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DNA Requirements *in Vivo* for Phage T4 Packaging

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Phage T4 terminase, comprising the products of genes 16 and 17, packages headfuls of DNA from a concatemer but its mechanism of DNA recognition remains to be determined. Phage T4 terminase gene sequences were introduced into prophage λ*imm*^{434} and plasmids in order to assess their effect on packaging as measured by transduction frequency and DNA content of T4-transducing particles. Multiple copy prophage λ*imm*^{434} genes were transduced at 100-fold higher frequency, and high copy plasmids were transduced at 1000-fold higher frequency than single copy prophage or chromosomal genes. T4 16 gene inserts enhanced both prophage and plasmid packaging; terminase gene-containing plasmid DNA in T4 transducing particles could exceed 10% of the total. Deletion or base change of the 24-bp gene 16 3′ region which is required for sequence specific amplification of terminase gene 17 (Hp 17 mutations) depressed these elevated plasmid transduction frequencies, suggesting that this is a preferred T4 *pac* sequence. Moreover, a specific gene 16-containing *pac* fragment could be detected in mature, packaged phage T4 DNA following restriction endonuclease digestion. We conclude that both the copy number of homologous sequences and the DNA *pac* sequence(s) themselves are important for packaging, consistent with a synapsis model for regulation of terminase cutting and packaging in phage T4.

INTRODUCTION

Most complex, tailed dsDNA phages containing linear DNA initiate packaging on DNA concatemers. In phages such as λ, packaging and cutting occur precisely at a single unique *cos* sequence (Murialdo, 1991; Feiss and Becker, 1983). In phages such as P1 and P22, packaging is initiated at a *pac* sequence, but cutting is variable around this site and packaging also occurs at distant sequences on the concatemer as a result of processive headful packaging and cutting on the concatemer (Sternberg and Coulby, 1987; Casjens *et al.,* 1992; cf. Black, 1989). Phage T4 is unusual because specific DNA packaging sequences have not been identified. Nevertheless, phage T4 terminase and *in vitro* packaging resemble that of other phages (Black *et al.,* 1994). Thus the T4 small terminase subunit gp16 resembles in many respects the analogous subunits of λ and SPP1 terminases, known to confer the terminase sequence-specific DNA binding of these phages. In addition, it forms novel ring and double ring structures (Lin *et al.,* 1997).

With the appropriate mutations, phage T4 (T4GT7) can act as a generalized transducing phage whose transduction efficiencies equal or exceed those of P1 (Wilson *et al.,* 1979). To analyze DNA packaging requirements *in vivo,* a number of constructs were analyzed using this phage T4-transducing derivative. Since DNA packaging is the last stage of phage development, both T4 replication and recombination pathways are expected to affect formation of the packaging substrate. Indeed, T4 transduction has been used to analyze both replication (Kreuzer and Morricol, 1994) and recombination (Mattson *et al.,* 1983; Kreuzer and Kreuzer, 1994). These analyses suggest that plasmid packaging can occur following plasmid concatemer formation resulting from T4-driven replication and recombination. In addition, packaging results from both homologous and illegitimate recombination of plasmid sequences into phage concatemers (Mattson *et al.,* 1983; Kreuzer and Kreuzer, 1994). Multiple, discontinuous plasmid monomers have also been reported to be packed to a headful under certain conditions (Leffers and Rao, 1996; Coren *et al.,* 1995). Because late protein synthesis and concatemer formation depend upon successful completion of T4 recombination and replication processes (Mosig, 1994), it is necessary to uncouple the effects on these earlier developmental stages from direct packaging effects.

T4-transducing particles were prepared in hosts containing T4 DNA sequences incorporated into plasmids and prophage. We focused on effects of the T4 terminase gene sequences for a number of reasons. First, these genes have not been found to contain T4 replication origins or recombination hot spots (Kreuzer and Alberts, 1986; Kreuzer and Morricol, 1994). Second, gene 16, encoding the small terminase subunit, is not lethal to *Escherichia coli* in high copy number plasmids (Wu *et al.,* 1995; Rao and Black, 1988). Third, in most phages *pac* or

