/T$ (a van't Hoff plot) to obtain the enthalpy of binding ($\Delta H°$) from the slope of the line (data not shown). The dependence of K_a on temperature was small,[2] an observation that has been previously made in the case of other protein–RNA interactions (Spierer et al., 1978; Mougel et al., 1986), and yielded $\Delta H°$ = +3.8 kcal/mol and, therefore, $\Delta S°$ = 55.8 cal·mol^{-1}·K^{-1}. Thus, the entropy change is the main driving force for the reaction. The value of $\Delta H°$ for binding of ρ to the dAL131 variant RNA was the same as for the wild-type RNA. However, the scatter in the data points for the weakly binding dAR70 RNA was too great to determine whether $\Delta H°$ was significantly different with it.

ρ ATPase Cofactor Properties of λ cro RNA. The termination of RNA synthesis by ρ factor is a process that is coupled to the hydrolysis of nucleoside triphosphates (Howard & de Crombrugghe, 1976; Galluppi et al., 1976). Since the hydrolysis reaction can occur uncoupled from termination by interactions between ρ and isolated RNA molecules (Lowery-Goldhammer & Richardson, 1974), it is possible to determine what segments of RNA are essential for NTPase activation by comparing ATP hydrolysis rates with variant forms of the RNA.

The ρ preparations used throughout this study could catalyze ATP hydrolysis with a rate of 16.1 nmol·min^{-1}·(μg of protein)$^{-1}$ when poly(C) was the activating RNA cofactor. Poly(C) is the most potent known activator of the ρ ATPase activity (Lowery & Richardson, 1977), but remarkably, *cro* RNA was almost as good. At saturation, it yielded a rate of 10.4 nmol·min^{-1}·(μg of protein)$^{-1}$ (Figure 5). On the other hand, the dAR70 RNA at saturation was one-tenth as active as wild-type *cro* RNA (Figure 5), and the variant *cro* RNA molecule synthesized from *Ava*I-cut pIF2 was inactive as an ATPase cofactor. Thus, those sequences in *cro* RNA that contribute important nonionic interactions in the binding reaction are also required for ATPase activation. The fact that the rate of ATP hydrolysis was lower with the variant RNAs at saturation than with wild-type *cro* RNA indicates that the mere binding of those RNAs is not sufficient for ATPase activation, as a defect in binding would be overcome by increasing the concentration of the RNA. The results with the dAL131 RNA show that a large segment of the RNA upstream from the *rut* sequences can be deleted without affecting cofactor activity. Thus, those sequences that appear to contribute some ionic contacts in the binding with wild-type *cro* RNA are not essential for activation of ATP hydrolysis.

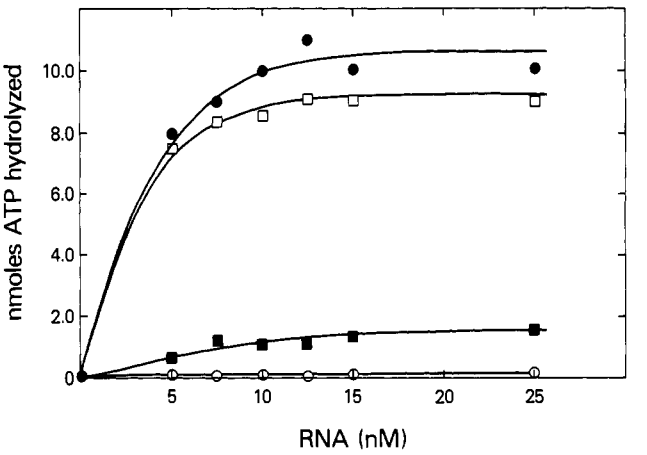

FIGURE 5: Activation of the RNA-dependent ρ ATPase by *cro* RNA. ATPase assays were performed by using a 45-min incubation as described under Materials and Methods. Activating RNA molecules: (●) pIF2/*Taq*I (w.t.) RNA; (○) pIF2/*Ava*I (*Ava*I–w.t.) RNA; (■) pIF2dAR70/*Taq*I (dAR70) RNA; (□) pIF2dAL131/*Taq*I (dAL131) RNA.

Tests of the effects of oligonucleotides that are complementary to various regions of wild-type *cro* RNA on ATPase activation showed, as expected, that those oligonucleotides that block termination and block binding (A and C) also inhibit ATP hydrolysis, whereas an oligonucleotide (F) that did not block termination had no effect on ATPase hydrolysis (data not shown).

