The small prohead RNA (pRNA) of the Bacillus subtilis bacteriophage φ29 is essential for ATP-dependent packaging of viral DNA. The 174-, 124-, and 120-residue forms of pRNA produced in vitro using T7 RNA polymerase were equivalent in prohead binding and DNA packaging activity to pRNAs produced in φ29-infected cells. pRNA binding to proheads, characterized by the use of Northern hybridization and filter binding assays, was specific, rapid, and irreversible in the presence of 10 mM Mg²⁺. Proheads produced in phage-infected cells carried 5.8 ± 2.7 copies of pRNA, and proheads assembled in the E. coli in the absence of pRNA bound 6.0 ± 3.5 copies of pRNA. Footprints of proheads on pRNA generated with the ribonucleases A, T1, and V1 showed that nucleotides 22-84, 5'-to 3', were protected from ribonuclease attack. Enhanced cleavage at nucleotides 37-40 with ribonuclease V1 suggested a conformational change of pRNA upon prohead binding.

An RNA molecule encoded by bacteriophage φ29 of Bacillus subtilis is an essential component of the viral precursor capsid or prohead into which the 19 kilobase double-stranded DNA-gene product 3 (DNA-gp3) complex is packaged with the aid of the ATPase gp16 and ATP hydrolysis (Guo et al., 1987b, 1987c). The prohead RNA (pRNA), initially isolated from purified proheads as a 120-residue molecule, can be detached from proheads and reattached with concomitant loss and restoration of DNA-gp3 packaging activity in the defined in vitro system (Guo et al., 1987c; Wichitwechkarn et al., 1989). The full-length molecule is 174 residues, and 54 residues are removed from the 3' end by adventitious nucleases during the prohead purification (Wichitwechkarn et al., 1989). The prohead is composed of the major head protein (gp9), the removable scaffolding protein (gp7), the head fibers (gp8.5), and the portal protein or connector (gp10) that serves as the attachment site for pRNA and as a unique vertex for DNA-gp3 packaging. Purified proheads contain approximately six copies of the 174-residue pRNA by Northern hybridization analysis, and in vitro DNA packaging activity in the defined system is maximal when RNA-free proheads are reconstituted with six copies of pRNA (Wichitwechkarn et al., 1989). The purified 120-residue pRNA molecule also reconstitutes RNA-free proheads for DNA-gp3 packaging (Guo et al., 1987c; Guo et al., 1987a).

pRNA secondary structure has been determined by phylogenetic analysis and is organized into two domains (Bailey et al., 1990). The larger 5' domain (domain I), composed of 117 residues and containing four helices, is necessary and sufficient for DNA packaging.

The ATPase activity of gp16 is dependent on DNA-gp3 and proheads in the defined in vitro DNA packaging system (Guo et al., 1987b). pRNA or purified proheads with pRNA stimulate the ATPase activity of gp16 in the absence of DNA-gp3, and the stimulation by proheads is pRNA-dependent (Grimes and Anderson, 1990).

Proheads with truncated pRNAs have been isolated and used to correlate pRNA size with DNA-gp3 packaging activity (Grimes and Anderson, 1989b). Cleaving pRNA alters the specificity to package left- and right-end restriction fragments of DNA-gp3. Residues 1-25 of pRNA contribute to a domain for DNA-gp3 interaction, while residues 26-49 contribute to a domain for prohead binding.

The role of pRNA in φ29 DNA packaging remains an intriguing question. To facilitate the study of pRNA structure and function, an in vitro T7 transcription system was developed that produces pRNA in quantity. The binding of pRNA to RNA-free proheads was characterized by Northern hybridization and nitrocellulose filter binding, and RNase footprinting was used to determine pRNA contacts with proheads.

