Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8070–8074, October 1990
Biochemistry

Cleavage of the bacteriophage P1 packaging site (*pac*) is regulated by adenine methylation

(DNA adenine methyltransferase/methylation/phage DNA packaging)

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Communicated by John Roth, June 25, 1990 (received for review February 9, 1990)

ABSTRACT **The packaging of bacteriophage P1 DNA is initiated when the phage packaging site (*pac*) is recognized and cleaved and continues until the phage head is full. We have previously shown that *pac* is a 162-base-pair segment of P1 DNA that contains seven DNA adenine methyltransferase methylation sites (5′-GATC). We show here that cleavage of *pac* is methylation sensitive. Both *in vivo* and *in vitro* experiments indicate that methylated *pac* is cleavable, whereas unmethylated *pac* is not. Moreover, DNA isolated from P1 phage and containing an uncut *pac* site was a poor substrate for *in vitro* cleavage until it was methylated by the *Escherichia coli* DNA adenine methyltransferase. Comparison of that uncut *pac* DNA with other viral DNA fragments by digestion with methylation-sensitive restriction enzymes indicated that the uncut *pac* DNA was preferentially undermethylated. In contrast, virion DNA containing a cut *pac* site was not undermethylated. We believe these results indicate that *pac* cleavage is regulated by adenine methylation during the phage lytic cycle.**

Bacteriophage P1 packages its DNA by a processive headful mechanism that uses a concatemeric DNA substrate consisting of repeating units of viral DNA (1–3). Packaging is initiated when a specific 162-base-pair (bp) *pac* sequence on P1 DNA (Fig. 1) is recognized and cleaved (1, 3, 4). The DNA is then packaged unidirectionally from the cleaved *pac* end into an empty phage prohead until that head is full (3, 5). When packaging has been completed, the DNA inside the head is separated from that outside of the head by a cutting process that appears independent of DNA sequence. The DNA end that remains outside of the head after the cut is then used to initiate the next P1 sequential packaging event (1, 3, 6). In this way packaging proceeds down the concatemer in a processive series with *pac* being recognized and cleaved only once, to initiate the series.

For P1, a headful contains ≈110–115 kilobase pairs (kbp) of DNA (7, 8) or ≈10–15% more DNA than is present in the viral genome. Because the virus packages this headful from a concatemer, the DNA present in each virus particle contains the same DNA sequences at both ends: it is terminally redundant (6, 9). That redundancy is critical for the vegetative growth of the virus because it permits the viral DNA to cyclize after its injection into a host cell. Cyclization is mediated by the homologous recombination system of the host (10, 11) or in a recombination-deficient host by the P1-*lox*–Cre site-specific recombination system, when *loxP* sites are present in the terminally redundant regions (12). For P1 to generate terminally redundant DNA it is important to prevent the cleavage of concatemeric P1 DNA at each *pac* site. We show here that *pac* cleavage depends on adenine methylation and suggest that this dependency allows the cleavage process to be regulated in the cell, so as to permit the production of terminally redundant viral molecules.

MATERIAL AND METHODS

Bacterial and Phage Strains. *Escherichia coli* strain N99 is sup^o (13) and strain NS2626 is N99Tn*9*::*dam*. Strain NS2626 was constructed by P1-mediated transduction of the Tn*9* insertion mutation in the *E. coli dam* gene from strain GM3808 (14) to strain N99. Strain NS2342 is N99 (λ*imm*434–P1:20b) (3). Strain NS2635 is NS2626 (λ*imm*434–P1:20b). Strain NS2634 is NS2626 (λ*imm*434–P1:20a). Strain NS3208 is MC1061 *recD*$^-$*hsdR*$^-$*mcrA*$^-$*B*$^-$ (P1*r*$^-$*m*$^-$*cm-2 c1.100 am10.1*); this strain was used to prepare the *pac* cleavage-proficient extract as described by Sternberg (8). The P1 wild-type phage used here is P1*r*$^-$*m*$^-$*Cm c1.100*, and the P1 *dam*$^-$ mutant is that same phage with an insertion of the aminoglycoside 3′-phosphotransferase (*kan*^{r}) gene from Tn*903* at the *Asu* II site in the N-terminal portion of the P1 *dam* gene (unpublished work). This insertion completely inactivates the *dam* gene. Phages λ*imm*434–P1:20a and λ*imm*434–P1:20b have been described (3). Each phage contains the 624-base pair (bp) P1 *pac*-containing *Eco*RI-20 fragment inserted in the *Eco*RI site at λ map coordinate 65 (Fig. 2*A*). In λ*imm*434–P1:20a, *Eco*RI-20 is oriented so that packaging from *pac* proceeds toward the attachment site of the λ vector. In λ*imm*434–P1:20b, *Eco*RI-20 is oriented in the opposite direction.

