Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6092–6096, August 1990
Biochemistry

In vitro maturation and encapsidation of the DNA of transposable Mu-like phage D108

(*in vitro* packaging/virus morphogenesis/molecular switch)

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Communicated by Dale Kaiser, May 21, 1990

ABSTRACT **Mu and D108 are related, temperate, transposable coliphages with unusual modes of DNA replication (transposition) and virion DNA maturation. These double-stranded DNA genomes replicate intrachromosomally and are matured and encapsidated linked to DNA sequences flanking the dispersed, integrated phage genomes. We have developed an *in vitro* system that employs crude lysates prepared from cells late in the Mu lytic cycle and that is proficient for both maturation and encapsidation of D108 DNA. Different forms of phage DNA were packaged at different efficiencies, with a circular pSC101::D108cts10 plasmid being most efficient, linearized plasmid less so, and mature virion DNA a poor substrate. The addition of purified D108 Ner protein to the reaction had no effect, whereas D108 repressor (c protein) inhibited the reaction. *Escherichia coli* integration host factor and D108 transposase proteins exerted an inhibitory effect on circular DNA substrates but had little effect on linear DNA packaging. This *in vitro* system, coupled with that developed for transposition, can now be used to biochemically dissect the protein and substrate requirements of these phages' DNA maturation pathway and the nature of the molecular switch between DNA transposition and encapsidation.**

Mu and D108 are related temperate phages of the bacterium *Escherichia coli* (1). They are unique among the coliphages in that their 37-kilobase pair (kbp) double-stranded DNA genomes are propagated as transposable elements (see refs. 2 and 3 for recent reviews). The large size and high transposition frequency of Mu and D108 have made these phages particularly amenable for *in vivo* genetic manipulations (4) and for studying the means by which mobile genetic elements transpose and induce mutations via molecular rearrangement reactions (see ref. 5 for a recent review).

Mu and D108 share >90% sequence homology at both the DNA (6) and protein (7) levels. However, they are not homologous in their left-end regulatory regions, which encode their respective repressors (c protein) (8), Ner (λ Cro-like proteins) (9), and the amino-terminal portions of the two phages' transposases (A proteins) (8–10).

Transposons are constantly flanked by host DNA sequences and this is also true of transposable phages Mu and D108, even when they are encapsidated within the virion (11). Unlike other temperate coliphages, the transposable phages must integrate regardless of the developmental pathway to be followed. During lytic growth, Mu DNA remains integrated, yet it is amplified and dispersed to ≈100 copies per cell genome by multiple cycles of replicative DNA transposition (7). For DNA encapsidation to occur, the phage genomes must be recognized and removed from the *E. coli* chromosome. This process is carried out by an as yet unidentified protein(s) termed "pacase" (12). This enzyme(s) recognizes a specific sequence (*pac*) located between bp 32 and 54 (13), which is itself nestled between the first two binding sites for the phage transposase protein at the left end of the phage DNA (14, 15). A double-stranded endonucleolytic cleavage is then made 56–144 bp in the upstream host sequences (16) and packaging is believed to continue by a headfull mechanism until the entire phage genome, plus 0.5–2.5 kbp of host DNA adjacent to the right end of the integrated phage, is cleaved and packaged (11, 12).

To date, though no *in vitro* system for either Mu or D108 DNA maturation and packaging has been described, several other double-stranded DNA bacteriophages [e.g., λ and T4 (17–19)] have been packaged *in vitro* (see refs. 19 and 20 for recent reviews). By utilizing the relatedness between Mu and D108 packaging enzymes and their differing repressors and immunity (21), we report here the development of an *in vitro* system, prepared from cells late in the Mu lytic cycle, capable of maturing and encapsidating D108 genomes with the Mu packaging machinery. This system takes advantage of the fact that Mu and D108 have identical *pac* sites and can package each other's DNA *in vivo* (22) but are heteroimmune, thus allowing the discrimination between endogenous mature Mu phage in the lysate and exogenously added D108 DNA that is matured and encapsidated *in vitro*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and DNAs. All bacterial strains used in this study are derivatives of *E. coli* K-12. Strain HB101 (*hsdS20, recA13, ara-14, proA2, lacY1, galK2, rpsL, xyl-5, mtl-1, supE44*)-derived indicator strains were LF4005 (D108c^+ lysogen of HB101; c^+ indicates wild-type gene encoding the repressor) and LF4017 (Muc$^+$ lysogen of HB101). Also used as indicator strains for plating of the *in vitro* packaging reactions were the isogenic strains BU252 [*met*$^-$, Mu^R, *recA*, F$^-$, Δ(*pro–lac*)] and BU2061 (*met*$^-$, *recA*, F$^-$, Δ(*pro–lac*)]. D108cts10 and Mucts62 phage lysates were prepared by thermal induction of strains LF4028 and HM8305, respectively, as described (7, 21). The plasmid pSZ53 (Tc^R, pSC101::D108cts10) (6), from the bacterial strain LF4053 (LF4005 transformed with pSZ53), was isolated according to Szatmari *et al.* (6). Linearized pSZ53 was prepared (9) by cleavage with *Xho* I (BRL) at a site 433 bp from the left end of the bacteriophage D108 insert and 8830 bp from the right end. Purified bacteriophage D108 DNA was prepared (7) from strain LF4028.