**EXPERIMENTAL PROCEDURES**

Bacteria and Plasmids—B. subtilis 12A (trp C2, spoOA12) was the host for bacteriophage φ29. Escherichia coli NM522 (recA⁺, supE, thi, Δlac-proAB), had 5, (F', proAB, lacI, lacZ Δm15)) was the ung⁺ host for the pBluescript KS⁺ phagemid (Stratagene). E. coli C236 (dut 1, ung 1, thi -1, rel A/tcJ105 cam' F') was the dut⁺ ung⁻ strain for preparation of uracil-containing single-stranded DNA from pBluescript derivatives. E. coli strains HMS174(DE3) (pART-8.8-5.10) and BL21(DE3)(pART-8.8-5.10) that produce pRNA-free φ29 proheads have been described (Guo et al., 1991a, 1991b).

Preparation of Proheads—Proheads were purified from B. subtilis 12A cells infected with the mutant sus16(300)-sus14(1241) that is defective for the DNA packaging ATPase gp16 as described (Wichitwechkarn et al., 1989). Most of the pRNA was removed from the proheads by a 100-fold dilution into 100 mM Tris borate (pH 8.3) and 2 mM EDTA (TBE buffer) for 10-15 min at room temperature, and the proheads were pelleted by centrifugation in a Beckman SW 55 rotor at 35,000 rpm for 5 h at 4°C.

Alternatively, proheads were produced in the absence of pRNA in E. coli strains HMS174(DE3) or BL21(DE3) containing the isopropyl-β-thiogalactopyranosidase-inducible plasmid pART-8.8-5.10 that encodes prohead structural proteins (Guo et al., 1991a). Cells were grown in Luria-Bertani broth (Maniatis et al., 1982) containing 100 μg/ml ampicillin at 37°C with shaking to an A₅₇₀ of 1.0. Isopropyl-β-thiogalactopyranoside was added to 0.5 mM, and incubation was continued for 3 h at 37°C. Cells were concentrated 100-fold by centrifugation and resuspended in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 10 mM MgCl₂ (TMS buffer) containing 30 units/ml RNase-free DNAse I (Boehringer Mannheim). The cells were disrupted by sonication for 6-8 min at 0°C,
Preparation of pRNA—pRNA was purified from *B. subtilis* 12a(pUM102) by electrophoresis in denaturing urea-acrylamide gels as described (Wichitwechkam et al., 1992). Individual pRNA bands were located by UV shadowing over polyethyleneimine/UV-\,-cellulose plates (Alltech Associates, Inc.) with a 254-nm light source. pRNA bands were excised and the pRNA eluted twice by diffusion into 500 mM acetic acid (pH 6), 0.1 mM EDTA, and 0.1% SDS for 3-6 h at 37 °C.

Alternatively, pRNA was produced by in vitro T7 transcription from plasmid pRT72. The transcription template was constructed by site-directed mutagenesis (Kunkel, 1985, 1987) of plasmid pBlue102 (Wichitwechkam et al., 1992). pBlue102 is a plasmid linearized with the Δ59 pRNA gene on a 320-base pair fragment and the pRNA promoter in the same orientation as the pBlueScript T7 promoter. The mutagenic oligonucleotide 5′-GCACTCACTATAGGAATGGTACGGTACTTC-3′ was used to delete 123 base pairs of plasmid DNA between the pBlueScript T7 consensus promoter and the pRNA transcription start site. The 30-mer juxtaposed the -12 to +2 sequence of the T7 promoter, numbering relative to the start of transcription, and the +3 to +18 sequence of the pRNA gene. The resulting plasmid pRT7Del, was mutagenized with the 38-mer 5′-GTGACCGCTACTTCTTAAAGTCTTACATGCGACACAG-3′ to insert a DdeI site at +120 and a BglII site at +124 with respect to the T7 promoter and produce pRT71. This also changed 116C→G and 117T→G in the pRNA gene to complement the changes 1A→C and 2G→C from the first round of mutagenesis and maintain pRNA secondary structure (Bailey et al., 1990). Then pRT71 was mutagenized with the 30-mer 5′-CTTGGTGCCTTTAAAATGTTGCTACATT-GACAAA-3′ to insert a DraI site at +174 and create pRT72. Clones were identified after each round of mutagenesis by DNA sequencing or restriction mapping. Restriction digests with DraI (Boehringer Mannheim) and with DdeI and BglII (Life Technologies, Inc.) were performed according to the manufacturers’ instructions.