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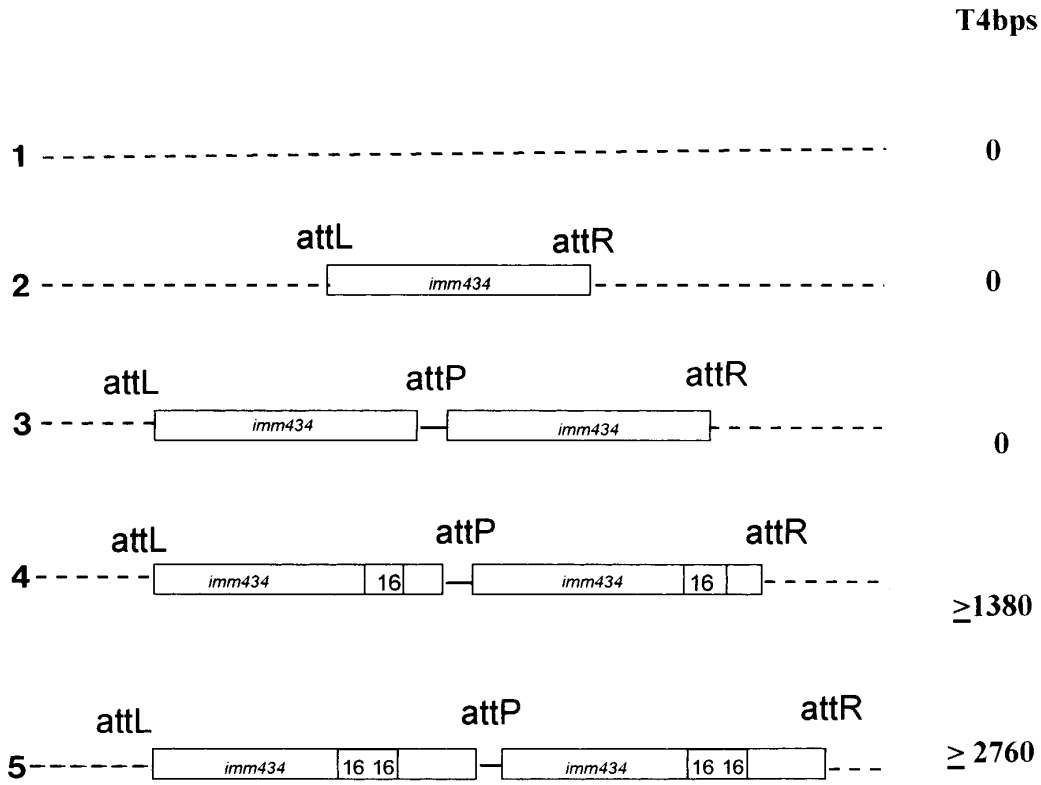

FIG. 1. T4 transduction of *E. coli* genes and λ*imm*^{434} mono- and polylysogens. Structures of the λ*imm*^{434} mono- and polylysogens are shown. The dotted lines indicate *E. coli* chromosomal DNA. The T4 gene 16 insert sizes correspond to two or more prophages. The transduction values of chromosomal genes and prophages shown are given on the corresponding lines of Table 1.

cos sequences lie within or close to the small terminase gene (cf. Black, 1989). Finally, we have shown a site-specific gene amplification of the T4 terminase region to depend upon a specific 3′ sequence in gene 16 and on the gene 16 product itself, and this amplification was supposed to reflect *pac*-directed DNA-binding activities of the small subunit of the T4 terminase acting upon its gene and upon homologous sequences in a flanking gene (Wu *et al.,* 1995; Wu and Black, 1995; Black, 1995). By changes in length, sequence, and copy number of the terminase gene-containing constructs (Fig. 1), our analysis shows that the number of both homologous DNA sequences and specific DNA sequences themselves are important for the efficiency of DNA packaging into phage T4 transducing particles.

RESULTS

The T4GT7 derivative of phage T4 contains a number of mutations, including a deletion of *rII,* which prevent destruction of host DNA and allow transduction of chromosomal and plasmid DNAs (Young *et al.,* 1982). Since λ*imm*^{434} prophage does not restrict *rII* mutants, T4GT7 can transduce mono- and polylysogenic derivatives of this prophage: the λ*imm*^{434}T4GT7 transducing phage can be titered on a λ lysogen on which T4GT7 itself does not grow. T4 sequences therefore can be inserted into the host chromosome in λ*imm*^{434} mono- or polylysogens, whereas plasmids can contain these same sequences in high or low copy numbers extrachromosomally. Figures 1 and 2 show the constructions used for our analyses of T4 packaging. These constructs were compared, except as noted, in the same hosts and under the same phage growth conditions to measure packaging effects.

Transduction of *E. coli* genes and T4 gene 16-containing λ*imm*^{434} lysogens

The transduction of four *E. coli* chromosomal genes tested was in the range of 10^{-8}–10^{-6}, which is comparable to those values reported previously (Table 1) (Young and Edlin, 1983). Construction of λ*imm*^{434} T4 gene 16 insert-containing derivatives allowed transduction measurements of chromosomal genes differing with respect both to copy number and to T4 DNA sequence content (Fig. 1). While the transduction value for the λ*imm*^{434} monolysogen was comparable to values for other chromosomal genes (ca. 10^{-6}), transduction values for the λ*imm*^{434} multilysogens were much higher (10^{-3} to 10^{-4}) (Table 1). Incorporation of two copies of T4 gene 16 into the prophage DNA led to a sixfold increase in transduction of the λ*imm*^{434} prophage. Transduction values were similar for multilysogens of the three λ*imm*^{434} derivatives of a number of hosts, including one (Schrenk