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Abbreviations: ATP[γS], adenosine 5′-[γ-thio]triphosphate; ddGTP, 2′,3′-dideoxyguanosine 5′-triphosphate; IHF, integration host factor; PFU, plaque-forming unit; PMSF, phenylmethylsulfonyl fluoride.

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Preparation of Cells. Five hundred milliliters of LB broth (23) containing 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ was inoculated with 10 ml of an overnight culture of strain LF999 (Mucts62 lysogen of HB101). After growth at 32°C to a titer of 2.5×10^8 cells per ml (OD_{550} = 0.4), the cells were heat-induced at 43°C for 40 min. The culture was then quickly chilled in a dry ice/ethanol bath and placed at 4°C for 25 min. The cells were then collected by centrifugation at 3300 × *g* for 15 min at 4°C, resuspended in 1 ml of resuspension buffer [10% (wt/vol) sucrose/50 mM Hepes·NaOH, pH 8.0), and stored at −70°C in 0.5-ml aliquots.

Preparation of Extracts. The cell preparations were thawed slowly at room temperature and then pooled. One-tenth volume of lysis buffer (6 mM Hepes·NaOH, pH 7.0/18 mM $MgCl_2$/30 mM 2-mercaptoethanol) was added to the cells, and the mixture was incubated on ice for 10 min and then sonicated on ice three times at full power for 5 sec at 15-sec intervals with a Vibra-cell sonicator. The sonicate was incubated on ice for 10 min and the unlysed cells and cell debris were sedimented in Nalgene Oak Ridge tubes by centrifugation at 32,500 × *g* for 20 min at 4°C in a Beckman type 40 rotor. The opalescent supernatant fluid ("extract") was immediately removed, frozen in a dry ice/ethanol bath, and stored at −70°C in 0.2 ml aliquots. These extracts were stable for at least 3 months when stored in this manner. Aliquots were thawed slowly at room temperature immediately before use. The protein concentrations of the extracts were determined by the method of Lowry *et al.* (24) and endogenous ATP levels were measured using a firefly luciferin–luciferase assay (Calbiochem).

DNA Maturation and Packaging Reactions. The standard *in vitro* packaging reaction consisted of 25 μl of cell extract, 6 mM spermidine hydrochloride (pH 7.0) (Sigma), 1 mM ATP, 5 μl of reaction buffer [20% (wt/vol) sucrose/130 mM Hepes·NaOH, pH 7.5/90 mM $MgCl_2$/150 mM 2-mercaptoethanol)], and 0.25 μg of circular pSZ53 DNA (in 10 mM Tris·HCl, pH 7.5/1 mM EDTA). Sterile deionized water was used to bring the final reaction volume to 50 μl. The reaction mixtures were incubated at 37°C for 60 min. Reactions were terminated by the addition of 12.5 μl of DNase I (bovine pancreatic DNase I at 200 μg/ml in 10 mM Hepes·NaOH, pH 7.4/100 mM $MgCl_2$), and the mixtures were diluted in Mu buffer (7), plated with indicator bacteria in 0.5% LB top agar on TCMG [10 g of trypticase peptone (Baltimore Biological Laboratory), 5 g of NaCl, and 8.5 g of agar per liter of H_2O with 10 mM $MgSO_4$] plates, and incubated overnight at 37°C prior to plaque quantitation. All results were confirmed in several independently performed reactions with different extracts. The other compounds examined (listed in Table 2) included DNase I and RNase A (Boehringer Mannheim Canada) and phenylmethylsulfonyl fluoride (PMSF) (Sigma).