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*In vitro* pRNA synthesis was based on the method of Cunningham and Ofengand (1990). Briefly, the template was digested with the appropriate restriction enzyme and purified with Prohead-A-Gene (Bio-Rad). Fifty-microliter reactions contained 0.9 pmol of template, 250 units of T7 RNA polymerase (Life Technologies, Inc.), and 40 units of human placental RNase inhibitor (Calbiochem) in 40 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 2 mM spermidine-(HCl)₃, 5 mM NaCl, 5 mM dithiothreitol, 5 units/ml inorganic pyrophosphatase (Sigma), and 2.5 mM of each nucleotide triphosphate. Reactions were incubated for 3-5 h at 37 °C and pRNA was purified by gel electrophoresis as described above.

pRNA was Δ59-labeled with T4 polynucleotide kinase (Life Technologies, Inc.) and \(γ[^32P]ATP\) (DuPont NEN) as described (Wichitwechkam et al., 1989) after removal of the 5′-terminal phosphate with calf intestine alkaline phosphatase (Boehringer Mannheim). Alternatively, pRNA was labeled by incorporating \([α[^32P]ATP\) during *in vitro* T7 transcription. Reactions were prepared as above except that 25 μC of \([α[^32P]ATP\) (DuPont NEN), 0.18 pmol of template, and 50 units of T7 RNA polymerase were added to a 50-μl reaction containing 10 μM ATP and 500 μM each of GTP, CTP, and UTP. Incubation was for 50 min at 37 °C. pRNA was purified by gel electrophoresis as described above, and bands were located by autoradiography.

**Binding of pRNA to Proheads**—Binding of labeled pRNA to proheads was determined quantitatively by nitrocellulose filter binding as described (Carey et al., 1983). Briefly, 10⁴ cpmp of \([32P]pRNA\) was diluted into 50 mM Tris-HCl (pH 7.5) and 10 mM MgCl₂ (TM buffer), heated to 75 °C for 3 min, cooled quickly to 0 °C, and mixed with RNA-free proheads in 50 μl of TM buffer. After incubation for 10 min at 25 °C, samples were diluted to 500 μl in TM buffer and loaded onto prewetted 25-mm diameter cellulose nitrate filters (Micro Filtration Systems) under constant suction in a filtration apparatus. After three washes of 2 ml each with TM buffer, the radiolabel retained on the filter was measured by scintillation counting.

For dot blot hybridization, 140 μg of RNA-free proheads were reconstituted with 8 μg of 174-residue pRNA (12 RNA/prohead) in TMS buffer for 15 min at room temperature. Proheads were separated from unbound RNA by centrifugation in a linear 5-20% sucrose gradient, concentrated by centrifugation in the SW 55 rotor at 35,000 rpm for 5 h at 4 °C, and quantified by protein assay (Bio-Rad). Hybridizations were performed as described (Wichitwechkarn et al., 1989).

For footprinting, 140 μg of RNA-free proheads were reconstituted with 2.8 μg of the Δ59-labeled, 124-residue \([32P]pRNA\) (6 RNA/prohead; 7 × 10⁴ cpmp/μg pRNA) in TM buffer for 15 min at room temperature. Proheads were then diluted to 5 ml with TM buffer at 4 °C, separated from unbound pRNA by centrifugation to pellet the particles as described above, and the fraction of pRNA bound determined by filter binding assay.

**RNase Footprinting Analysis**—Proheads with bound \([32P]pRNA\) (3-5 × 10⁴ rpm) were digested with varying concentrations of RNase A (Sigma), RNase T1 (Boehringer Mannheim) or RNase V1 (Pharmacia LKB Biotechnology Inc.) in 10-μl reactions for 15 min at room temperature. pRNA was extracted with 200 μl of a mixture containing 1 part 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 20 μg/ml tRNA and 1 part phenol

![FIG. 1. pRNA content of proheads determined by Northern hybridization. 5 μg (lane 1), 2.5 μg (lane 2), 1.3 μg (lane 3), and 0.6 μg (lane 4) of each purified prohead preparation with associated 174-residue pRNA was spotted onto a membrane and hybridized with a pRNA-specific [32P]-labeled probe as described (see "Experimental Procedures"). A, proheads purified from *sus16(3000)-sus14(1241)-infected* cells; B, proheads from *A* after EDTA treatment and repurification; C, proheads from *B* incubated with 174-residue pRNA and repurified; D, RNA-free proheads assembled in *E. coli*, incubated with 174-residue pRNA, and repurified.](image)