General Methods. Restriction enzyme digestions were done as described by the vendor. Fragments generated by those digests were end-labeled by treating them first with calf intestinal alkaline phosphatase (New England Nuclear) and then with [γ-^{32}P]ATP and polynucleotide kinase (New England Biolabs) as described in Maniatis *et al.* (15). Methylation by the *E. coli* DNA adenine methyltransferase (Dam) (New England Biolabs) was done in 10 mM Tris·HCl, pH 8.0/10 mM EDTA/0.1 mM *S*-adenosylmethionine. Preparation of λ and P1 lysates (3), purification of phage by banding in CsCl gradients (16), fractionation of DNA fragments by agarose and acrylamide gel electrophoresis, and Southern hybridization analyses (17) have been described. The intensity of DNA bands in gels was determined by density scanning of exposed films with a Hoefer G3000 scanning densitometer.

***In Vivo pac* Cleavage Assay.** Details of the assay have been described (3). Briefly, cultures of strains containing λ*imm*434–P1:20a prophage are infected with either λ*imm*434–P1:20b or with P1, or with both phages, and total cellular DNA is isolated from the infected cells at various times after infection. The DNAs are digested with *Bam*HI, fractionated by agarose gel electrophoresis, transferred to nitrocellulose filters, and analyzed by hybridization to nick-

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Abbreviations: Dam, DNA adenine methyltransferase; pacase, protein necessary for *pac* cleavage; λ·dam$^-$, λ grown in *dam*$^-$ host; λ·*dam*$^+$, λ grown in *dam*$^+$ host.

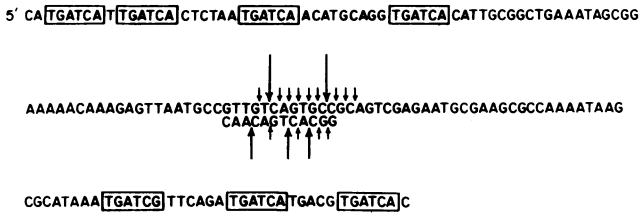

FIG. 1. Sequence of P1 *pac*. Sequence of the 162-bp *pac* site is sufficient to promote *pac* cleavage and P1 packaging (4). Boxed sequences, seven hexanucleotide (TGATCA) elements of *pac*; large arrows, positions of frequent *pac* cleavage events; and small arrows, positions of rare *pac* cleavage events (4).

translated probes containing λ sequences flanking the cloned P1 *pac* fragment (see Fig. 2).

***In Vitro pac* Cleavage.** The substrate for the assay is P1 *Eco*RI fragment 20. It was prepared by digesting plasmid pRH92 [pBR322 with *Eco*RI fragment 20 cloned into the vector *Eco*RI site (3)] or P1 DNA with *Eco*RI. The fragment was purified by agarose gel electrophoresis and end-labeled as described above. The *in vitro* cleavage reaction was done as described by Sternberg (8) with the use of an extract prepared by the induction of strain NS3208.

RESULTS

Cleavage of the Bacteriophage P1 *pac* Site Is Blocked in a Bacterial Host that Lacks Dam Methylase Activity. The P1 *pac* site is a 162-bp segment of DNA (Fig. 1) that contains seven hexanucleotide elements (5'-TGATCA) the integrity of which must be maintained for *pac* to be cleaved (4). Because each of these elements is also a Dam methylation site (5'-GATC), it was of interest to determine whether adenine methylation affects *pac* cleavage. The initial assay used either wild-type or *dam* mutant strains containing λ prophage with a cloned P1 *pac* fragment (λ*imm*434–P1:20b, Fig. 2*A*). These cells were infected with P1, cellular DNA was isolated at various times after infection, that DNA was digested at *Bam*HI restriction sites in λ DNA that flank the chromosomal *pac* site, and *pac* cleavage was assessed by Southern hybridization analysis with a probe (probe B, Fig. 2*A*) for the flanking λ sequences. If *pac* is not cleaved, a single 7.4-kbp fragment should be detected. If it is cleaved, a smaller 3.2-kbp fragment should also be detected. Comparing results in the two strains used (Fig. 2*C*), the onset of *pac* cleavage is clearly delayed by ≈30 min in the *dam*$^-$ host (lanes 7–12) compared with the wild-type host (lanes 1–6), but cleavage eventually reaches the same level (≈40–50% of the substrate is cleaved) in the two strains. Because P1 replication, as measured by the increase in P1 DNA in the gel used to prepare the filter for the hybridization analysis, is the same in the two infections (Fig. 2*B*), the delay in *pac* cleavage seen in the *dam*$^-$ host probably does not reflect a general phage growth defect in this host.