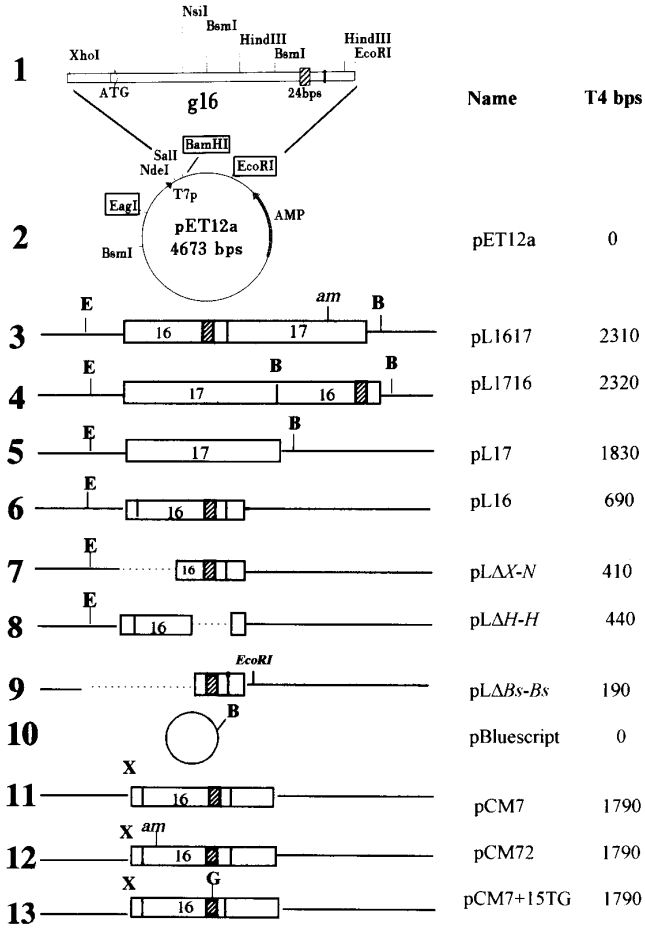

FIG. 2. T4 transduction of plasmids containing T4 terminase gene 16 and 17 inserts. The restriction endonuclease sites of gene 16 and the 5′ portion of gene 17 and of pET12a used to construct plasmid deletions are given on lines 1 and 2. The shaded box in gene 16 is the 24-bp region of partial homology to a 24-bp region in gene 19. Recombination between these two sequences leads to terminase Hp17 gene amplification mutations in T4. *am* (amber) and site directed base change (15T → G) mutation (G) sites are indicated. Restriction sites shown on lines 3–13 are B, *Bam*HI; E, *Eag*I; and X, *Xho*I. The plasmid constructs shown on lines 2–9 (pET12a derivatives) and 10–13 (pBluescript derivatives) yielded the T4 transduction values shown on the corresponding lines 2–13 of Table 2.

and Weisberg, 1975) which is deleted for the λ attachment site, suggesting that the high transduction values of the tandem inserted polylysogenic prophages are independent of the chromosomal integration site.

Transduction of T4 terminase gene-containing plasmids

Phage T4GT7 grown in a host containing the *bla* gene-containing plasmid constructions shown in Fig. 2 transduced ampicillin resistance to recipient bacteria. The transduction of plasmid pET12a (6×10^{-3}) or pBluescript (2×10^{-3}) is about 10-fold higher than that of prophage polylysogens (2×10^{-4}) and about three orders of magnitude higher than monolysogens or host chromosomal genes (ca. 10^{-6}) (Table 2). Since plasmid transduction requires that the recipient contain a functional *recA* gene, this apparently occurs following homologous recombination in the recipient to regenerate circular plasmid DNA from the injected multimer (data not shown) (Takahashi and Saito, 1982). Significantly, we find that high frequency transduction of plasmids is strongly dependent upon the copy number of the plasmid. A host which reduces pBR322-derived plasmid copy numbers to 5–10% normal (He *et al.,* 1993), GBE180, yields about 50-fold less T4GT7 *bla* transductants than the isogenic DH5α. The reduction is about ten-fold when the plasmid carries a gene 16 insert (Table 2, lines 14–17).

TABLE 1

T4 Transduction of *E. coli* Chromosomal Genes

Gene(s)[a]	Transduction efficiency$^{(Exps)b}$	Strain(s)
1, *nadA*	$4.0 \times 10^{-8\,(3)}$	c
1, *bioA*	$6.0 \times 10^{-7\,(3)}$	c
1, *trpA trpE*	$5.0 \times 10^{-7\,(3)}$	c
2, (λ*imm*^{434})1	$1.7 \times 10^{-6\,(3)}$	d
3, (λ*imm*^{434}) ≥ 2	$2.4 \times 10^{-4\,(6)}$	e
4, (λ*imm*^{434}*g*16) ≥ 2	$3.8 \times 10^{-4\,(6)}$	f
5, (λ*imm*^{434}*g*16*g*16) ≥ 2	$1.5 \times 10^{-3\,(6)}$	e

[a] See Fig. 1 for chromosome structures of 1 to 5.
[b] Transduction efficiency, transductants/plaque forming unit of T4GT7. The number of determinations is shown as a superscript.
[c] CES200.
[d] MC1000.
[e] CES200, CES201, and MC1000.
[f] CES200 and CES201.

TABLE 2

T4 Transduction of *E. coli* Plasmids

Plasmids[a]	Strain	Transduction efficiency (SE)^{Exps}[b]
2, pET12a	HMS174	6.0×10^{-3} $(1.7 \times 10^{-3})^4$
3, pL16-17*am*	HMS174	8.8×10^{-2} $(2.5 \times 10^{-3})^3$
4, pL17-16	HMS174	7.5×10^{-2} $(3.1 \times 10^{-2})^3$
5, pL17	HMS174	3.3×10^{-2} $(5.9 \times 10^{-3})^8$
6, pL16	HMS174	2.6×10^{-2} $(3.4 \times 10^{-3})^6$
7, pLΔ*X-N*	HMS174	1.8×10^{-2} $(3.4 \times 10^{-3})^5$
8, pLΔ*H-H*	HMS174	6.4×10^{-3} $(1.6 \times 10^{-3})^5$
9, pLΔ*Bs-Bs*	HMS174	1.7×10^{-2} $(2.0 \times 10^{-3})^5$
10, pBluescript	HMS174	2.4×10^{-3} $(7.4 \times 10^{-4})^3$
11, pCM7	HMS174	1.7×10^{-2} $(2.7 \times 10^{-3})^4$
12, pCM72	HMS174	1.0×10^{-2} $(5.0 \times 10^{-3})^3$
13, pCM7 + 15TG	HMS174	4.0×10^{-3} $(1.5 \times 10^{-3})^4$
14, pL16	GBE180	2.0×10^{-4} $(5.5 \times 10^{-5})^2$
15, pL16	DH5α	1.9×10^{-3} $(5.0 \times 10^{-4})^2$
16, pET12a	GBE180	1.1×10^{-5} $(3.2 \times 10^{-6})^4$
17, pET12a	DH5α	6.6×10^{-4} $(2.2 \times 10^{-4})^4$

[a] See Fig. 2 for plasmid constructions, 2–13.
[b] Transduction efficiency, transductants/plaque-forming unit of T4GT7. The number of experiments is given following the standard error.

