The terminase of bacteriophage SPP1, constituted by a large (G2P) and a small (G1P) subunit, is essential for the initiation of DNA packaging. A hexa-histidine G2P (H6-G2P), which is functional in vivo, possesses endonuclease, ATPase, and double-stranded DNA binding activities. H6-G2P encounters a cut with preference at the 5′-RCCG↓CW-3′ sequence. Distamycin A, which is a minor groove binder that mimics the architectural structure generated by GIP at pac, enhances the specific cut at both bona fide 5′-CTATTGCGG↓C-3′ sequences within pacC of SPP1 and SF6 phages. H6-G2P hydrolyzes rATP or dATP to the corresponding rADP or dADP and P₃. H6-G2P interacts with two discrete GIP domains (I and II). Full-length GIP and G1PΔN62 (lacking domain I) stimulate 3.5- and 1.9-fold, respectively, the ATPase activity of H6-G2P. The results presented suggest that a DNA structure, artificially promoted by distamycin A or facilitated by the assembly of GIP at pacL and/or pacR, stimulates H6-G2P cleavage at both target sites within pacC. In the presence of two GIP decamers per H6-G2P monomer, the H6-G2P endonuclease is repressed, and the ATPase activity stimulated. Based on these results, we propose a model that can account for the role of terminase in headful packaging.

Initiation of packaging of double-stranded viral DNA (dsDNA) involves the specific interaction of the prohead with viral DNA in a process mediated by a phage-encoded terminase protein (1–4). The terminase enzymes are usually hetero-oligomers composed of a small and a large subunit. The role of the terminase small subunit is to specifically recognize the packaging initiation site (cos or pac) and to form a nucleoprotein structure that helps to position the terminase large subunit to cleave at the pac or cos site (1–6). Two general principles for packaging of concatemeric DNA into a virus head have been proposed. The first implies a site-specific packaging that shows some constraint in DNA size, in which the recognition sequence (termed cos in phage λ) plays an important role in initiation and termination of packaging. This packaging process is well-characterized in coliphages λ, T3, and T7 (1–6). The second principle implies headful packaging, in which the packaging initiates at a specific site (termed pac in phage SPP1) but with the capacity of the prohead playing a predominant role in the termination step. Within the poorly characterized headful packaging mechanism, phages P1, P22, T4, and SPP1 are included (1, 3, 7).

The terminase enzyme of Bacillus subtilis phages SPP1 and SF6 is composed of a small (G1P) and a large (G2P) subunit (8). The terminase initiates unidirectional DNA packaging from a concatemeric DNA substrate by binding to pac DNA, introducing a 1-bp staggered cut, within the 83-bp pacC subsite, and encapsidating the DNA from the cleaved end into an empty prohead until no more DNA can be inserted (headful) (8–11). DNA packaging terminates when the DNA inside the prohead is separated from the concatemer by a cutting process (headful cut). The headful cleavage generates a new end that serves as the starting point for the second round of DNA packaging. The pacC cut is precise, and the headful cut is imprecise and could be spread over a 2.5-kb region of DNA (8, 9, 12, 13).

SPP1 and SF6 wt GIP are 184 and 151 residues long, respectively, and share 71% identity clustered in three discrete regions (domains I, II, and III) (14, 15). Within domain I lies the B-type nucleotide-binding motif, the DNA-binding motif, and a GIP/G2P interacting region, and within domain II lies the A-type putative phosphate-binding loop (AXXXGKI/A), the GIP/G2P interacting domain, and a GIP/G2P interacting region (11, 14, 15). No apparent biological role can be assigned to domain III and the extended SPP1 GIP C-terminal region (see Refs. 14–16). GIP, which is unable to cleave DNA, binds but does not hydrolyze ATP (14, 15).

The SPP1 and SF6 GIP (native molecular masses of ~200 and ~170 kDa, respectively) consist of a specific ring containing 10 monomers of GIP (11). A ring-like structure was also observed in the terminase small subunit, gp16, of phage T4 (17). GIP, which is an abundant protein, binds cooperatively to two discrete (pacL (non-encapsidated left DNA end) and pacR (the encapsidated right DNA end)) subsites and holds the two binding sites together in a DNA loop of about 20 turns of the DNA helix (11, 14–16).

The SPP1 and SF6 G2P, which has a predicted molecular mass of 48,840 kDa and is a long product of 422 residues, contains a putative B-type nucleotide-binding motif preceded by hydrophobic amino acids between residues 106 and 114. An A-type NTP-binding loop (see Ref. 18), however, was not found in the G2P protein (8). The presence of a histidine-rich metal-
binding motif, identified in the terminase large subunit of phages λ, T3, T7, and T4, and a leucine zipper region are not obvious from the primary sequence of SPP1 and SF6 G2P (8).

We have previously observed that a plasmid-borne SPP1 H6-G2P fully complements the growth defect of different SPP1 conditional lethal mutants in gene 2 (15). The results presented here provide the first evidence that the SPP1 terminase large subunit, in a DNA structure-dependent manner, introduces double strand nicks within both target sites within pacC.