As an added control in these experiments, we determined the methylation state of the DNAs recovered from the infected cells by measuring their sensitivity to restriction enzymes the activities of which are sensitive to adenine methylation (*Bcl* I, *Mbo* I, and *Dpn* I) (18, 19). To our surprise we discovered that host DNA recovered at least 20 min after P1 infection of the *dam*$^-$ mutant was largely methylated. This DNA was resistant to *Bcl* I and *Mbo* I and sensitive to *Dpn* I (data not shown). The simplest interpretation of this result is that P1 encodes its own Dam methylase, which is expressed about midway through the viral life cycle. Activity of that enzyme presumably accounts for the *pac* cleavage seen after phage infection of a *dam*$^-$ host (Fig. 2*C*). We recently localized the P1 *dam* gene and have generated an insertion mutation that inactivates its function (see *Material and Methods*). When that mutant infects a *dam*$^-$ host containing a chromosomal *pac* site, cleavage of *pac* is undetected (Fig. 2*D*, lanes 7–12). Control experiments indicate that P1 *dam*$^-$ mutant cleaves *pac* normally in a *dam*$^+$ host (Fig. 2*D*, lanes 1–6) and that P1 *dam*$^-$ lytic replication is normal in a *dam*$^-$ host (data not shown). These results indicate that *pac* cleavage absolutely depends on adenine methylation.