Plasmid transduction was enhanced more than 10-fold by T4 terminase gene inserts. The T4 gene-containing plasmid transductants appear to contain the entire plasmid and insert sequences as judged by T4 gene marker rescue tests and by restriction endonuclease digestion (data not shown). Taking into consideration T4 sequence length in these constructs (Fig. 2, lines 2–6), the analysis suggests that the predominant terminase enhancement of plasmid transduction is due to a gene 16 sequence (Table 2). Deletion analysis of the pET12a derivatives shows that the most significant portion of the transduction enhancement conferred by the 16 gene appears to reside at its 3′ end and that the amount of T4 sequence required for this elevation can be low. Less than 200 bp of T4 gene 16 DNA at the 3′ end yields about a 3-fold increase relative to pET12a, whereas a 250-bp deletion of this end of gene 16 shows only a slight increase over the parent plasmid (Table 2, lines 6–9; Fig. 2). This 200-bp portion of gene 16 contains the 24-bp box implicated in reiterated terminase gene amplifications (Wu *et al.,* 1991).

Analysis of site-directed mutant sequences confirms the effect of the specific 3′ end sequences in gene 16. Transduction is enhanced about 7-fold by gene 16 (and a fragment of gene 17) sequences in pBluescript (Table 2, lines 10 and 11). Although a site-directed gene 16 *am* mutation in this construct only marginally reduces transduction efficiency (line 12), a site-directed mutation in the 24-bp box (15T → G) reduces the enhancement of transduction to less than twofold (Table 2, line 13). This 15T → G mutation, which leaves the amino acid sequence of gp16 unaltered, when introduced into the plasmid or recombined back into T4, had the most significant effect of the five site-directed mutations in the 24-bp box in gene 16. It resulted in elimination of Hp17 amplification mutants when recombined into T4 phage (>40-fold reduction) as well as eliminating a PCR-generated fragment which results from the initiating Hp17 recombination event as assayed in phage DNA (Wu and Black, 1995; Wu *et al.,* 1995). Overall, although the full enhancement of packaging efficiency of the terminase genes may require more extended sequences in this region, the analysis strongly suggests that the Hp17 amplifications and effects on packaging are correlated with both the 24-bp gene 16 recombination box sequence and with a functional gp16.

DNA analysis of the transducing phage

Restriction digestion of purified transducing phage DNAs allows an independent measure of DNA packaging. Since T4 DNA (170 kb) contains a single *Bam*HI site, and only two *Eag*I sites, most of the T4 sequences should remain of high molecular weight (>25 kb) following digestion with these restriction endonucleases, whereas the plasmid DNA is expected to be released as unique 4.6- to 7-kb monomers (Fig. 2) by cutting at the single *Bam*HI, *Eag*I, and *Eco*RI sites in the plasmids (the latter enzyme digests the T4GT7 cytosine-containing DNA to smaller fragments) if the plasmid DNAs are packaged as linear multimers or circular monomers. However, monomeric pET12a plasmid DNA constructs recombined into the T4 DNA (e.g., at the 16 or 17 genes by homologous recombination or elsewhere by illegitimate recombination) should not be released as specific fragments by *Bam*HI or *Eag*I digestion. Multiple tandem copies of plasmid DNA inserted into T4 DNA cointegrates would still leave behind one copy or ~5% (5–7/170 kb) in a phage/plasmid hybrid concatemer packaged to one headful.

Plasmid DNA sequences are readily detected by EtBr staining of the digested transducing particle DNAs (Fig. 3). In fact, quantitative densitometry of the stained plasmid DNA band for one gene 16–17 construct preparation (Fig. 2, line 3), following *Bam*HI digestion of the transducing phage DNA, yielded 14% plasmid to phage DNA, compared to 13% *bla* transduction. These are comparable values. This and other plasmid DNAs released by digestion of the phage DNAs have the size expected of the respective plasmids, and plasmid-size DNA in the transducing phages is not found without digestion (Fig. 3, lanes 10–12; also cf. Fig. 5).