The available information suggested that the roles of GIP in the holoenzyme is to promote initiation of headful packaging by forming a nucleoprotein structure (8, 11) that helps to position G2P to cleave at both cognate sites, to impose directionally to DNA packaging, and to modulate DNA translocation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Bacterial Strains and Plasmids—Escherichia coli strain BL21(DE3) was described previously (19). pUC18 was used as a vehicle of our constructs, because it does not contain any supercoiling-dependent structure (20). Plasmids pBT115, pBT419, and pBT363 (14); pCB191 and pCB53 (15); and pQE10 and pQE9 (Qiagen) have been described previously. Plasmids pCB236, pCB220, and pCB31, containing GIPαN62, GIPN64-C141, and GIPαC55, respectively, were constructed by fusing the indicated DNA sequence of GIP to the last His codon of the His-Tag pQE vector.

Endonuclease—Ultraviolet acrylamide was purchased from Serva; molecular weight markers were from Bio-Rad; rifampicin was from BioChemika; DTE and proteinase K were from Sigma; trizma base was from Biomedics, Inc.; isopropyl-1-thio-β-D-galactopyranoside was from Calbiochem; and DNA restriction and modification enzymes, phenylmethylsulfonyl fluoride, poly(dC), and poly(dI-C) were purchased from Roche Molecular Biochemicals. All were used as recommended by the suppliers. All chemicals used were of reagent grade, from Merck; and DNA restriction and modification enzymes, [α-32P]dATP, [α-32P]TP, [γ-32P]ATP were from Amersham Pharmacia Biotech.

**Methods**

**DNA Manipulations**—Covalently closed circular plasmid DNA was purified by using the SDS lysis method (21), followed by purification on a cesium chloride/ETBr gradient. Gel-purified DNA fragments were end-labeled by filling in the restriction sites with the large fragment of DNA polymerase 1 in the presence of dTTP, dCTP, dGTP, and [α-32P]dATP.

Analytical and preparative gel electrophoreses of plasmid DNAs and restriction fragments were carried out either in 0.8% (w/v) agarose/Tris-acetate-EDTA/EBBr horizontal slab gels or on 6% (w/v) urea/polyacrylamide/Tris-borate gels.

The concentration of DNA was determined using molar extinction coefficients of 6500 x 10⁻³ cm⁻¹ at 260 nm. DNA was expressed as moles of DNA molecules.

**Protein Purification**—SPP1 wt GIP and the truncated GIP products either lacking the first 62 residues (GIPα262 formerly termed GIPα) or the last 48 residues (GIPαC136 formerly termed Chi) (14) were purified as described previously (14). B. subtilis chromatin-associated protein Hbsu was purified as previously described (22). GIPαN55, GIPN64-C141, and H6-G2P were purified from BL21(DE3) cells carrying plasmids pCB231, pCB220, and pCB191, respectively, after induction with isopropyl-1-thio-β-D-galactopyranoside (1 mM). After a 30-min induction, rifampicin was added to the final concentration of 200 μg/ml. Cells were harvested by centrifugation at 10,000 g for 10 min at 30°C and the pellets were frozen at −80°C. For the purification procedure, the pellet was thawed, resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 2 mM DTE, and 0.2 mM phenylmethylsulfonyl fluoride, 5% glyc- erol) containing 50 mM NaCl, and lyzed with a French press. The crude extract was centrifuged at 12,000 x g for 30 min, and the supernatant was used for a posterior purification using a Hitrap nickel- cheating column according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

The molar extinction coefficient for the different proteins was calculated as described by Gill and von Hippel (23). Protein concentration was determined by UV absorbance, using the corresponding molar extinction coefficients at 280 nm. Spectra were recorded on a 4054 UV-visible spectrophotometer LKB Biochrom Ultraspec Plus. The wt GIP, GIPα262, and GIPαC136 concentrations are expressed as moles of protein decamers, whereas the H6-GIPαN55, H6-GIPN64-C141, and H6-G2P concentrations are expressed as moles of protein monomers.

**Protein Manipulations**—The native molecular mass of G2P was determined by gel filtration fast protein liquid chromatography using a Superox 12 HR 10/30 column (Amersham Pharmacia Biotech). A chromatography was carried out on a TSK-Gel Superose 12 column (Knoll, Tris-HCl, pH 7.5, 100 mM NaCl, and 5% glycerol) at 4°C with a flow rate of 0.1 ml/min, and the band was measured. G2P (200 μg) was applied onto the column. A standard curve of Kav versus log10 of relative mobility was determined as recommended by Amersham Pharmacia Biotech. Protein standards were obtained from the manufacturer (ribonuclease A, 13.7 kDa; chymotrypsinogen A, 25 kDa; catalase, 43 kDa; bovine serum albumin, 67 kDa; aldolase, 158 kDa; and catalase, 232 kDa).

For protein cross-linking, the pure proteins were incubated in the presence or absence of 0.04% glutaraldehyde at room temperature in buffer D (60 mM NaHPO4, pH 7.2, 30 mM MgCl2, and 15 mM DTE), containing either 25 or 100 mM NaCl. Aliquots were collected at different times, and the reactions were stopped by precipitating the proteins with 25% (w/v) trichloroacetic acid, followed by two washing steps with acetone. The pellets were dissolved in loading buffer and loaded in a 7–15% SDS-PAGE.