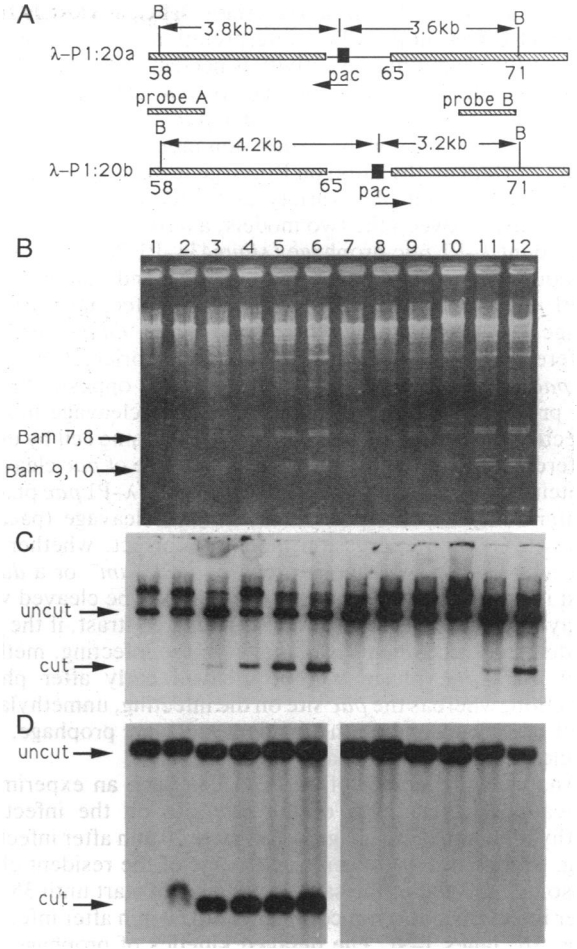

FIG. 2. *pac* cleavage in *dam*$^+$ and *dam*$^-$ bacteria. (*A*) Structure of λ–P1:20a and λ–P1:20b phages (3). The phages contain P1 *Eco*RI-20 (thin lines) with its *pac* site (■) cloned into λ DNA (thick cross-hatched lines) at λ map coordinate 65. The P1 fragment is cloned in opposite orientations in the two phages. Digestion of λ DNA with *Bam*HI (*B*) cleaves the DNA at λ map coordinates 58 and 71 and generates a 7.4-kbp fragment. This fragment is converted to two smaller fragments when *pac* is also cleaved. Arrows under the *pac* sites indicate direction of P1 packaging. The location of DNAs used as probes to detect the *Bam*HI-*pac* fragments are derived from plasmids pKA6 (probe A) and pRAL (probe B) (3). Note that while two fragments are generated when the *pac* site in λ DNA is cleaved, only the fragment destined to be packaged is detected in our assay; the other fragment is destroyed by cellular nucleases (3). (*B*) DNAs isolated from P1-infected cells were digested with *Bam*HI and fractionated in 1% agarose gel that was then stained with ethidium bromide. Lanes: 1–6, DNAs from cell line NS2342 (*dam*$^+$) infected with P1 wild type that were isolated 5, 10, 15, 30, 45, and 60 min after infection, respectively; 7–12, DNAs from cell line NS2635 (*dam*$^-$) infected with P1 wild type also isolated 5, 10, 15, 30, 45, and 60 min after infection, respectively. Positions of P1 *Bam*HI fragments 7–10 are shown by arrows. (*C*) The gel in *B* was transferred to nitrocellulose and hybridized with nick-translated probe B to detect the 7.4-kbp uncut and 3.2-kbp *pac*-cut fragments. (*D*) DNAs isolated 15, 25, 35, 45, 60, and 90 min after infection of NS2342 *dam*$^+$ (lanes 1–6) or NS2635 *dam*$^-$ (lanes 7–12) with P1 *dam*$^-$ were analyzed by Southern transfer hybridization using nick-translated probe B. Positions of the uncut and *pac*-cut fragments are shown.

***pac* Cleavage Defect in a Methylase-Deficient Host Is from a Direct Effect on *pac*.** Two interpretations follow from the data of Fig. 2: methylase activity is needed (*i*) for expression of P1-encoded proteins essential for *pac* cleavage (3) (the expression model) or (*ii*) to render *pac* cleavable (the *pac* model). Precedent for the expression model comes from the effect of Dam methylation on P1 *cre* gene expression (12) and on the expression of a variety of bacterial genes (18). To distinguish between the two models, a *dam*$^-$ host containing a resident λ–P1 *pac* prophage (λ*imm*434–P1:20a) was simultaneously infected with P1 and with a second homoimmune λ–P1 *pac* phage (λ*imm*434–P1:20b). The infecting λ–P1 *pac* phage was prepared in either a *dam*$^-$ or a *dam*$^+$ host and differed from the λ–P1 *pac* prophage in that orientation of the P1 *pac* fragment in the infecting phage was opposite that in the prophage, allowing us to measure *pac* cleavage in both infecting phage and prophage DNAs in the same cell by using different λ probes (Fig. 2*A*). The only source of *pac* cleavage proteins in this experiment is P1 because the λ–P1 *pac* phages contain only a small portion of the *pac* cleavage (pacase) genes (3). If the expression model is correct, whether the infecting λ–P1 *pac* phage is prepared in a *dam*$^-$ or a *dam*$^+$ host is immaterial; in both cases *pac* should be cleaved with delayed kinetics, as shown in Fig. 2*C*. In contrast, if the *pac* model is correct, then the *pac* site on the infecting, methylated λ–P1 *pac* phage will be cleaved early after phage infection, whereas the *pac* site on the infecting, unmethylated λ–P1 *pac* phage and on the resident λ–P1 *pac* prophage, will be cleaved late in the phage infection.

Analyses of the fate of *pac* DNA in such an experiment indicates that cleavage of the *pac* site on the infecting, methylated λ–P1 *pac* phage is complete 20 min after infection (Fig. 3*A*, lanes 7–9), whereas cleavage of the resident chromosomal *pac* site in the same cell does not start until 35 min after infection and is not complete until 60 min after infection (Fig. 3*B*, lanes 1–9). The delayed kinetics of prophage *pac* cleavage is consistent with the idea that synthesis of the P1 methylase occurs late in the infection and must methylate *pac* before it can be cleaved. In contrast, if the *pac* site is introduced into the cell on an unmethylated λ–P1 *pac* phage, cleavage of that site is not detected at all during the experiment (Fig. 3*A*, lanes 4–6), despite the fact that the initially unmethylated chromosomal *pac* site in the same cell is cleaved by 35 min after infection. Failure to cleave the infecting, unmethylated phage is not from a general failure of this phage to infect cells because it can infect a *dam*$^+$ host and be cleaved with an efficiency comparable to that of an infecting, methylated λ–P1 *pac* phage (Fig. 3*C*, lanes 1–4). These results strongly support a model in which *pac* needs to be methylated before it can be cleaved.