![FIG. 2. pRNA binds specifically to proheads in the presence of Mg²⁺. Proheads were mixed with pRNA (760 pmol/ml) in TM buffer without (●) or with (○) tRNA (36.6 μmol/ml) as a nonspecific competitor, or in TE buffer without (×) or with (□) tRNA. Proheads bound 2.3 and 1.9 pRNA in TM buffer without and with tRNA, and 1.2 and 0.1 pRNA in TE buffer without and with tRNA, respectively.](image)
at 50 °C, and the aqueous phases were precipitated with 2,5 volumes of ethanol containing 20 μg/ml glycogen. Pellets were resuspended in 20% glycerol, 0.05% xylene cyanol, 0.05% bromphenol blue, and 4 M urea, and the digested pRNA was separated by electrophoresis on a 10% polyacrylamide gel containing 8.3 M urea. The gel was fixed in H2O/ acetone/methanol (8/1/1), dried, and placed on x-ray film.

DNA Packaging Reactions—In vitro DNA packaging assays were performed as described (Grimes and Anderson, 1989a). Briefly, purified proheads or proheads reconstituted with pRNA were mixed with purified DNA-gp3 in reaction buffer containing 10 mM ATP, 6 mM spermidine, and 3 mM 2-mercaptopethanol in TMS buffer. The ATPase gp16 in 4 M guanidinium chloride was renatured by a 25-fold dilution into 2 mM Tris (pH 7.5), 0.4 mM CHAPS, and 5 mM dithiothreitol for 40 min on ice. Reaction mixtures containing proheads, DNA-gp3, and gp16 (2:1:50) were incubated for 30 min at room temperature. Unpackaged DNA was digested with 5 μg/ml DNase I, the DNase inactivated with 10 mM EDTA, and the packaged DNA extracted from filled heads for 60 min at 65 °C and quantified by agarose gel electrophoresis.

RESULTS AND DISCUSSION

In Vitro pRNA Synthesis and Activity—In vitro transcription by T7 RNA polymerase can provide large quantities of RNA for biochemical and biophysical analyses (Lowary et al., 1986; Studier et al., 1990). Three rounds of oligonucleotide-directed mutagenesis on plasmid pBlue102rev, which contains the pRNA gene, produced the in vitro transcription template pRTT2 (see “Experimental Procedures”). First, the pRNA promoter was deleted to align the T7 promoter for pRNA expression. Second, changes of nucleotides 1A→C and 2G→C in the pRNA gene were made to accommodate requirements of the T7 polymerase (Milligan et al., 1987; Chapman and Burgess, 1987; Dunn and Studier, 1983). Nucleotides 1 and 2 of pRNA are at the end of a helix (see Bailey et al. (1990) and Fig. 5), so the compensating mutations 116C+G and 117T-+G were made in the pRNA gene to restore base pairing in the transcripts. Finally, because a 3′-truncated 120-nucleotide form of the 174-residue pRNA is sufficient for packaging (Guo et al., 1987a, 1987c), three restriction sites were inserted to permit the production of 174-, 124-, or 120-nucleotide pRNA. The prohead binding and in vitro DNA packaging activity of each of these pRNAs on proheads was indistinguishable from wild-type pRNA (data not shown). Moreover, the changes in pRNA nucleotides 1 and 2, along with the compensating changes at 116 and 117 to maintain secondary structure, did not affect pRNA function.