Significantly more plasmid DNA is seen in the gene 16 construct plasmids compared to the pET12a or gene 17-transducing phage DNA, determined by EtBr staining (Fig. 3A, lane 6 compared to lanes 3 and 4). Southern blots of these digests show that the linearized plasmid-size bands contain pET12a DNA sequences and that the relative amounts of plasmid DNA measured by transduction and EtBr staining are also reflected by Southern blots *in situ* in the agarose gel (Fig. 3a). Gel analysis also shows more DNA in the gene 16 constructs containing the 3′ end of the gene compared to the other deleted gene 16 plasmids (compare lanes 6, 7, 8, and 9 of Fig. 3a). In support of the interpretation that most of the plasmid sequences result from packaging of plasmid-only concatemers, relatively little hybridization with plasmid sequences is seen in the T4-size DNA region of the gel following digestion, whereas before digestion all of the plasmid sequences are T4 size (Fig. 3, lanes 4–12). Clearly, some residual high molecular weight plasmid DNA sequences are found in the phage DNA band, particularly for the gene 17-containing derivative (lanes 4 and 12), indicating that either tandem or single copies of gene 17 have recombined into the phage DNA. However, these amounts appear lower than 5% by densitometry scanning for the gene 16 constructs, suggesting packaging predominantly of multimerized plasmid DNA (data not shown) (lanes 6–9). Taken together with knowledge that there are many T4 genes lethal to *E. coli,* the Southern blotting results strongly suggest that most of the

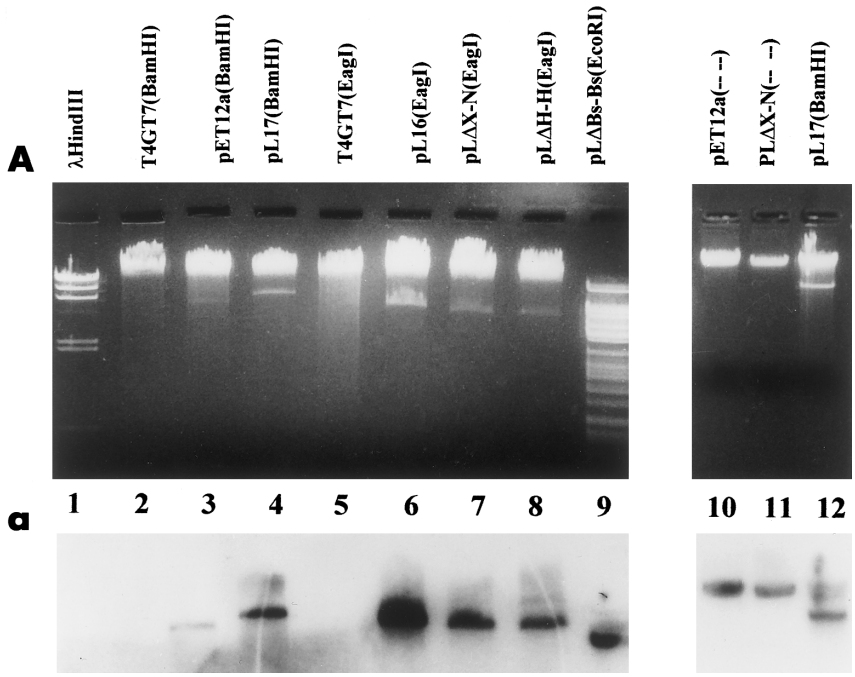

FIG. 3. Plasmid content of transducing phage DNA determined by restriction endonuclease digestion. 0.5 μg of DNA, prepared from CsCl-purified phage T4 transducing particles grown in *E. coli* HMS174 with or without the plasmids shown in Fig. 2 was electrophoresed on an agarose gel following restriction endonuclease digestion. A, EtBr stained gels; a, Southern blots using nick-translated pET12a DNA as probe. Samples: (1) λ*Hin*dIII size standard; (2) no plasmid, *Bam*HI; (3) pET12a, *Bam*HI; (4) pL17, *Bam*HI; (5) no plasmid, *Eag*I; (6) pL16, *Eag*I; (7) pLΔ*X-N,* EagI; (8) pLΔ*H-H, Eag*I; (9) pLΔ*Bs-Bs, Eco*RI; (10) pET12a, undigested; (11) plΔ*X-N,* undigested; (12) pL17, *Bam*HI.

bla-transducing phages carry plasmid-only DNA molecules, rather than phage–plasmid cointegrate molecules.

DNA content of phage-transducing particles: Relationship to DNA concentration in infected and uninfected bacteria

The effects of the T4 terminase gene sequences on transduction could be due to T4-induced replication of T4 DNA sequence-containing plasmids and prophages following infection, rather than to effects on packaging per se, although it would be difficult to explain, by this hypothesis, the differences found among plasmids containing small deletions or site-directed mutations in the 24-bp box found in gene 16. Nevertheless, in order to investigate the replication hypothesis, the pET12a and prophage DNA contents were compared in the bacteria before infection, in the infected bacteria at the time of phage purification and in purified phage particles yielding transduction results comparable to those reported in Tables 1 and 2. In fact, phage T4GT7 infection was found to decrease the amount of plasmid DNA rather than increasing it, and there was no significant difference between the level of plasmid only and of gene 16 insert plasmid DNA following infection (Figs. 4A and 4B). Moreover, the amount of packaged plasmid DNA appears to be greater in purified phage particles than in the infected bacteria (Compare Figs. 3 and 4A and 4B).

Similarly, measurement of the quantity of prophage DNA in the mono- and polylysogen showed that T4GT7 infection does not selectively enhance the amount of either prophage DNA as a consequence of T4-promoted replication. In fact, the amounts of both prophage sequences are comparably lowered, presumably because enhanced T4 DNA synthesis reduces the relative amount of λ*imm*^{434} DNA compared to the total in the cell (Fig. 4C). Overall, we conclude that the higher transduction values of the tandem inserted polylysogenic prophages and the gene 16-containing plasmid (Tables 1 and 2) reflect direct effects on packaging rather than on DNA abundance following T4-promoted replication.