***pac* Cleavage *in Vitro* Is Methylation Dependent.** We have recently demonstrated that an extract prepared from a P1-induced lysogen ("pacase extract") can cleave *pac in vitro* (8). To determine whether that cleavage process also is affected by the methylation state of *pac* we isolated from plasmid pRH92 methylated and unmethylated P1 DNA fragments containing *pac* (*Eco*RI-20) and measured cleavage *in vitro*. As expected, the methylated fragment was cleaved into two smaller fragments, whereas the unmethylated fragment was not (Fig. 4, lanes 2 and 4). If the unmethylated *pac* fragment were methylated *in vitro* with the *E. coli* Dam methylase before being exposed to the P1 pacase extract, it could now be cleaved with the same efficiency as the methylated *pac* fragment (Fig. 4*A*, lane 3). Similar treatment *in vitro* of the methylated fragment had no effect on the cleavage reaction (Fig. 4*A*, lane 5).

Uncut *pac* DNA Isolated from P1 Virions Is Defective for *pac* Cleavage *in Vitro* Because It Is Preferentially Undermethylated. We have previously suggested that *pac* cleavage of P1 concatemeric DNA must be limited to ensure that packaged

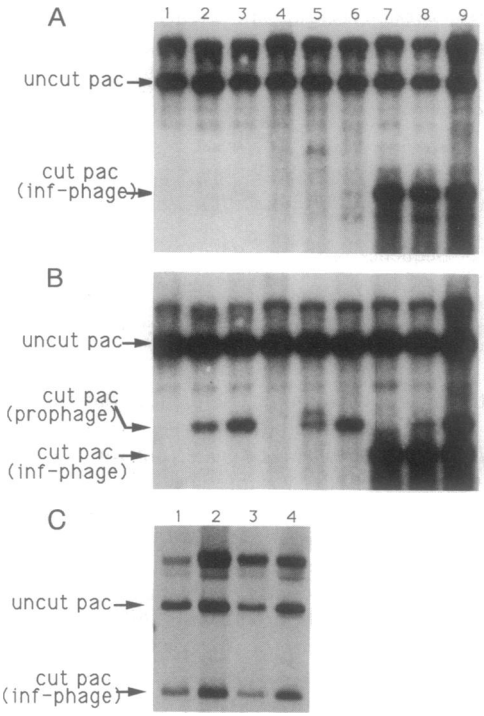

FIG. 3. Cleavage of methylated and unmethylated *pac* DNA in *dam*$^-$ bacteria. (*A* and *B*) Gels containing DNAs from *dam*$^-$ lysogen NS2634 (λ–P1:20a) infected with P1 wild type (lanes 1–3), P1 wild type and λ–P1:20b·dam$^-$ (λ grown in a *dam*$^-$ host; lanes 4–6) or P1 wild type and λ–P1:20b·dam$^+$ (λ grown in a *dam*$^+$ host; lanes 7–9). DNAs in lanes 1, 4, and 7 were isolated 20 min after infection (inf), DNAs in lanes 2, 5, and 8 were isolated 35 min after infection, and DNAs isolated in lanes 3, 6, and 9 were isolated 50 min after infection. DNAs were digested with *Bam*HI, fractionated in 1% agarose gels, transferred to nitrocellulose, and hybridized first with probe B to detect the 3.2-kbp cut *pac* fragment from the infecting λ–P1:20b phage (*A*) and then with probe A to detect the 3.8-kbp λ–P1:20a prophage cut *pac* fragment (*B*). (*C*) DNA from *dam*$^+$ strain N99 was infected either with P1 wild type and λ–P1:20b·dam$^+$ (lanes 1 and 2) or with P1 wild type and λ–P1:20b·*dam*$^-$ (lanes 3 and 4). DNAs were isolated 20 and 35 min after infection and treated as in *A* and *B*; filters were hybridized with nick-translated probe B.

DNA will be terminally redundant and, therefore, can cyclize after infections. Thus, it may be important to regulate *pac* cleavage *in vivo* by methylation so as to ensure that *pac* cleavage is used only to initiate the packaging. If this is correct, uncleaved *pac* DNA isolated from virions would be predicted to be a poor *pac* substrate *in vitro* because it is preferentially undermethylated; this prediction is supported by the following results.