**Electrophoretic Mobility Shift Assay**—The DNA fragment used to analyze the binding of H6-G2P to DNA through EMSA was the SPP1 242-bp (α2P)XhoI-Infl fragment (containing part of pacI, pacC, and pacR sites). The radiolabeled DNA (1 nm) was incubated with increasing concentrations of H6-G2P in buffer B (50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 2 mM DTE, 5% glycerol), containing 10 mM CaCl2 (in a 20-μl reaction) for 30 min at 37°C. When indicated, increased concentrations of poly(dI-C) (nonspecific) or cold SPP1 242-bp XhoI-Infl DNA fragment (specific) were added as competitor DNA. The reaction was stopped (by the addition of 3 μl of loading buffer (50 mM Tris-HCl, pH 7.8, glycerol 30%, bromphenol blue 0.25%, xylene cyanol 0.25%), immediately loaded onto a 1X TAE, and separated in a 4% non-denaturing PAGE. Gels were run for 3 h at 150 V and room temperature and dried prior to autoradiography.

**Endonuclease Activity Assay**—Supercoiled pBT363 DNA containing the SPP1 pac site (10 nm) was incubated with H6-G2P (20 nm) in buffer B containing 10 mM MgCl2 at 37°C for a variable time. The reaction was stopped by addition of 2 μl of loading buffer containing 0.4 μM EDTA, and the resultant products were then separated in 0.8% agarose gel.

The SPP1 242-bp XhoI-Infl DNA fragment obtained as a 320-bp EcoRI-PstI DNA segment from plasmid pBT363 or the SF6 233-bp Earl-Infl (coordinates 223–456) obtained as a 311-bp EcoRI-PstI DNA fragment were end-labeled at the 5′-end of each fragment and the resultant products were then separated in 6% denaturing PAGE. Autoradiograms of the dried gel were subsequently taken.

**ATPase Activity Measurement**—The ATPase standard reactions were incubated in buffer B containing 10 mM MgCl2 in a final volume of 20 μl. The reactions were initiated by addition of the substrate after a preincubation of 5 min at 37°C and allowed to proceed for 15 min more at the same temperature. ATPase activity was determined by measuring the amount of phosphate set free upon hydrolysis as described previously (24). Initial velocity studies were performed using 20 nm H6-G2P and an ATP concentration range of 0.5 μM to 10 μM (10–0.05 μC/ml). The initial velocity of ATP hydrolysis was determined within the linear range of each reaction, and the kinetic constants were determined by non-linear regression analysis of the experimental data using the KaleidaGraph version 3.0.2 software program (Abelbeck).
RESULTS

H6-G2P, G1P, and G1P Derivatives: Purification and Activities—SPP1 20-kDa wt G1P (predicted molecular mass 20.7 kDa), G1P variants with deletions at its N-terminal (13-kDa G1PΔN62) or C-terminal moiety (15-kDa G1PΔC136) (14) or isolated G1P domains (7-kDa H6-G1PΔC55 and 9-kDa H6-G1PΔN62-C141) (15) and the 49-kDa H6-G2P (predicted molecular mass 49.7 (H6, 0.9 + G2P, 48.8 kDa) were purified from the E. coli BL21(DE3) strain bearing a plasmid-borne SPP1 gene 1, gene 1C136, His-tagged domain I or domain II of gene 1, or His-tagged gene 2 as described previously (14, 15). The purified polypeptides (G1P, G1PΔC136, G1PΔN62, H6-G1PΔC55, H6-G1PΔN62-C141, and H6-G2P) were more than 99% pure, as judged by SDS-PAGE (data not shown). N-terminal protein sequence analysis of the first 10 N-terminal residues of the purified proteins were in full agreement with the amino acid sequence deduced from the nucleotide sequences.

Previously, it has been shown that G1P and G1PΔC136 specifically bind dsDNA and are not able to hydrolyze ATP (11, 14, 15). G1PΔN62, H6-G1PΔC55, and H6-G1PΔN62-C141 do not bind DNA or hydrolyze ATP (14, 15, data not shown). The abilities of H6-G2P to act as dsDNA nuclease (exo- or endonuclease), ATPase in the presence or absence of ssDNA or dsDNA, and DNA helicase and to bind to dsDNA or ssDNA were assayed. As shown below H6-G2P possesses an endonuclease and a modest ATPase and is able to bind dsDNA. All these activities co-purified in a Ni-agarose column with H6-G2P and exhibited the same kinetics of heat inactivation (data not shown). Furthermore, such activities were not observed when the same protocol and a Ni-agarose column was used to purify proteins from BL21(DE3) plasmid-free strain (data not shown).