We have shown in this report that changes in temperature have only a small effect on the ρ–*cro* RNA binding interaction. We also found that the binding constant (K_a) was independent of the Mg^{2+} concentration in the range from 0 to 10 mM (data not shown). In contrast, the ATPase activity was very sensitive to temperature and $MgCl_2$ concentration (Figure 6). These results parallel previous findings with T7 RNA as cofactor and differ from those obtained with poly(C). ρ ATPase with poly(C) is maximally stimulated with 4–10 mM $MgCl_2$ and increased only 2-fold for every 10 °C increase in temperature from 0 to 30 °C, as compared to 3- to 4-fold with *cro* RNA (Richardson & Macy, 1981). Thus, with *cro* RNA, as with T7 RNA, ATP hydrolysis depends on subsequent interactions with the RNA that are particularly sensitive to temperature and Mg^{2+} concentration. One important difference between poly(C) and *cro* RNA that could account for the differences in the Mg^{2+} ion and temperature dependence is that poly(C)

[2] Previous results from our laboratory (Ceruzzi et al., 1985) indicate that ρ binds to *cro* RNA with a 20-fold lower affinity at 4 °C than at 37 °C. Although the affinity we measured for the 378-nt *cro* RNA at 37 °C was the same as that determine previously under the same ionic conditions, we did not reproduce the earlier results for binding at 4 °C.

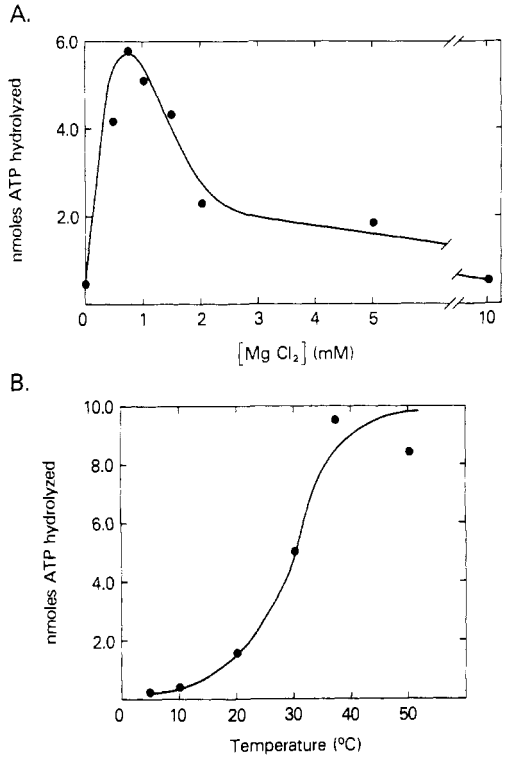

FIGURE 6: Effects of (A) Mg^{2+} and (B) temperature on ρ ATPase activity with *cro* RNA. ATPase assays were performed as described under Materials and Methods by using 20 nM pIF2/*Taq*I *cro* RNA. Incubations were for 30 min (A) and 45 min (B).

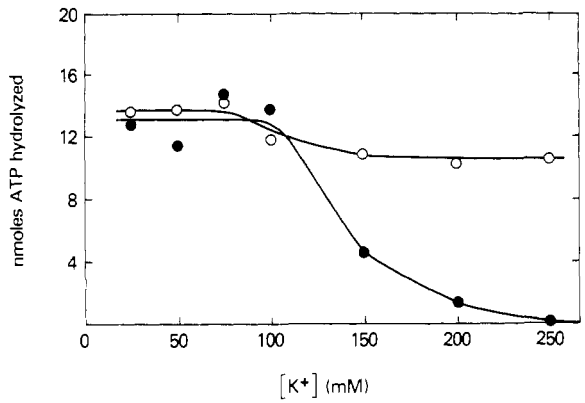

FIGURE 7: Effects of KCl and potassium glutamate on ρ ATPase activity with *cro* RNA. ATPase assays were performed as described, for 30 min at 37 °C, with 30 nM *cro* RNA and 0.18 pmol of ρ (●) with KCl or (○) with potassium glutamate.

lacks base pairing. Since higher temperature and lower Mg^{2+} concentration would both reduce the extent of base pairing in *cro* RNA, the subsequent interactions needed for ATP hydrolysis may be directly coupled to the breaking of base-paired structures in the RNA.

As expected, however, the effects of KCl and potassium glutamate concentrations on binding to *cro* RNA were apparent on ρ ATPase activity as well. At the lower concentrations (<100 mM) the hydrolysis rates with the two salts were about the same, but at higher concentrations, there was significantly less activity in reaction mixtures with KCl than in mixtures with potassium glutamate (Figure 7).

Discussion

The results presented in this paper further extend the analysis of Chen et al. (1986) and Chen and Richardson (1987), who showed that two single-stranded segments near the 3′ end of *cro* RNA called *rut*A and *rut*B are required for ρ function at λ tR_1. We have shown in this report that these regions of *cro* RNA are very important for tight binding of ρ to the isolated transcripts and for eliciting the high level of ATPase activity that is characteristic of normal *cro* RNA. We also demonstrate that these critical binding contacts between ρ and the *rut* segments involve almost exclusively nonelectrostatic interactions.