First, we isolated P1 *pac* fragment *Eco*RI-20 from viral DNA and assessed its ability to be cleaved *in vitro*. Although some cleavage of the native fragment was detectable, cleavage was stimulated ≈3-fold by premethylating the fragment with *E. coli* Dam methylase before introducing it into the pacase reaction (Fig. 4, lanes 9–13). Thus, ≈75% of the potentially cleavable viral *pac* DNA could not be cleaved until it was methylated *in vitro*. The same result was obtained with a *pac*-containing fragment isolated from a second preparation of P1 virions (Fig. 4*A*, lanes 7 and 8). To measure directly the methylation state of *pac* DNA we isolated three classes of DNA fragments from P1 DNA: the 624-bp *pac*-containing *Eco*RI-20 fragment, *Eco*RI fragments 21, 22, 23, and 24 (the sizes of which vary from 180–450 bp), and the 145- to 150-bp *pac*–*Eco*RI fragment, which contains the cut viral *pac* end. These DNAs were end-labeled, digested with *Bcl* I (which only cuts fully unmethylated 5′-TGATCA sites), and the products were analyzed on denaturing sequencing gels

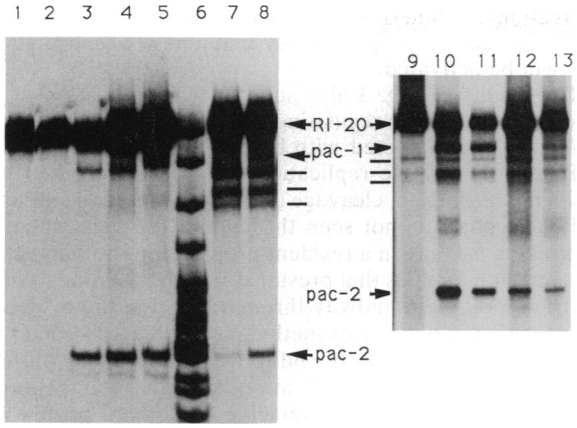

FIG. 4. *In vitro pac* cleavage is methylation dependent. All fragments used here were labeled at both ends with [γ-^{32}P]ATP and polynucleotide kinase. The fragments were then either methylated with the *E. coli* Dam methylase or not, incubated with the pacase extract, and then fractionated in 5% acrylamide gel. Lane 1 contains *Eco*RI-20 isolated from pRH92·*dam*$^-$. That same DNA was incubated in the pacase reaction unmethylated (lane 2) or after *in vitro* methylation (lane 3). Positions of the two *pac* cleavage fragments (the 475-bp *pac*-1 fragment and the 150-bp *pac*-2 fragment) are shown in lane 3. Lanes 4 and 5 contain *Eco*RI-20 isolated from pRH92·*dam*$^+$ incubated in the complete pacase reaction without *in vitro* methylation (lane 4) or after *in vitro* methylation (lane 5). Lanes 7 and 8 contain *Eco*RI-20 isolated from P1 phage DNA and either untreated (lane 7) or premethylated with Dam (lane 8) before being incubated with pacase extract. This fragment is partially contaminated with the smaller P1 *Eco*RI fragments 21–23; their positions in the gel are indicated by lines to right of lane 8. Lane 9 contains *Eco*RI-20 isolated from a second P1 phage preparation; it was incubated in the *pac* cleavage reaction without prior *in vitro* Dam methylation (lanes 12 and 13, respectively) or after methylation (lanes 10 and 11, respectively). Lanes 10 and 12 contain 3 times as much DNA as lanes 11 and 13. Lane 6 contains a *Hpa* II digest of pBR322 DNA. The labeled fragments were detected after gel was dried by exposure to Kodak XAR film.

(Fig. 5). Four classes of single-stranded DNA cleavage products should be generated when the six *Bcl* I sites in *pac* are digested (Fig. 5*A*). Despite the fact that *Eco*RI-20 is contaminated with a small amount of *Eco*RI-21, -22, and -23, *Bcl* I fragment classes 1, 2, and 3 were readily detected in the gels (lanes 5, 6, 8, and 9). Class 4 fragments were too large to be distinguished from uncut *Eco*RI-20. The results clearly indicate that *Bcl* I can digest a significant fraction of the *pac* site in *Eco*RI-20. Based on yield of class 1 and class 3 cleavage products (Fig. 5*B*, lanes 6 and 9) ≈30% of the *Bcl* I sites in *pac* could be cleaved (see Fig. 5 legend). In contrast, yield of class 2 cleavage products (Fig. 5*B*, lane 6) was more variable—cleavage ranged from 6 to 30% depending on site. At first approximation these two results appear contradictory because class 2 and 3 cleavage products are generated by cutting the same *Bcl* I restriction sites. However, the two products differ in that production of class 2 fragments depends on failure to cleave class 1 sites. All results are self-consistent if DNA molecules containing unmethylated class 2 *Bcl* I sites frequently contain unmethylated class 1 sites. Why this should be so, when only 30% of the DNA contains unmethylated *Bcl* I sites, is not clear, but the high degree of undermethylation of uncut *pac* is undeniable. In contrast, when the cut *pac* fragment was incubated with *Bcl* I, <2% of the DNA was digested (Fig. 5*B*, lanes 10–15). Note that this fragment can only generate class 1 *Bcl* I products because it contains only the right half of *pac*. When the cluster of *Eco*RI-21–24 fragments is digested with *Bcl* I, the single *Bcl* I site in *Eco*RI-22 is poorly digested. From the intensity of the Bc-22 fragments (Fig. 5*B*, lane 3), we estimate