Protein Binding Reactions. Protein binding reactions were carried out in 15 μl at 37°C for 10 min prior to addition of the binding mixtures to the *in vitro* packaging reactions. Binding assays for purified (25) bacteriophage D108 transposase (A protein) were done according to Cameron *et al.* (26). *E. coli* integration host factor (IHF), D108 Ner, and D108 repressor (c protein) were purified and used in protein binding assays according to Nash and Robertson (27), Kukolj *et al.* (28), and Kukolj and M.S.D. (unpublished protocol), respectively. In all cases, the test protein was allowed to bind to the DNA for 10 min at 37°C prior to addition of the rest of the reaction components. The DNA-binding proteins were added at concentrations to cover the range where both strong and weak sites are bound (on linear DNA fragments) as determined by electrophoretic band retardation and DNase I protection assays. These binding reactions were carried out in the presence of slightly different buffers, which were found to have little effect on the overall extent of maturation and packaging of pSZ53 DNA (data not shown).

RESULTS

Characterization of the *in Vitro* Packaging Reaction. An *in vitro* packaging system for phage D108 DNA was prepared using crude sonicates from thermally induced Mucts62 lysogens of *E. coli* strain HB101 (*recA* to eliminate recombination) late (40 min) in the lytic cycle, as such lysates should contain mature Mu phages plus the various precursor proteins necessary for Mu, and thus D108 (22), DNA maturation and particle morphogenesis (29). The protein concentrations of the sonicates varied between 15 and 25 mg/ml, with ATP concentrations in the range of 10 μM. The primary substrate used in these reactions was a circular pSC101 derivative (pSZ53) containing an entire D108*cts*10 prophage (6). The differing immunity (repressor specificity) displayed between Mu and D108 was exploited to discriminate between Mu phages in the lysate and *de novo* generated D108 immunity-type phages by plating the resulting reaction products on isogenic D108 lysogens (to titer endogenous Mu phage) and Mu lysogens (to quantitate *in vitro* matured and encapsidated D108 DNA).

The evidence that pSZ53 plasmid DNA was matured, and the D108 genome encapsidated, into Mu phage particles *in vitro* is fivefold. (*i*) Plating of the reaction mixtures on Mu- and D108-lysogenic strains indicated that phage particles with the immune specificity of the exogenously added DNA (D108) were generated (Table 1). (*ii*) Extracts prepared from identically treated nonlysogenic HB101 cells did not produce any Mu or D108 PFUs *in vitro* from circular pSZ53 DNA (data not shown). (*iii*) Addition of DNase I prior to, but not following, incubation of the reaction totally inhibited formation of D108 immunity-type phage particles (Table 2). (*iv*) No plaques from the reaction were observed on a strain that is resistant to Mu but sensitive to D108 infection, indicating that all PFUs present in the reaction had the adsorption specificity (30) of Mu phage particles (Table 1). (*v*) The relationship between the quantity of pSZ53 DNA added to the reaction mixture and the number of D108 immunity-type PFUs generated *in vitro* was found to be virtually linear in the range 0–5 μg of input DNA per reaction (Fig. 1*A*). Moreover, formation of D108 immunity-type phage increased exponentially with time of incubation in the range 0–90 min at 37°C (Fig. 1*B*), as previously observed for *in vitro* λ packaging (31).

We next examined the effects of changing and/or adding several key components to the standard reaction mixture using circular pSZ53 DNA. In all cases, phage yield was compared with that of a reaction identical in all respects except for the test compound. The polyamine spermidine and divalent metal cations were absolutely required for the reaction, as the addition of EDTA completely inhibited D108 immunity-type phage formation (Table 2). PMSF had little effect on the *in vitro* reaction (Table 2), suggesting that any virion protein maturation events requiring proteolytic cleavage are not sensitive to this compound *in vitro* (32). However, the addition of RNase A dramatically reduced D108 immunity-type plaque formation (Table 2), a result similar to that observed for encapsidation of *Bacillus subtilis* phage φ29 DNA (33).