H6-G2P Binds in a Sequence-independent Manner to Linear DNA—EMSA had indicated that the highly purified H6-G2P interacts with a linear intrinsically curved 320-bp DNA (1 nM) fragment (containing the SPP1 242-bp XhoII-HinflII segment (part of pacL, pacC, and pacR sites) from plasmid pCB363), provided that a divalent cation (e.g. Mg$^{2+}$, Ca$^{2+}$) is present in the reaction mixture. In the presence of Mg$^{2+}$, the DNA substrate is hydrolyzed (see below), thus we performed the experiment in buffer containing 10 mM CaCl$_2$. The protein-DNA complex was separated in a 4% non-denaturing PAGE. The 320-bp DNA segment, which migrates as a 620-bp fragment, is intrinsically curved (11). Two discrete H6-G2P-DNA complexes (CI and CII) were seen on addition of 20 and 40 nM (in monomers) of H6-G2P (Fig. 1, lanes 5–4). A slowly migrating form (CIII) and material that did not enter in the gel was observed in the presence of 80 and 160 nM of H6-G2P (Fig. 1, lanes 3 and 2). The $K_{\text{app}}$ of the H6-G2P-DNA complex was estimated to be ~35 nM at pH 7.8 and 37 °C. When the H6-G2P was present in limiting amounts (35 nM), the addition of 5-fold excess of a nonspecific poly[d(l-c)] DNA fully competed for the formation of H6-G2P-pac DNA complex (data not shown). Furthermore, when non-curved DNA was used the H6-G2P-DNA complex did not enter in the gel (data not shown). It is likely, therefore, that H6-G2P binds curved dsDNA with low sequence specificity.

H6-G2P Exhibits a pac-independent Endonucleolytic Activity—The supercoiled (form I) pBT363-borne pac site, containing a small amount (<10%) of relaxed circular DNA (form II), was incubated with a fixed amount of H6-G2P for varying times (Fig. 2). The resulting products were separated in a 0.8% agarose gel (Fig. 2). At early times of incubation (Fig. 2, lanes 2–4) ~20% of form I DNA was converted to form II DNA, with a small fraction as form III DNA. The H6-G2P nicking of both DNA strands was uncoupled. At later times the reaction seemed to be saturated (Fig. 2, lanes 6–12), because no further conversion of form I DNA into form II, then into form III, was observed. A fine mapping of pac-containing form II DNA revealed that H6-G2P introduced many discrete endonucleolytic nicks (data not shown). Under these experimental conditions, we were not able to identify a specific cut at pacC. Similar results were obtained when the pUC18 DNA control was used (data not shown).

A time courses for cleavage using various concentrations of H6-G2P (80–240 nM) at a constant concentration of pBT363 DNA revealed that, at high enzyme/DNA ratios, the data are biphasic and yield a fast and slow phase of the reaction. A reduction in the enzyme concentration not only resulted in the complete disappearance of the fast rate but also substantially limited the extent of nicking (cleaving) of pBT363 DNA (data not shown). These data suggested that the reaction did not have a catalytic turnover.

No H6-G2P nuclease activity was seen in the absence of Mg$^{2+}$ or in the presence of a 2-fold excess of EDTA with respect to the metal ion. Varying the MgCl$_2$ concentration from 0.5 to 20 mM revealed a similar pattern of cleavage by H6-G2P. Increasing concentrations of Mg$^{2+}$ correlated with an increasing endonucleolytic activity of H6-G2P with an optimum at ~10 mM. Mn$^{2+}$ partially replaced Mg$^{2+}$ (an optimal concentration gave ~50% of the activity observed with Mg$^{2+}$), but when Ca$^{2+}$ or Zn$^{2+}$ was used no H6-G2P endonuclease activity was observed.

The presence of a nucleotide cofactor (0.1–1 mM ATP), which exerts a negative effect in H6-G2P endonuclease activity, did not increase the cleavage specificity of H6-G2P (data not shown).

The endonuclease activity exhibited a relatively narrow pH optimum, which was maximal at pH 7.8. The activity decreased rapidly below pH 7.0 and above pH 8.5. Increasing concentrations of NaCl correlated with an increasing endonucleolytic activity of H6-G2P until an optimum was reached at ~50 mM, with a sharp decrease beyond 120 mM NaCl.

H6-G2P Introduces Many Discrete Cuts on pac-containing DNA—The SPP1 pacC subsite contains two directly oriented Box b repeats (10-bp in length) (Fig. 3). DNA encapsidation
initiates upon terminase cleavage at the Box b site proximal to the pacR subsite (8, 9, Fig. 3A). Hence, terminase has to discriminate between the two Box b sites at the 83-bp pacC sub-site and to introduce a 1-bp staggered cut within one of them (5′-CTATTGCGG\(G\)\(C\)\(-3′\)) (8–10, Fig. 3B). On the basis of genome organization and sequence identity, we predict that the SF6 terminase upon recognition of pacL (non-encapsidated) and pacR (encapsidated end) directs the terminase large subunit toward pacC (Fig. 3). To initiate unidirectional encapsidation, terminase introduces a double strand cleavage within the 13-bp Box b site (5′-GCTATTGCGGG\(C\)\(-AG-3′\)) proximal to pacR (9, see Fig. 3). Alternatively, terminase cleaves both Box b sites, but by an uncharacterized mechanism only, the cut at the Box b proximal at pacR is used as a substrate for \textit{in vivo} DNA encapsidation. Previously, Chai \textit{et al.} (8) have shown that the pac DNA end that is not the substrate for encapsidation is nucleolytically degraded, and such effect is independent of the major host-encoded nuclease (Exo V enzyme).

The SPP1 or SF6 pacC subsite located on a 242-bp Xhol\-HinfII DNA fragment (coordinates 157–399 of SPP1, obtained as a 320-bp EcoRI-PstI DNA segment from pBT363) or on a 233-bp Earl-HinfII (coordinates 223–456 of SF6, obtained as a 311-bp EcoRI-PstI DNA segment from pCB53) was used for \textit{in vitro} cleavage assays (see Ref. 8).