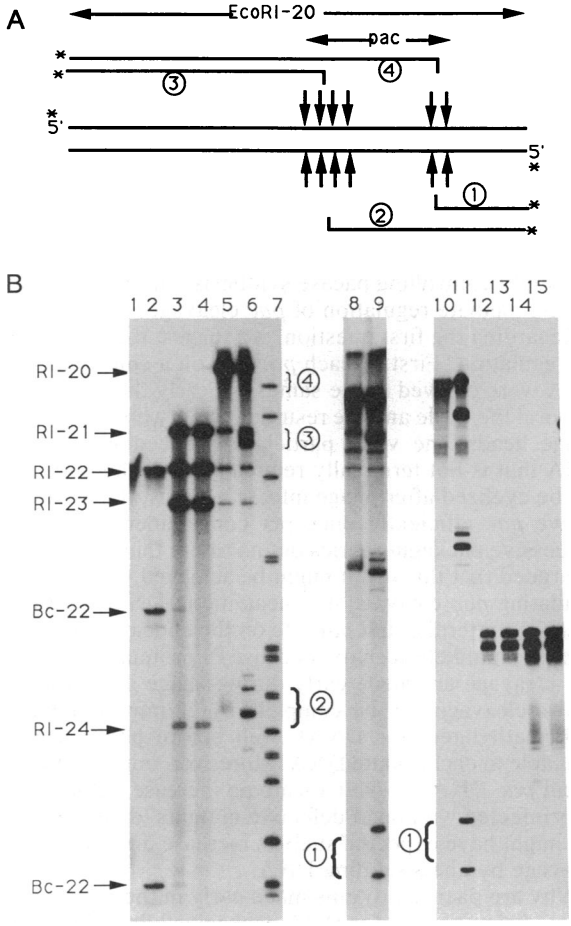

FIG. 5. *Bcl* I digestion of *pac* fragments isolated from P1 DNA. *Eco*RI fragments were isolated from P1 virus DNA, labeled with [γ-^{32}P]ATP and polynucleotide kinase, digested with restriction enzyme *Bcl* I, heat-denatured, and then fractionated in 8 M urea/6% acrylamide gels. (*A*) Four classes of restriction fragment that should be generated when unmethylated, end-labeled *Eco*RI-20 DNA is cut with *Bcl* I. →, *Bcl* I restriction sites; *, DNA ends labeled with ^{32}P. (*B*) Lanes: 1, Unmethylated P1 *Eco*RI-22 DNA; 2, unmethylated P1 *Eco*RI-22 DNA digested with *Bcl* I. Two smaller fragments (Bc-22) are generated; 4, *Eco*RI-22–25 fragments isolated from P1 virion DNA; 3, same DNA as in lane 4 after *Bcl* I digestion; 5 and 8, *Eco*RI-20 isolated from P1 virion DNA contaminated with small amounts of *Eco*RI 21–23; 6 and 9, that same fragment after *Bcl* I digestion. DNAs in lanes 8 and 9 were fractionated by gel electrophoresis for a shorter time than those in lanes 5 and 6. Positions of class 1–4 *Bcl* I restriction fragments are indicated by the brackets between lanes 7 and 8. The class 4 restriction fragments are difficult to distinguish in these gels from *Eco*RI-20. Lanes: 10 and 11, similar to lanes 8 and 9, except that the *Eco*RI-20 fragment was from a different P1 virion preparation; 12 and 14, 145- to 150-bp *pac*–*Eco*RI fragments isolated from two different P1 virion preparations; 13 and 15, *Bcl* I digests of these latter DNAs.

that <4% of this site is cut. The same result was obtained with virion *Bam*HI fragment 9, which contains three clustered *Bcl* I restriction sites (18; data not shown). We conclude that uncleaved *pac* DNA in virus particles is a poor substrate for *pac* cleavage because it is preferentially undermethylated.