Table 1. Virion behavior and immunity type of phages generated in the *in vitro* packaging reaction

Indicator strain*	PFUs†
LF4005 (D108c^+ lysogen)	1.50×10^5
LF4017 (Muc$^+$ lysogen)	2.23×10^5
BU2061 (Mu^S/D108^S)	1.27×10^5
BU252 (Mu^R/D108^S)	0.0

*Superscript S, sensitive; superscript R, resistant.
†Plaque-forming units per reaction.

Table 2. Effects of inhibitors and other requirements of *in vitro* packaging

Omission or addition	Phage yield, %
− Spermidine	<1
+ EDTA (25 mM)	<1
+ PMSF (2 μg/ml)	115
+ Glycerol (5%)	220
+ Glycerol (10%)	425
+ DNase I (4 μg/ml)	0
+ RNase A (40 μg/ml)	13
+ Linear pSZ53 DNA (0.25 μg/rxn)	45
+ Virion DNA (0.25 μg/rxn)	5

Components listed were omitted or added at the given final concentration. rxn, Reaction mixture.

To determine the DNA substrate requirements for the reaction, different DNA forms that may represent *in vivo* substrates, or intermediates, in the DNA maturation pathway were examined (34): circular plasmid pSZ53, linearized pSZ53 DNA, and mature D108 DNA extracted from purified virions. Maximal efficiency of packaging was obtained with circular plasmid DNA. Linear pSZ53 DNA was matured and packaged at 45% of the extent of circular plasmid DNA, whereas mature phage DNA, packaged at 5% the extent of circular pSZ53 DNA, was the least efficient substrate (Table 2). Similar results were obtained in the packaging of a pSC101::Mucts62 (pMC321) plasmid (35) using a lysate prepared from an induced D108*cts*10 lysogen of HB101 (data not shown).

Effects of Nucleoside Triphosphate Cofactors. The effect of varying the ATP concentration was examined using all three D108 DNA substrates as described above. The addition of ATP at final concentrations of up to 1.5 mM promoted the maturation and packaging of circular plasmid pSZ53, whereas the encapsidation of mature D108 DNA was inhibited at all concentrations tested (Fig. 2*A*). Linearized pSZ53 DNA gave a relative yield of D108 immunity-type phage similar to that obtained with the circular form at 0.5 mM ATP, but from 1.0 to 2.0 mM final ATP concentrations, the yield closely matched that of mature DNA (Fig. 2*A*). The addition of ATP[γS], a nonhydrolyzable ATP analogue, markedly inhibited the *in vitro* packaging reaction (with circular pSZ53 DNA) (Fig. 2*B*). These results indicate that most energy requirements for the formation of phage particles can be met by the extract, as is the case for *in vitro* λ packaging (17), and that exogenously supplied ATP has differing effects on DNA maturation and encapsidation dependent upon the DNA substrate added to the reaction mixture. These results may be due to competition between encapsidation and other ATP-dependent reactions (e.g., those catalyzed by nucleases and proteases) present in the crude extracts. The addition of dATP, GTP, or ddGTP was found to inhibit the *in vitro* reaction, but to differing overall extents (Fig. 2*B*).

Effects of Purified Proteins on *in Vitro* D108 Packaging. The effects of purified early phage gene products on DNA maturation and packaging were determined. The addition of D108 Ner had no significant effect on the *in vitro* maturation and packaging reaction at any of the concentrations tested (Fig. 3). However, D108 repressor (c protein) markedly inhibited the maturation and/or encapsidation of D108 DNA from the plasmid pSZ53 at the higher concentrations tested (Fig. 3). Additionally, the heterodimeric host protein, IHF (27), which is known to bind to specific sequences in the D108 early operator region (21), was found to be slightly stimulatory at the lowest concentration, yet inhibitory at the higher concentrations tested (Fig. 3). To examine whether this effect was due to DNA binding and steric hindrance of *in vitro* maturation and packaging, identical concentrations of IHF were added to linear pSZ53 DNA (Fig. 3). Only slight inhibition of *in vitro* packaging, at the highest concentration tested, was observed, suggesting that IHF effects may be dependent on DNA substrate structure (36, 37).