Evidence for a phage T4 gene 16 *pac* fragment in mature packaged T4 DNA

If the 3′ end of gene 16 is a preferred phage T4 *pac* site, then T4 mature DNA might be expected to contain abundant ends in the vicinity of this *pac* site. This is indeed the case as is shown by restriction endonuclease digestion of T4GT7 and T4GT7 plasmid-transducing phage DNAs. The analysis makes use of the fact that there is a single *Bam*HI site in the T4 genome (in gene 7, sequence position 86,441, about 10.23 kb from the 3′ end of gene 16). When DNA from mature T4GT7 particles is digested with *Bam*HI, a band of ~10 kb can be visualized by EtBr staining within the smear of fragments which presumably result from other, dispersed ends of the permuted DNA (Fig. 5A). When the restriction digest is

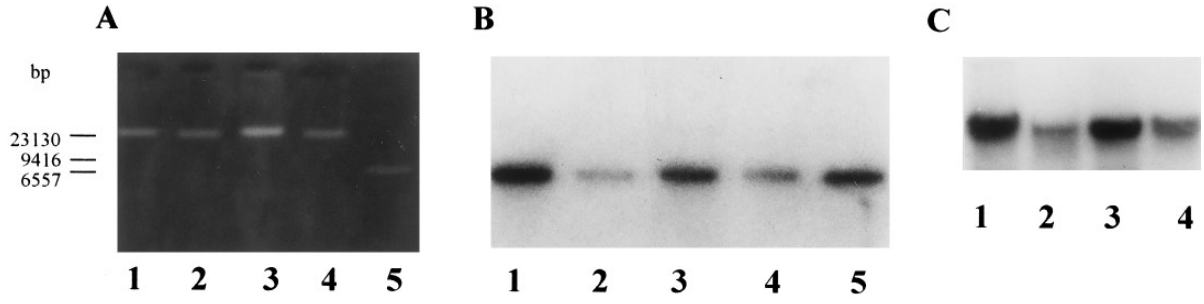

FIG. 4. Plasmid and prophage DNA content in *E. coli* before and after infection by T4GT7 to prepare transducing phage particles. (A) An EtBr-stained agarose gel with 0.2 μg of total cell DNA (lanes 1–4) or 0.02 μg of pL16 plasmid DNA (lane 5). Lanes 1 and 2, total cell DNA extracted from uninfected or T4GT7-infected pET12a-containing bacteria, respectively. Lanes 3 and 4, total cell DNA extracted from uninfected and T4GT7-infected pL16-containing bacteria, respectively. Lane 5, pL16 plasmid DNA digested with *Xho*I endonuclease. (B) Southern blot of *Bam*HI-digested total cell DNA from A, lanes 1 to 4, using pBluescript plasmid DNA as probe. Lane 5, contains pL16 plasmid following *Xho*I digestion shown as a positive control. (C) Southern blot of *Hin*dIII digest of total cell DNA extracted from SBM361 (lanes 1 and 2) and MC1000 (λ*imm*^{434}) (lanes 3 and 4). Radioactive λ*imm*^{434} DNA was used as the probe. Lanes 1 and 3 represent the total DNA from the lysogen before infection, and lanes 2 and 4 are the total DNA following T4GT7 infection. Only the 23-kb *Hin*dIII bands are shown here. 5 μg of total cell DNA was applied to each lane.

probed with gene 16–17 (Fig. 5B) or with gene 12–16 DNA (Fig. 5C), the ~10-kb fragment is detected (arrows) together with the linearized 7-kb plasmid, high (>30 kb) molecular weight phage DNAs, and the smear of sequences which are expected to arise from other T4 end fragments containing genes 12–16. Of course, the ~10-kb fragment should not arise from the transduced plasmid DNA (*Bam*HI digestion, 7 kb), and indeed, it is still detected in the absence of plasmid (Fig. 5C, lanes 2 and 6). It is not found in the absence of restriction endonuclease digestion (Fig. 5B, lane 1 vs lane 2, and Fig. 5C, lanes 1 and 2 vs lanes 3 and 4). Significantly, however, more ~10-kb fragment is detected in the transducing particle phage DNA prepared from bacteria with the terminase gene-containing plasmid, than from bacteria without plasmid (Fig. 5C, lane 1 vs lane 2 and lane 5 vs lane 6), suggesting that cutting at this *pac* sequence in the phage concatemer is promoted by the multiple copies of the *pac*-containing plasmid.

DISCUSSION

Transduction of *E. coli* chromosomal and prophage genes, and of plasmids lacking T4 sequences, by T4GT7