DISCUSSION

The phage P1 packaging site (*pac*) is a 162-bp segment of P1 DNA that contains four hexanucleotide elements (5′-TGATCR) at one end, three hexanucleotide elements at the other end, and a segment of 90 bp separating these elements (Fig. 1). Cleavage of this DNA within the 90-bp spacer region

is an initial step in the P1 headful packaging process (4). Each hexanucleotide element of *pac* contains a Dam methylation site, and we show here that methylation of at least some of those elements is necessary for *pac* cleavage. In addition, we show that methylation probably plays a role in regulating *pac* cleavage because uncleaved *pac* DNA isolated from virions is undermethylated and is a poor substrate for *pac* cleavage *in vitro*, whereas cleaved *pac* DNA from virions is more extensively methylated. Questions still remaining are why does *pac* cleavage have to be regulated, why isn't it regulated by simply controlling pacase synthesis, and how does methylation mediate regulation of *pac* cleavage?

Regarding the first question, we suggest that there is need for regulation. First, if each *pac* site on a concatemer of P1 DNA were cleaved at the same, or nearly the same, time in the viral life cycle and the resulting DNA were packaged into phage heads, the virus particles produced would contain DNA that is not terminally redundant and, therefore, could not be cyclized after phage infection. Thus, it is desirable to cleave *pac* efficiently once per concatemer to initiate the processive packaging series but not more than once. It could be argued that this result might be achieved without directly regulating *pac* cleavage if concatemeric DNA were rapidly packaged after the first *pac* site on the concatemer were cut. This is an unlikely scenario because P1 proteins necessary for *pac* cleavage are made early during phage infection (Fig. 3 shows cleavage can be complete by 20 min after infection with methylated *pac* DNA), well before phage heads are available to encapsidate DNA. Moreover, our measurements of cut *pac* DNA *in vivo* indicate no increase in that DNA in cells infected with head-defective mutants (data not shown). We might have expected such an increase if heads block *pac* cleavage by encapsidating DNA.

Why are pacase proteins made early in the viral life cycle before being needed for DNA packaging? Probably they are necessary for a function other than packaging. Perhaps they play a role in initiating rolling circle replication by nicking circular DNA at *pac*.

How is *pac* regulated by methylation? We believe a key point in the regulation process is the replication of *pac* DNA. That replication temporarily generates hemimethylated or even unmethylated *pac* DNA (18, 19). Normally the host and/or the P1 methylase would rapidly methylate the newly replicated *pac* DNA, rendering it cleavable. Regulation might be achieved by a competing reaction in which either a P1 or a host-encoded protein binds to the undermethylated *pac* site and prevents further methylation and subsequent cleavage. Our recent demonstration that pacase can bind to, but cannot cleave, hemimethylated *pac* DNA (the primary product of *pac* replication) suggests that pacase itself may be responsible for inhibiting *pac* methylation (N.S., unpublished work). To account for the *pac* cleavage that must occur to initiate P1 packaging one need only argue that the competition between methylation and protection is designed to produce one functional *pac* site for every three to five nonfunctional sites. Alternatively, the methylation function is enhanced or the protection function is diminished at some point in the viral life cycle, increasing the probability that a newly replicated *pac* site will be activated.

The results of Fig. 3 also suggest that replication of *pac* DNA is important in regulating *pac* availability. Thus, when a *dam*$^-$ host is infected with both P1 and an unmethylated λ–P1 *pac* phage, the replication of which is blocked by a resident λ prophage, cleavage of the *pac* site on the infection λ–P1 *pac* phage is not seen throughout the experiment. In contrast, a *pac* site in a resident prophage in the same cell is cleaved with kinetics that presumably reflect synthesis of the P1 Dam methylase midway through the viral life cycle. We propose that in both cases methylation and activation of *pac* is blocked by protein(s) bound to that site. For the chromosomal site the host's replication machinery occasionally moves through the site, displacing the bound protein and permitting methylation and subsequent cleavage of the site. The extrachromosomal λ *pac* substrate is never replicated during the experiment and is thus never cleaved.

Growth of P1 *dam*$^-$ phage is normal in a *dam*$^+$ host but severely restricted in a *dam*$^-$ host. Under fully Dam$^-$ conditions, P1 produces only ≈5% as much phage as when either phage or host methylase is active (unpublished work). Moreover, the phage produced under Dam$^-$ conditions do not have *pac* ends (G. Lucey and N.S., unpublished work). These results support our conclusions and suggest that packaging of P1 DNA under Dam$^-$ conditions, occurs by a mechanism that does not recognize and cleave *pac*.

We thank Drs. R. Menzel and G. Cohen for their critical reading of this manuscript.

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