The effects of the presence of the transposase (A protein) on the *in vitro* maturation and packaging reaction were also examined. In either the absence or the presence of exogenously supplied ATP, the A protein was found to inhibit the *in vitro* maturation and packaging reaction in a dose-dependent manner, with added ATP (1 mM) resulting in a greater relative inhibition (Fig. 4). However, when linearized plasmid pSZ53 DNA was used, inhibition of DNA maturation and packaging was observed at only the highest concentration tested, suggesting that A-protein binding to linear DNA is not sufficient to block DNA maturation and subsequent encapsidation, or reflecting the strength of transposase binding to supercoiled versus linear DNA (38). Additionally, the formation of protein–DNA complexes with the phage B protein (39) and/or host proteins such as HU (40) and IHF (37) present in the extracts may also play a role in the observed effects.

The addition of equivalent concentrations of acidic (bovine serum albumin, ovalbumin) or neutral (β-galactosidase) control proteins stimulated the reaction (data not shown), as did glycerol when present at 5% or 10% of the reaction volume (Table 2), a result previously observed with *in vitro* T7 DNA packaging (32). The addition of basic proteins (cytochrome *c*, lysozyme), like Ner (Fig. 3), had little effect on the *in vitro* reaction, with only 20% inhibition observed with lysozyme at the highest concentration (63 μg/ml) tested (data not shown).

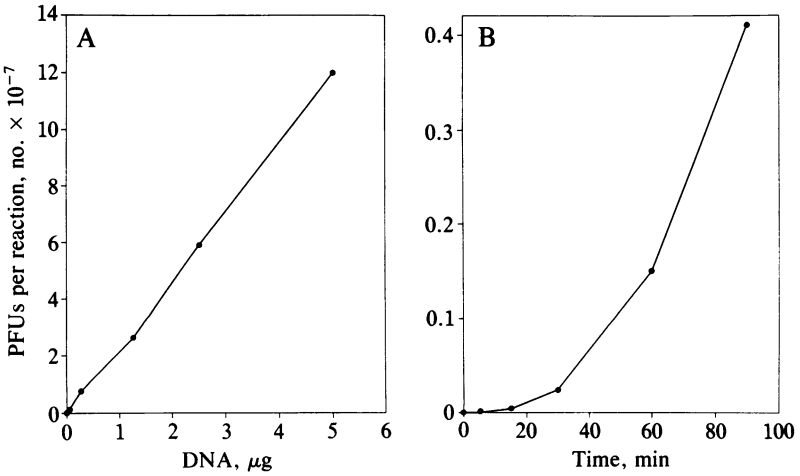

FIG. 1. Time course and pSZ53 DNA dependence of *in vitro* D108 packaging. The standard reaction conditions were as described in *Materials and Methods* except that the time of incubation was varied (*B*) or the indicated quantities of circular pSZ53 DNA, ranging from 0 to 5 μg, were used as the packaging substrate (*A*).

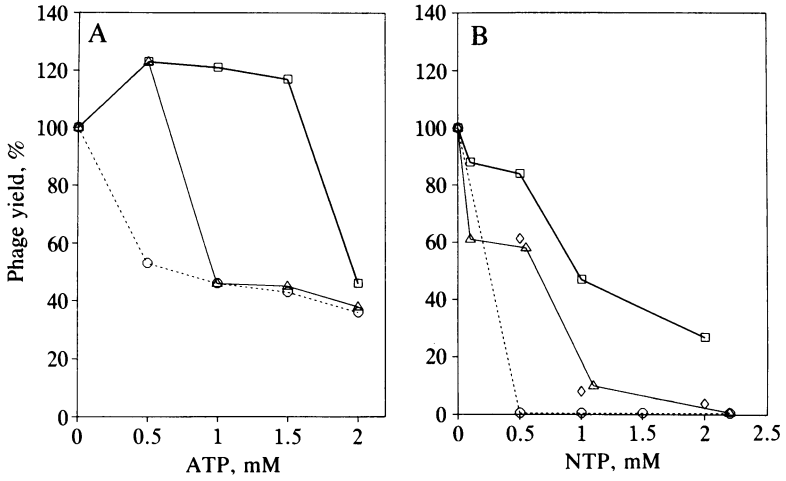

FIG. 2. Effects of nucleoside triphosphates on *in vitro* D108 DNA packaging. Standard reaction conditions and DNA substrates were used except as indicated. (*A*) The amount of ATP added was varied from 0 to 2 mM with 0.25 μg of various D108 DNA substrates: □, circular pSZ53; △, linear pSZ53; ○, mature phage DNA. (*B*) Various nucleoside triphosphates were added (0–2.2 mM) to reaction mixtures containing 0.25 μg of circular pSZ53 DNA: □, GTP; △, dATP; ◇, 2′,3′-dideoxyguanosine 5′-triphosphate (ddGTP); ○, adenosine 5′-[γ-thio]triphosphate (ATP[γS]).