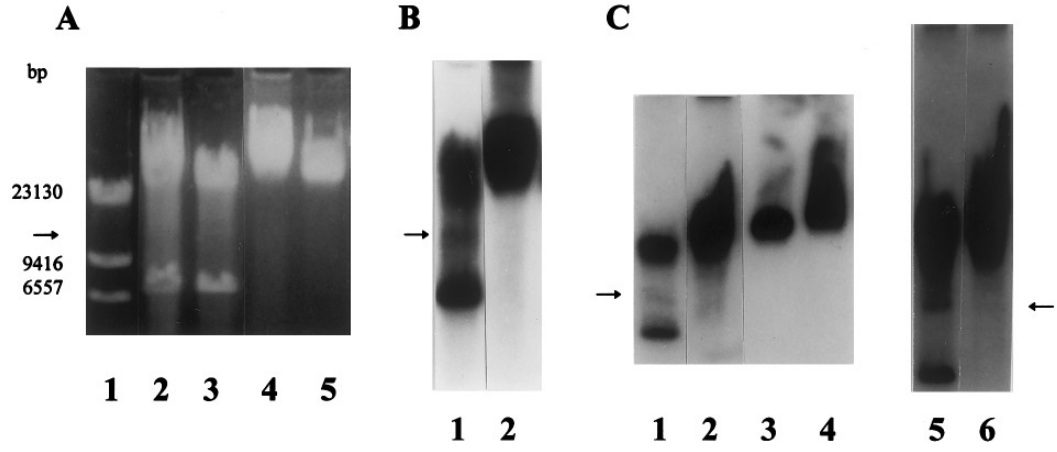

FIG. 5. Detection of a gene 16 *pac*-specific fragment in packaged, mature phage T4 DNA. (A) EtBr-stained gel. DNAs were isolated from CsCl-purified phage T4-transducing particles grown in either pL1617*am*- (lanes 2 and 4) or pL1716 (lanes 3 and 5)-containing *E. coli* HMS174. 5 μg of DNA was used in each lane. Lane 1, λ*Hin*dIII fragments. Lanes 2 and 3, *Bam*HI-digested transducing phage DNA. Lanes 4 and 5, the same transducing phage DNAs without digestion. (B) Southern blot of DNAs extracted from phage T4-transducing particles grown in pL16-17*am*-containing *E. coli* HMS174 probed with radioactive pL1617*am* DNA. Lanes 1 and 2, *Bam*HI-digested and intact transducing phage DNA, respectively. (C) Southern blot of transducing phage DNA probed with p659 DNA which contains a T4 gene 12-gene 16 DNA fragment. Transducing phage were grown in HMS174 either with pL16-17*am* (lanes 1, 3, and 5) or without plasmid (lanes 2, 4, and 6). Lanes 1 and 3, DNAs with and without *Bam*HI endonuclease digestion. Lanes 2 and 4, *Bam*HI digest and intact transducing phage DNA. Lanes 5 and 6 were *Bam*HI digests run longer in comparison to lanes 1 and 2. 5 μg of transducing phage DNAs was used except for 2 μg of DNAs used in lanes 1 and 3. The arrows indicate the 10.6 ± 0.2-kb *pac* fragment DNA located between the linearized plasmid and phage DNAs. The fragment size was estimated by mixing with a 10-kb ladder marker standard (Boehringer Mannheim Co.).

shows that specific T4 sequences are not required for packaging, in agreement with previous observations (cf. Black *et al.,* 1994). The same is true for generalized transducing phages such as P1 and P22 which are known to package their DNAs from a *pac* site. For example, introduction of the P1 *pac* site into the *E. coli* chromosome results in a 40- to 80-fold increase in transduction of flanking genes (Sternberg and Coulby, 1987). Like T4GT7 transduction (Young and Edlin, 1983), efficiency of P1 transduction of *E. coli* genes is not closely related to gene location or sequence, suggesting that neither P1 nor T4 packaging of chromosome fragments is dependent upon *pac*-resembling sites. However, a significant difference from T4 is that P1 transduces 6- to 7-kb plasmids with much lower frequencies (10^{-6} to 10^{-7}) than T4GT7 (10^{-1}–10^{-3}, cf. Table 2) (Yarmolinsky and Sternberg, 1988).

Phage T4 DNA packaging specificity is suggested by the observation that T4-transducing particle frequencies range over more than six orders of magnitude and that T4 terminase sequences enhance packaging 6- to 15-fold (Tables 1 and 2). High level packaging (>10%) of plasmids apparently results from phage T4 recombination activities in the infected *recA* host carrying the plasmid to yield plasmid multimers from the high copy number plasmids employed. In support of this interpretation, lowering the plasmid copy number greatly reduces the transduction, even with a full gene 16-containing plasmid, suggesting that it is not primarily dependent upon T4 replication, but results from a recombinational plasmid concatemer generated by homologous T4 recombination pathways (Kreuzer and Kreuzer, 1994). Phage T4-driven replication of plasmid DNA leading to concatemer formation is expected to be low in our gene 16 constructs or in pET12a (Kreuzer and Morricol, 1994). In fact, direct measurements of DNA content in bacteria before and after infection shows that plasmid DNA concentration differences do not account for the differences in transduction efficiency (Fig. 4).

Host genes, prophage inserted into the host chromosome, and multimerized plasmid segments should all be present in more than headful lengths (>170 kb) of DNA to allow efficient headful packaging. Although plasmid monomers have been reported to be packaged into phage particles *in vitro* and *in vivo* (Leffers and Rao, 1996; Coren *et al.,* 1995), this apparently occurs at significantly lower levels than we can detect by Southern blotting under our T4-transducing particle growth conditions *in vivo* (Fig. 3, lanes 10–12; Fig. 5B, lane 2; and Fig. 5C, lanes 3 and 4). As analyzed previously (Mattson *et al.,* 1983; Kreuzer and Kreuzer, 1994), transducing particles may contain a complex mixture of phage and/or plasmid intramolecular and intermolecular recombinant products. However, the Southern blotting and *bla* transduction measurements suggest that all-plasmid DNA concatemers predominate under our growth conditions. These yield the viable *bla* transductants we measure as well as most of the packaged plasmid DNA.