In the absence of H6-G2P, three faint DNA bands (denoted by arrows in Fig. 4A) were reproducibly observed. The 320-bp EcoRI-PstI SPP1 DNA segment or the 311-bp EcoRI-PstI SF6 DNA segment (1 nM), labeled at the top strand, was incubated with increasing concentrations of H6-G2P (5–40 nM) in buffer B containing 10 mM MgCl\(_2\), for 60 min at 37 °C, and the resulting products were then separated in 6% denaturing PAGE (Fig. 4A, data not shown). The endonuclease activity of the enzyme seems to be stoichiometric rather than catalytic. Six major SF6 ssDNA fragments (denoted Z, Y, 3, 4, 2, and 1, see Fig. 3) and many minor discrete ssDNA segments were observed (Fig. 4A).

A fine mapping of SF6 pac-containing DNA revealed that the end of two of those fragments map outside (Z, Y) and four inside (3, 4, 2, and 1) pacC (Fig. 3B). The fragments 1 and 2 end within the two Box b sites. The addition of a nucleotide cofactor seems to exert a negative rather than a stimulatory effect in H6-G2P nuclease activity (see below). Similar results were obtained when SPP1 pac DNA was used, except that here only four major DNA fragments (Y and 4 are missing) were observed (see Fig. 3B).

A nucleotide sequence analysis of H6-G2P-generated DNA ends revealed that the enzyme introduces, with certain preference, a nick in the top strand within the 5′-RCGG\(C\)\(-CW-3′\) nucleotide sequence (5′-agca\(\downarrow\)ca-3′ (Z) 5′-aacg\(\downarrow\)ct-3′ (Y), 5′-GCCG\(\downarrow\)C-3′ (3), 5′-GCCG\(\downarrow\)CA-3′ (2), 5′-TCGG\(\downarrow\)C-3′ (4), and 5′-GGGG\(\downarrow\)CA-3′ (1)) (see Fig. 3B). A low amount of the DNA segments ending at the 3 and 4 sites were also observed in the absence of H6-G2P.

We have investigated the effect of adding increasing GIP to the H6-G2P pattern of cleavage. The 320-bp EcoRI-PstI SPP1 pac DNA segment (1 nM) was incubated with a constant amount of H6-G2P and various amounts of GIP (ratios: 1:25, 1:5, 1:1, 1:5, 1:10, and 1:50). At low GIP concentration, the H6-G2P cleavage pattern is similar to the one reported in Fig. 3B, and at higher GIP concentrations (>1:5) an inhibition of the nicking reaction was observed (data not shown). It is like that when decameric GIP was in a molar ratio of 1:5 or greater, the DNA is unavailable for H6-G2P cleavage. Alternatively, GIP exerts an inhibitory effect of H6-G2P endonuclease activity (see below).

\textit{H6-G2P Introduces Discrete Cleavage on Structured pac-containing DNA}—Previously, we have shown that distamycin A (Dis), a minor groove binder that induces local distortions of the DNA, competes with GIP for pacL DNA binding, whereas other minor groove binders, such as spermine and Hoechst 33258, which do not distort DNA, failed to compete with GIP for pacL DNA binding (26). It was proposed that Dis mimics the DNA structure promoted by GIP upon binding to pac DNA (11, 26).

The 311-bp EcoRI-PstI SF6 pac DNA segment (1 nM), labeled at the top strand, was preincubated with either 1 μM (Fig. 4B) or 100 μM Dis (Fig. 4C) for 20 min at room temperature and then incubated with increasing concentrations of H6-G2P. The resulting products were then separated in 6% denaturing PAGE. In the presence of 1 μM Dis, a DNA fragment ending at the Y site was not observed (Fig. 4B, lanes 8–11), when compared with the cleavage products generated by H6-G2P (40 nM) in the absence of Dis (Fig. 4B, lane 7). In the presence of the 100 μM Dis, only five DNA fragments ending at sites 3, 2, 4, 1, and A were observed. The DNA fragments ending at site 1 (5′-GCCG\(\downarrow\)CA-3′) coincided with the \textit{bona fide} terminase packaging initiation site (9, Fig. 4C). H6-G2P also introduced a nick at the second Box b site (5′-GCCG\(\downarrow\)CA-3′, site 2). In the presence of 100 μM Dis, the DNA fragments ending at positions 3, 4, and A co-migrate with the DNA bands observed in the absence of H6-G2P (Fig. 4C, lanes 12 and 14). It is likely that (i) H6-G2P recognized the sequence 5′-RCGG\(C\)\(-CW-3′\) and preferentially cleaved the 5′-GCCG\(\downarrow\)CA-3′ sequence embedded in a special architecture, and (ii) the repertoire of DNA structures generated by Dis partially mimicked the ones generated by GIP.