DISCUSSION

We have developed an *in vitro* system for maturing and encapsidating phage D108 DNA with the Mu phage packaging machinery provided by crude extracts prepared from thermally induced lysogens late in the lytic cycle. Strong evidence, including phage immune specificity, DNase I resistance, and host cell surface receptor specificity, suggests that the D108 immunity-type phages are *de novo* generated *in vitro*. That this system is proficient for DNA maturation as well as encapsidation is supported by the fact that nonmatured DNA forms are readily converted to mature phage particles. We thus conclude that these procedures provide a valid *in vitro* system for examination of both phage DNA maturation and morphogenetic events required by these two unusual *E. coli* phages. Mu and D108 maturation differs markedly from that of other known double-stranded DNA phages in that maturation of the (left) end requires the recognition of a sequence (13) within the integrated phage genome (*pac*), and subsequent cleavage and encapsidation of adjacent host sequences beginning at least 54 bp from the left end (16). Mu and D108 are also unusual in that their *pac* site (13) is located within their functional origin of replication (*attL*), and thus, for these two processes to occur in the correct temporal sequence in the Mu lytic cycle, a switch between the two pathways must occur. Lastly, unlike other known phages, which replicate extrachromosomally (20), Mu and D108 replicate while integrated within host DNA and the dispersed prophages must be recognized and cleaved (matured) from the *E. coli* chromosome (34), which in this case behaves as a discontinuous concatemer during headfull virion morphogenesis (19).

We found that the optimal DNA substrate for *in vitro* packaging of D108 DNA was a circular pSC101::D108*cts*10 plasmid, followed by linearized plasmid and mature phage DNA. The differences between the three DNA forms suggest that the unprocessed flanking sequences may act in a stimulatory manner, possibly as substrates for phage maturase and/or prohead attachment (12, 20) or for the stabilization of these protein–DNA interactions in the DNA forms observed late in the Mu lytic cycle (41). Experiments with ATP[γS] indicate that ATP is absolutely required for the reaction, whereas the lack of complete inhibition of the reaction by ddGTP, along with a lack of [α-^{32}P]dATP incorporation into

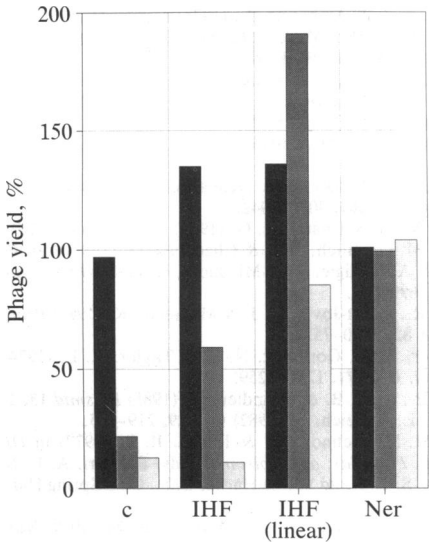

FIG. 3. Effects of D108 repressor (c protein), D108 Ner, and *E. coli* IHF on *in vitro* D108 packaging. Protein binding and *in vitro* packaging reaction conditions (with circular pSZ53 unless otherwise indicated) were as described in *Materials and Methods*. Initial protein concentrations were as follows: D108 repressor (c), 0.40, 20.0, and 60.0 μg/ml (black, dark stippled, and light stippled bars, respectively); D108 Ner, 0.20, 9.3, and 26.7 μg/ml; *E. coli* IHF, 0.43, 21.7, and 63.0 μg/ml. IHF was tested with both circular and linear pSZ53.