Characterization of pRNA Binding to Proheads—Northern hybridization and filter binding assays were used to determine the extent, specificity, rate, and ionic requirements of [32P]pRNA binding to proheads stripped of pRNA with EDTA or to proheads assembled in E. coli in the absence of pRNA. Northern hybridization was used to determine the extent of pRNA binding to proheads, and the results of a typical experiment are illustrated in Fig. 1. Proheads produced in phage-infected cells carried 5.8 ± 2.0 (n = 8) copies of pRNA. Proheads treated with EDTA retained 2.8 ± 1.0 (n = 6) copies of pRNA, and after reconstitution these proheads carried 5.4 ± 3.2 (n = 2) pRNA/prohead. Proheads assembled in E. coli bound 6.0 ± 2.5 (n = 5) pRNA/prohead. The pRNA content or binding per prohead was more variable than the value of 5.5 ± 0.9 pRNA/prohead determined by electron microscopic counts of particles (Wichtweck-karn et al., 1989). Possibly proheads of some preparations can bind more than the mean of 5–6 pRNA. The demonstration that proheads produced in E. coli in the absence of pRNA and pro-

**Fig. 3. Prohead footprint on pRNA with RNase A.** Denaturing gel electrophoresis and digestions with RNase A were performed as described (see “Experimental Procedures”). [32P]pRNA (−) and
**Fig. 4. Prohead footprint on pRNA with RNases V1 and T1.** Denaturing gel electrophoresis of [³²P]pRNA (−) and [³²P]pRNA-prohead complexes (+) treated with no RNase (lanes 1 and 2), 600 units/ml RNase T1 (lanes 3 and 4), 60 units/ml RNase T1 (lanes 5 and 6), 6 units/ml RNase T1 (lanes 7 and 8), 0.3 units/ml RNase V1 (lanes 9 and 10), 0.003 units/ml RNase V1 (lanes 11 and 12), and 0.03 units/ml RNase V1 (lanes 13 and 14). Markers were generated as described in the Fig. 3 legend.
heads produced in phage-infected cells bound the same amount of pRNA is consistent with the finding that DNA packaging by these two types of proheads in the defined in vitro system is comparable (Guo et al., 1993). Between 60 and 80% of the input pRNA was competent to bind to proheads. Similar variability in the fraction of RNA that can bind a protein has been observed with other small RNAs, such as the R17 coat protein binding sequence (Carey et al., 1983). The unbound pRNA was intact, and therefore structural variations may exist among pRNA molecules.

Varying amounts of proheads were incubated with a constant amount of [32P]pRNA in TM buffer or 50 mM Tris-HCl (pH 7.5), 10 mM EDTA (TE buffer) to determine the specificity of pRNA binding in the presence or absence of Mg^{2+}, with or without competitor tRNA (Fig. 2). In the presence of 10 mM Mg^{2+} each prohead bound 2.3 pRNA without tRNA and 1.9 pRNA with tRNA as determined by linear regression analysis. The relationship between proheads added and pRNA bound was linear, demonstrating stoichiometric binding, although the extent of binding was generally lower than that observed in the hybridization experiments, perhaps due to lability of the RNA-free proheads upon storage. Proheads in TE buffer bound 1.2 pRNA/prohead without tRNA and 0.1 pRNA/prohead with excess tRNA. Thus proheads have a general affinity for RNA in the absence of Mg^{2+} but bind pRNA specifically in the presence of Mg^{2+}.

To test for reversibility of binding, [32P]pRNA (116 pmol/ml) was incubated with proheads (23.2 pmol/ml), and the filter assay showed 1.5 mol pRNA bound/mol of proheads. Unlabeled pRNA (1.16 nmol/ml) was then added, and the amount of labeled pRNA bound was assayed over 1 h. No decrease in the amount of labeled pRNA bound was observed, showing that bound pRNA did not exchange with unbound pRNA (data not shown). Additionally, the prohead-pRNA complex was stable upon dilution and ultracentrifugation.

To determine the rate of pRNA binding, proheads produced in E. coli (48 pmol/ml) were incubated with pRNA (510 pmol/ml) and tRNA (59 nmol/ml), and the amount of pRNA bound was determined at 3-min intervals. Proheads bound approximately 1.3 pRNA within 3 min at 0, 25, or 37 °C, with no increase thereafter, and approximately 80% of the ultimate binding occurred within 1 min (data not shown). The amount of pRNA bound per prohead varied over time of storage of the proheads at −20 °C and also among prohead preparations.