\textit{Dis Enhances H6-G2P Double Strand Cleavage within pacC}—SPP1 399-bp pac DNA (5 nM), labeled at the bottom strand (see Fig. 5A), was preincubated with increasing concen-
G2P Hydrolyzes ATP—H6-G2P has a putative nucleotide-binding motif (8, 18). To determine whether H6-G2P is able to display an adenosine triphosphatase (ATPase) activity we measured ATP hydrolysis in the presence or absence of ATP-containing DNA (pBT363) (1 nM). H6-G2P (20 nM) was able to hydrolyze the [γ-32P]rATP or -dATP present in the reaction to rADP or dADP and P1 (data not shown). The rATP behaved as a Michaelis-Menten type substrate with the kinetic parameters of $K_m = 994 \pm 60 \mu M$, $k_{cat} = 27 \text{ min}^{-1}$, $V_{max} = 0.54 \pm 0.02 \mu M \text{ mol min}^{-1} \text{ mg}^{-1}$ of H6-G2P (Fig. 6; data not shown). Such an ATPase activity was slightly increased by the addition of DNA (1.8-fold), but it was not affected by the presence of pacC-containing dsDNA or ssDNA linear DNA (data not shown).

Previously, it had been reported that GIP is unable to hydrolyze ATP (14, 15, data not shown). The effect of GIP on the ATPase activity of H6-G2P was investigated. The rate of H6-G2P-catalyzed ATP hydrolysis, in the presence of an optimal concentration of GIP (95 nM), as a function of ATP concentration is shown in Fig. 6. In the presence of GIP, H6-G2P (20 nM) was more active, with a $K_m = 386 \pm 50 \mu M$, $k_{cat} = 90 \text{ min}^{-1}$, $V_{max} = 1.8 \pm 0.05 \mu M \text{ mol min}^{-1} \text{ mg}^{-1}$ of H6-G2P (see Fig. 6). Identical results were observed when wild-type G2P was replaced by GIPΔC136 (lacking the last 47 residues, domain III; see Fig. 7).

Like the endonuclease activity of the enzyme (see above), the ATPase activity exhibits an optimum at pH 7.8 and is inhibited by the addition of EDTA or when Mg2+ is replaced by Ca2+ or Zn2+ (<5% of the ATPase activity). When Mn2+ (10 mM MnCl2) was used instead of Mg2+, ~55% of the H6-G2P ATPase activity was detected.

G2P is able to hydrolyze [γ-32P]rATP or [γ-32P]dATP but fails to hydrolyze the remaining rNTPs and dNTPs (data not shown).

GIP Interacts with H6-G2P—Previously, it has been shown that H6-G2P interacts with wt GIP and GIPΔC136 but fails to interact with GIPΔN62 (lacking the first 62 residues, domain I) when polyclonal antibodies raised against purified GIP or GIPΔN62 immobilized in a protein A-Sepharose column were used (15). Using affinity chromatography (the protein cross-linked to a gel matrix (Affi-Gel, Bio-Rad) or the His-tagged protein metal chelated to the column) and protein:protein cross-linking, we confirmed that H6-G2P interacts with wt GIP and GIPΔC136 and showed that H6-G2P has a weak interaction with GIPΔN62, H6-GIPΔC55 (containing the first 54 residues, domain I) and H6-GIPN64-C141 (containing from residue 65 to 141, domain II) (18, Fig. 7B).

Using protein cross-linking techniques, we failed to detect a direct H6-G2P:H6-G2P interaction (Fig. 7B). The molecular mass of native H6-G2P, deduced from the amino acid sequence as 49,771 kDa, was then determined by gel filtration chromatography. From the elution profile of H6-G2P and of a number of protein standards, we estimate that the $M_1$ of H6-G2P is ~50,000. The single H6-G2P peak in the chromatogram coincided with the eluted ATPase activity (data not shown). It is likely, therefore, that H6-G2P is a monomer in solution.

In the previous section we showed that wt GIP stimulated ~3.5-fold the ATPase activity of H6-G2P. Alternatively, in the presence of H6-G2P, a cryptic ATPase activity associated with GIP could be detected. To address this question, different variants of GIP were incubated with H6-G2P and its ATPase activity measured. When GIPΔ62, which lacks the putative Walker’s motif B (8, 18, Fig. 7), was incubated with H6-G2P, the H6-G2P ATPase activity was stimulated ~1.9-fold. The addition of GIPΔC55 (domain I containing Walker’s motif B) (18), GIPN64-C141 (domain II containing the putative ADP and/or ATP-binding loop, Walker’s motif A), or both proteins together, however, exerted a negative effect in the H6-G2P ATPase activity. (H6-G2P retained ~34% of its ATPase activity in the presence of H6-GIPΔC55, no activity was detected in H6-GIPΔN64-C141, or ~10% of the H6-G2P ATPase activity was detected in the presence of both H6-GIPΔC55 and H6-GIPN64-C141 polypeptides.)

The Amount of GIP Alters the Relative Activities of H6-G2P—In a previous section we showed that the presence of...
increasing concentrations of G2P stimulated the ATPase activity of H6-G2P, but G2P might exert a negative effect on the H6-G2P endonuclease activity. We have investigated the effect of increasing amounts of G2P on the ATPase and endonuclease activities of H6-G2P. For the measurement of the endonuclease activity of H6-G2P, we used form I pBT363 DNA and measured the generation of form II and form III DNA. H6-G2P was incubated with increasing concentrations of G2P and then with supercoiled pac-containing pBT163 DNA. The ATPase activity was assayed as follows: H6-G2P was incubated with increasing concentrations of G2P and then with pBT363 DNA and 2.5 mM $[^{32}P]$ATP. An identical ATPase activity was observed when DNA was omitted from the reaction mixture.