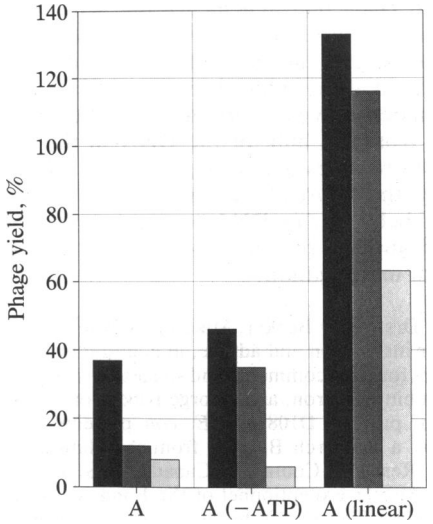

FIG. 4. Effects of the D108 transposase (A protein) on *in vitro* D108 packaging. Protein binding and *in vitro* packaging reaction conditions were as described except that ATP was omitted where indicated and linear pSZ53 DNA was the packaging substrate where indicated. Initial concentrations of the transposase were 1.13 μg/ml (black bars), 44.7 μg/ml (dark stippled bars), and 178.7 μg/ml (light stippled bars).

acid-insoluble material (data not shown), strongly suggests that DNA replication is not required for Mu and D108 DNA encapsidation.

Purified D108 transposase (A) protein was found to be inhibitory, yet little inhibition was observed if the DNA was in a linearized form, suggesting that mere binding of transposase to the DNA (15) was not responsible for the observed inhibition, or that the A protein may bind preferentially to supercoiled DNA, as is seen with Mu repressor (42). The transposase protein is produced early in the lytic cycle in small quantities (9, 25, 28), is used stoichiometrically, and is unstable *in vivo* (43). It is thus entirely possible that the effective concentration of active transposase protein determines whether a prophage is to transpose or, late in the lytic cycle, to be matured and encapsidated. During DNA encapsidation, the left end [which enters the capsid first (44)] could be either inaccessible to the A protein, or, once linearized by the phage "pacase," less susceptible to its inhibition during subsequent packaging steps.

The Mu repressor protein has a weak, but definite, affinity for binding to the left-end transposase binding sites and repressor operator of the phage genomes (10, 14, 45), and the observed inhibition by D108 repressor at these high concentrations may reflect repressor binding to these sites. The host protein IHF (27) binds to the left end of Mu and D108 DNA, where it stimulates early gene transcription, inhibits transcription of the repressor gene (36, 46), and stimulates *in vitro* transposition of a mini-Mu when the DNA is not optimally supercoiled (37). The addition of IHF to the *in vitro* reaction mixture had a slight stimulatory effect on linear (as opposed to circular) DNA and displayed inhibition only at high concentrations, where it may bind nonspecifically and wrap DNA (47).

The development and characterization of this *in vitro* maturation and encapsidation system will allow several interesting features of these phages' life cycles to be elucidated, such as the nature of the enzyme(s) that recognizes the *pac* signal and catalyzes the unusual cleavages seen at the left end of mature Mu and D108 DNA, which also appear to occur among transposable phages from the genus *Pseudomonas* (1). It should also not be overlooked here that this *in vitro* system for DNA maturation and morphogenesis, coupled with the *in vitro* transposition reaction (48), will allow all the benefits of Mu-induced DNA rearrangements and gene fusions (40) to now be carried out on any DNA *in vitro* without regard to phage host range. The ease of purification of the phage particles would allow induced rearrangements of Mu, D108, or their miniderivatives *in vitro* to be purified and characterized away from the bulk of the DNA in the reactions as, unlike λ, Mu requires only a single site for DNA encapsidation. Lastly, the nature of the molecular switch that occurs at the left end between one DNA-processing reaction (transposition) and another (maturation) can now be amenable to biochemical investigation.

We thank Drs. Andy Becker, Mike Feiss, Marv Gold, and Helios Murialdo for inspiration and advice, in addition to Dale Kaiser and the reviewers for their comments and suggestions. We thank Chantal Autexier, Robin Cameron, and George Kukolj of our laboratory for their gifts of purified D108 and *E. coli* proteins. H.L.B.C. was supported by a Research Bursary from the Natural Sciences and Engineering Research Council of Canada. M.S.D. is a Chercheur-Boursier de Mérite Exceptionnel of the Fonds de la Recherche en Santé du Québec. This research was supported by a grant (MA 6751) from the Medical Research Council of Canada.

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