Ionic requirements for pRNA binding were determined by varying NaCl or MgCl₂ concentrations in binding mixtures containing proheads (54 pmol/ml), pRNA (420 pmol/ml), and tRNA (8.2 nmol/ml). Varying NaCl concentration from 0 to 300 mM with 10 mM MgCl₂ had no effect on the amount of pRNA bound (data not shown). When MgCl₂ concentration was varied from 0 to 25 mM, the amount of pRNA bound increased with increasing MgCl₂ concentration to 10 mM and then remained constant.

In summary, pRNA bound to proheads specifically, rapidly, and irreversibly in the presence of 10 mM Mg^{2+}. Mg^{2+} has both structural and catalytic roles for RNA enzymes (for review see Yarus (1993)). Mg^{2+} likely stabilizes pRNA secondary and tertiary structure to allow for specific binding to proheads.

**RNase Footprint Analyses—** Proheads with 2–3 copies of 124-residue [32P]pRNA were separated from unbound pRNA by centrifugation and used to produce footprints with ribonucleases A, T1, and V1 (Figs. 3 and 4). The purified pRNA-prohead complexes showed greater than 90% pRNA bound by filter binding assays.

pRNA bound to proheads was protected from RNase A digestion at residues 25–84, 5' to 3', and residues 4–19 and 110–120 were not protected (Fig. 3). Footprints produced with RNase V1 showed protection of nucleotides 22–69, with no protection of nucleotides 12–16 or from nucleotide 93 to the 3' end. Enhanced cleavages of pRNA with RNase V1 occurred at nucleotides 37–40. RNase T1 digests of pRNA-prohead complexes showed protection of residues 51–82, supporting the results shown with RNases A and V1 (Fig. 4). Cleavages by the single- or double-strand specific RNases agreed generally with previous digests of pRNA alone that supported the model of secondary structure (Bailey et al., 1990). The composite ribonuclease footprint presented in Fig. 5 shows that a 63-nucleotide region that subdends residues 22–84 within the 120-residue domain of pRNA interacts with the proheads. The footprinting results are consistent with the results of Grimes and Anderson (1989b), which showed that a 95-residue pRNA molecule that included nucleotides 26–120 bound to proheads and that further truncations of the 5' end to nucleotide 50 abolished prohead binding. Since nucleotides 26–49 constitute approximately one-half of the prohead footprinted region, deletion of these residues would be expected to prevent prohead binding.

The enhanced cleavage at residues 37–40 by RNase V1 might be due to enrichment of cleavable pRNA from a pool of heterogeneous molecules by prohead binding, since unbound pRNA was removed prior to ribonuclease treatment. However, similar enhanced cleavages at residues 37–40 by RNase V1 were observed when unbound pRNA was not removed (data not shown). Therefore, irreversible binding of pRNA to proheads apparently stabilized pRNA secondary structure or resulted in a conformational change of pRNA to allow enhanced cleavage by RNase V1. Some background cleavage at nucleotides 17, 18, 39, and 40, likely due to contaminating nucleases, was noted upon addition of pRNA to proheads (Figs. 3 and 4, lane 2); this cleavage was reduced by a second gradient purification of the prohead preparations (data not shown).

The 63-residue prohead binding region of φ29 pRNA seems relatively large when compared to protein interactive sites of certain other RNAs, for example, HIV TAR RNA (Churcher et al., 1993) and the bacteriophage R17 coat protein binding sequence (Carey et al., 1980). This may be due in part to the fact that ribonuclease probes are subject to sterio constraints for cleavage near the prohead binding site. Other evidence sug-
gests that much of the sequence is essential for prohead binding. A number of oligonucleotide-directed mutants within the 63-nucleotide prohead footprint region of pRNA have been produced.\(^3\) pRNAs of several deletion mutants, which together cover 44 residues of the footprinted region, do not compete with wild-type \(^{32}\)P-pRNA for binding to proheads when added in a 10-fold molar excess.

Our goal is to analyze the higher order structure of pRNA as it interacts with the \(\phi 29\) portal protein and the ATPase gp16, both of which have RNA recognition motifs (Grimes and Anderson, 1990), to constitute the DNA packaging machine.

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