In the presence of 2 G2P decamers per H6-G2P monomer, a 1.8-fold reduction of the endonuclease activity (measured as the accumulation of form II and form III DNA) with a concomitant stimulation of its ATPase activity was observed (Fig. 8). In the presence of 4–12 G2P decamers per H6-G2P monomer, G2P fully stimulated the ATPase activity and drastically reduced the H6-G2P endonuclease activity (Fig. 8). The inhibitory effect of G2P in the endonuclease and stimulatory effect on the ATPase activity of H6-G2P were fully reversible (data not shown). It is likely, therefore, that G2P modulated the G2P activities.

**DISCUSSION**

The mechanism of site-specific packaging, in which the recognition sequence plays an important role in initiation and termination of packaging, has been well characterized (1–6). The mechanism of headful packaging, in which the packaging head plays a predominant role in the termination, is...
poorly understood (1, 3, 7). The present study is our first step toward an understanding of terminally redundant and partially circularly permuted packaged molecules (headful packaging).

Previously we presented evidence that a plasmid-borne wt SPP1 gene 2 (G2P) or His-gene 2 (H6-G2P) fully complemented the amplification of SPP1 conditional lethal mutants in gene 2 (15). It is likely, therefore, that the addition of (His)_6 at the N terminus of the terminase might enhance its packaging activity.

FIG. 6. Kinetic analysis of the ATPase activity of H6-G2P and terminase by initial velocity versus substrate concentration. The reaction conditions were as described under “Experimental Procedures.” Each point represents the average of experiments done at least in triplicate. Kinetic parameters were obtained from the fitting of the experimental data to the Michaelis-Menten equation. The ATPase activity of H6-G2P (○) and SPP1 terminase (○○) are indicated.

FIG. 7. Schematic representation of SPP1 G1P. wt G1P and deletion derivatives are shown as solid boxes. The thin lines denote non-G1P residues, and the asterisks indicate the hexa-histidine extension. Domains I (39 residues in length), II (~56 residues), and III (~15 residues) are boxed. The polypeptide length is indicated. The putative Walker B nucleotide-binding motif and Walker A phosphate-binding loop (AXXXXGK/L/A) are indicated as solid boxes in domains I and II, respectively. The physical interaction of H6-G2P with itself and with wt and the different variants of G1P is indicated. ++, denotes a positive interaction at 100 mM NaCl; +, indicates a positive interaction at 25 mM, but a weak interaction at 100 mM NaCl; and −, no interaction.

FIG. 8. An increasing concentration of G1P stimulates the H6-G2P ATPase and exerts a negative effect on its endonuclease activity. Supercoiled pCB363 DNA (20 nM in the endonuclease and 5 nM in the ATPase assay) was incubated with increasing concentrations of G1P (19, 28, 38, 47, 95, 190, 300, 500, 640, 800, and 1000 nM in the endonuclease and 5.1, 9.4, 23.7, 41.5, 71.4, 95, 119, 190, and 275 nM in the ATPase assay) and a constant amount of H6-G2P (80 nM endonuclease and 20 nM ATPase) in buffer B containing 10 mM MgCl₂ for 30 min at 37 °C. The percentage of relaxed plus linearized supercoiled pBT163 DNA (v) and the ATPase activity of H6-G2P (V) are indicated.
terminus of G2P does not affect the biological activity of the polypeptide. We have failed to overexpressed G2P or H6-G2P, but, taking advantage of affinity chromatography, we have purified H6-G2P from poorly expressing cells toward homogeneity (15). In this report we have biochemically characterized H6-G2P. H6-G2P possesses an endonuclease and ATPase that require Mg2+ and a neutral or slightly basic reaction pH. In the presence of Ca2+ H6-G2P binds dsDNA rather nonspecifically but is unable to degrade DNA or to hydrolyze ATP.

Using a supercoiled DNA substrate, H6-G2P nicks and then linearizes DNA with poor sequence specificity. Using a linear DNA substrate and denaturing PAGE, we observed that the H6-G2P endonuclease showed a certain preference for the sequence 5'-RCOG 3'-CW-3'. The endonuclease activity and the cleavage specificity of H6-G2P were not stimulated by the presence of a nucleotide cofactor (ATP or dATP) or the presence of the DNA-binding Hbsu protein (5–400 nM) (B. subtilis counterpart of E. coli IHF). A gene coding for the sequence-specific DNA-binding IHF protein cannot be identified in B. subtilis. Unlike the H6-G2P of SPP1, faithful cleavage of the P1 pac site requires phage-encoded terminase (PacA and PacB) and two E. coli chromatin-associated proteins (IHF and HU) (27). Furthermore, in the case of phage λ, the endonuclease activity of the terminase large subunit, gpA, is stimulated by the presence of the E. coli IHF protein (28, 29) and ATP (29, 30).