From the results of binding studies with the variant RNA molecules and with normal *cro* RNA under different ionic conditions, a distinct correlation can be made between binding affinity and termination function (Table II). ρ is very effective at termination under those conditions in which K_a for binding of ρ to the isolated transcript is (5–10) × 10^8 M^{-1} but is essentially inactive under conditions in which K_a is $\leq 1 \times 10^8$ M^{-1}. This correlation includes the difference in effects of chloride ions and glutamate ions on binding activity. The difference between a functional and a nonfunctional interaction appears to involve only 1–2 kcal/mol binding energy out of a total of 12.6 kcal/mol. This total depends on the contributions from both ionic and nonelectrostatic interactions. The importance of the nonelectrostatic interactions is evident from the difference in binding properties of wild-type RNA and the dAR70 RNA. The loss of the sequence deleted in the dAR70 variant reduced the nonelectrostatic component of the binding energy by 1 kcal/mol without affecting the ionic component. The importance of the ionic interaction is evident from the effects of KCl concentration on the binding to wild-type RNA. The loss of binding energy in going from 0.05 to 0.15 M KCl is 1.4 kcal/mol, but in this case, ionic interactions are lost due to the competition with counterions.

The fact that loss of termination function correlates with less than 10% loss of binding energy seems surprising at first. However, that decrease in binding energy is a reflection of a 6-fold difference of binding affinity. Since transcription is a dynamic process, the interaction between ρ and nascent RNA has to be rapid enough for ρ to dissociate the transcript before RNA polymerase has traversed the termination region. A 6-fold decrease in binding affinity could be enough to render the interaction inadequate for this dynamic process.

In spite of the rough correlation between binding affinity and termination, there is more to a functional interaction between ρ and the nascent RNA than mere binding. This distinction was revealed by the ATP hydrolysis activation studies, which showed that the maximum rates of hydrolysis at RNA saturation were lower with the variant RNAs that had lower binding affinity than with the normal RNA. If the alterations were only affecting the binding affinity (K_a) for the RNA cofactor, the V_{max} achieved at cofactor saturation should be the same, which is not the case. One explanation for this observation is that the residual binding that is detected with the variants lacking the *rut* sequence reflects the contribution of a large number of nonproductive weak complexes involving different sites on the RNA. None of these individual weak complexes would have a lifetime long enough to allow activation of ATP hydrolysis. However, there is also another aspect of the cofactor activity of RNA that is revealed in the different effect of temperature and Mg^{2+} concentration on binding and ATP hydrolysis. In our model for RNA cofactor activity (Galluppi & Richardson, 1980), ATP hydrolysis is coupled to secondary interaction between ρ and RNA. These secondary interactions may involve the breaking of base pairs, as is evident in the ATP hydrolysis dependent ρ helicase activity (Brennan et al., 1987). The sharp thermal activation and extreme Mg^{2+} ion sensitivity of the ATP hydrolysis re-

action with *cro* RNA could be a reflection of an effect of RNA structural stability on the V_{max} of the ρ ATPase reaction. It is also possible that one reason why the V_{max} with dAR70 RNA is lower than with wild-type *cro* RNA is that the sequences that are missing in the dAR70 RNA may be involved in secondary (ATP hydrolysis dependent) interactions as well as the primary interactions.

What is therefore recognized by ρ in an RNA molecule? Lau and Roberts (1985) found that there was a sequential loss of termination efficiency at tR_1 as they deleted larger segments of the *cro* gene. They interpreted these results to mean that there is a minimum transcript length that is required for a functional interaction. On the other hand, Chen and Richardson (1987) found that some deletions in the *cro* gene sequences were more deleterious for ρ function than others. These results implied that there is some degree of sequence specificity in the ρ–*cro* RNA interaction.

We propose that *both* a minimum transcript length and specific sequences in the nascent transcript are important for ρ function. We have shown that both ionic and nonelectrostatic contacts are important for a functional ρ–*cro* RNA interaction. The ionic contacts could be sequence-independent contacts between basic residues in ρ and backbone phosphates. Hence, a minimum transcript length might be required for sufficient ionic contacts. In fact, we have shown that deleting 131 nucleotides in *cro* RNA reduces the number of ion pairs formed in the interaction from 3 to 1. But the specificity of binding appears to be mediated by the ability of ρ to make nonelectrostatic contacts with residues near the 3′ end of the molecule. Structural studies on *cro* RNA indicate that the segments of RNA important for ρ function are single stranded (I. Faus and J. P. Richardson, unpublished results). Contacts between ρ and these single-stranded regions of λ *cro* RNA are the interactions that are important for ATPase activation. Since the ρ–poly(C) interaction is salt-insensitive (Galluppi & Richardson, 1980) and since it is likely that cytosine residues in the primary ρ interaction site play a role in activating the NTPase activity, it is not surprising that deletion of this region greatly diminishes the nonelectrostatic component of the ρ–*cro* RNA interaction.

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