Previously we have shown that SPP1 G1P interacts with the pacL and pacR subsites and forms a loop that brings them together (11). Furthermore, Dis, which induces local distortions of the DNA, competes with G1P-pac complex formation (11, 26). When Dis was used instead of G1P, H6-G2P introduced ssDNA nicks and dsDNA breaks with high specificity at pacC. Our results suggest that Dis alters the conformation of pac DNA generating a repertoire of DNA structures that mimic the one generated by G1P. This is consistent with the fact that, in the presence of Dis, H6-G2P specifically nicks both DNA strands at the bona fide pacC subsite. Our results do not explain, however, how cleavage at other pacC subsites, except the one used for the initiation of DNA packaging, was prevented by the SPP1 terminase. In the P1 case Sternberg and coworkers (31) have shown that cleavage at the pac site is regulated by adenosine methylation and that the P1 terminase “small” subunit (PacA) can discriminate between methylated and hemi- or unmethylated DNA, and accordingly, they proposed that pac cleavage is regulated by methylation of the DNA (27). Unlike P1, the SPP1 pac site does not contain DNA methylation sites for host DNA methyltransferases and SPP1 does not code for any DNA methyltransferase product (32).

Packaging of concatemeric DNA into a preformed prohead is believed to occur by a conserved mechanism, but certain details can vary from system to system. The terminase enzyme is directly involved in the initiation of packaging as well as in the ATP-driven translocation of DNA into a prohead (4–7). Previously we presented evidence that G1P binds but does not hydrolyze ATP (14). We show that H6-G2P hydrolyzes rATP and dATP to the corresponding diphosphates and inorganic phosphate with a low affinity for the nucleotide (Km of 994 μM) and a kcat of 27 min-1. H6-G2P failed to hydrolyze the remaining rNTPs and dNTPs. ATP hydrolysis was marginally increased.
by the addition of DNA (1.8-fold). Unlike SPP1 H6-G2P ATPase, the phage λ gpA ATPase is capable of hydrolyzing different nucleoside triphosphates (28, 29) and is active in the presence of Ca$^{2+}$ as a divalent metal (29, 33). Both λ gpA (28, 29, 33) and H6-G2P (this work) display an in vitro ATPase activity independent of proheads, whereas such hydrolysis is dependent of proheads in the φ29, T3, and T7 packaging systems (see Ref. 6).

The presence of two to four molecules of GIP per H6-G2P molecule lowered the $K_m$ (386 μM) and increased $k_{cat}$ (90 min$^{-1}$) of H6-G2P ATPase ~3.5-fold, but the endonuclease was repressed. What could be the functional implications of the different stoichiometry of terminase on SPP1 headful packaging? GIP specifically binds, with high cooperativity, to two discrete (pacL and pacR) subsites, separated from each other by a stretch of 140-bp and bends the DNA (11, Fig. 9A). The interaction between a GIP decamer bound to pacL and a GIP decamer bound to pacR forms a specialized nucleoprotein structure that gives rise to a DNA loop of 204 bp in length (11, Fig. 9B). GIP interacts with H6-G2P (15, this work), and the H6-G2P target site (pacC) is located between the pacL and pacR subsites (8–10, Fig. 9). We show that the DNA architecture artificially generated by Dis stimulates the cleavage of H6-G2P to the bona fide pacC subsite and predict that the DNA distorsion mediated by GIP could do so. GIP forms a nucleoprotein complex at the pacL and pacR subsites and interacts with GIP (11, 15). We hypothesize that the GIP-promoted architectural element facilitates the recognition and specific cleavage of GIP at both Box b sites as illustrated in a model presented in Fig. 9B. The interaction of two GIP decamers (bound to pacL and pacR) with GIP bound to the Box b proximal to pacR might induce a conformational change in GIP. “Modified GIP” has a stimulated ATPase with a shut off of nuclease activity, hence this DNA end is not degraded. G2P bound to Box b proximal to pacL, which is freed of GIP and not subjected to such a conformational change, degrades this DNA end that is not a substrate for encapsidation (Fig. 9B). We hypothesize that the overlapping of pacC and pacR might impart the unique directionality of packaging. The terminase, composed of 2 GIP and 1 G2P molecules, then participates in the translocation of the non-degraded DNA end into an empty prohead through a unique portal vertex structure of the prohead.

GIP, which is an abundant protein (16), bound to a second pac site in the concentrator, further stimulating the ATPase activity of “modified G2P.” When the head becomes full, the speed of translocation could slow down and an “unknown signal” reversed modified G2P to its endonuclease form. When G2P finds a 5'-RGG ↓ CW-3' site, it introduces a “headful” cut. In the absence of such a signal, the ATPase form of “G2P” remains active (translocase). The “conformational change” hypothesis is consistent with the biochemical data present here and with the previously published genetic data (8–10). In short, SPP1 encapsidates its DNA from concatemeric DNA molecules by a processive headful packaging mechanism. The pac site is used only once per packaging series, and the non-encapsidated pacC DNA end is subject to nucleolytic degradation. Furthermore, two different concatemeric plasmid molecules (each of them smaller in length than a mature SPP1 particle) could be encapsidated into a single SPP1 head before the headful signal is triggered and G2P endonuclease is activated (8–16, 34). We assume that the same hypothesis holds true for the highly related phage SF6 and that the same process is operative in other phages that package their DNA by a headful mechanism (see Refs. 35, 36).

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