What is important for efficient packaging in addition to headful DNA length? It appears from our analysis that both multiple homologous sequences and specific DNA sequence play a significant role in promoting packaging. Certain sequences, such as found at the 3′ end of gene 16, appear to be preferred *pac* sequences. If there are ~50 copies of 5–7 kb pBR322-derived plasmids containing genes 16 and 17 (Fig. 2), then in the absence of T4-driven replication, these sequences comprise only one to two potential *bla*-transducing phage particles containing 170 kb of DNA, compared to a yield of about 40 T4GT7 per cell. Since these gene 16-containing plasmid-transducing particles are found at a frequency of from 2 to 10% (Table 2), these concatemerized sequences may be packaged more efficiently than T4 concatemers in the same infected bacterium. Transduction values are 10^6-fold lower if the DNA copy number is one, as expected of a single copy chromosomal gene, despite the fact that such a gene should have a nearly equal chance to constitute a DNA headful. Synapsis of the homologous plasmid sequences to promote packaging initiation would be one explanation for this apparent dependence of high frequency packaging upon gene copy number. Addition of the terminase gene sequences to the homologous multicopy sequences further enhances transduction values 6- to 15-fold, values which possibly reflect packaging of all the available plasmid DNA (Tables 1 and 2).

Evidence for synapsis in packaging came from our analysis of a sequence-specific amplification of the terminase gene 17 and flanking sequences (Wu *et al.,* 1995). Site-directed mutagenesis of a 24-bp region in the 3′ end of gene 16 and other experiments showed that both this sequence and the gene 16 protein itself were required for recombination with a region of partial homology in gene 19. These experiments suggested that gene 16 product participated in synapsis of the homologous gene 16 and 19 sequences, and we suggested that this reflected a terminase mechanism designed to measure concatemer formation to control packaging initiation. Could such an activity also reflect the evolution of the phage chromosome by accretion of modular plasmid sequences by the terminase? Our present work supports the earlier site-directed mutagenesis experiments in suggesting that the 3′ end of gene 16 is a preferred *pac* site, by showing that a mutation in this site which reduces amplification also reduces packaging. In fact, direct study of gp16 shows it to be a dsDNA-binding protein with preferential binding to gene 16 DNA (Lin *et al.,* 1997). Interestingly, there is recent support for an analogous synaptic mechanism in phage λ from evidence that packaging of one λ *cos* site requires a second *cos* site in *trans* in the cell (Thomason *et al.,* 1997). We have detected in T4GT7-transducing phage particles

a gene 16 *pac*-specific DNA fragment of ~10 kb following *Bam*HI digestion and Southern blotting (Fig. 5). The elevation in the level of this fragment observed in terminase gene-containing plasmid-containing bacteria during growth of T4GT7 is consistent with the proposed synapsis mechanism. Although mature T4 DNA ends are not confined to such a gene 16 *pac* fragment (Fig. 5), judging from the smear of nonspecific fragments in the endonuclease digest, this should also be the case for other *pac* phages such as P1 and P22. Imprecise terminase cleavage occurs to yield multiple P1 and P22 cutting sites around their *pac* sites. Additional, distant ends are generated by processive movement of these terminases to downstream headful cutting and packaging sites (Casjens *et al.,* 1992; Sternberg and Coulby, 1987; Skorupski *et al.,* 1992). In fact, although the mature T4 DNA ends are thought to be widely distributed over the genome, the most complete heteroduplex analysis of this distribution concluded that these ends were not found in all regions, suggesting that packaging was initiated from specific sequences in the T4 DNA (Grossi *et al.,* 1983).

Similar to P1 and P22, the T4 terminase might initiate packaging elsewhere on the concatemer after responding to gene 16 *pac* sites, but moving more distantly to single-stranded or transcriptionally active regions (Bhattacharyya and Rao, 1994; Franklin and Mosig, 1996). Gp16 might be less DNA sequence specific in its binding than other *pac* phage terminase small subunits, and it might bind and synapse homologous DNA segments at alternative gene 16 *pac* site-resembling sequences in the T4 DNA. Although the gene 16 *pac* site may not be eliminated by the site-directed mutations we have tested, the fact that the T4 gene 16 15T → G mutation is not lethal suggests that alternative packaging mechanisms exist. T4 terminase could package from dsDNA ends formed in the concatemer by other mechanisms to yield a complex distribution of end sequences. Indeed, *in vitro* packaging data strongly suggest that such a packaging mode (utilization of dsDNA ends) operates in both T4 and other phage DNA packaging systems (cf. Black, 1989).

MATERIALS AND METHODS

Purification of phage and phage DNA-transducing plasmids

E. coli HMS174 (F-*recA hsdR* (r_k^- m_k^+)Rif^r or other bacteria (Table 2) containing the plasmid constructs shown in Fig. 2 were grown in M9S medium containing ampicillin (150 μg/ml) to $OD_{600\ nm}$ = 0.4 and then infected with T4GT7 at a multiplicity of infection of 0.1. After 3 h of growth at 37°C, lysis was induced by addition of chloroform. The bacterial debris was removed by low speed centrifugation (5900*g* for 10 min) and the phage were concentrated by high speed centrifugation (35,000*g* for 60 min). After incubation with pancreatic DNase (from USB Co.) in phage dilution buffer, the phage were further purified by centrifugation on CsCl step gradients for DNA analysis (Fig. 3). The phage were titered on LE392 (F-e14- (*McrA*-) *hsd*R514(r_k^- m_k^+)*supE44 supF58 lacY1* or Δ(*lacIZY*)6 *galK2 galT22 metB1 trpR55*) and used to transduce *E. coli* B834 (*hsd*R_B*hsd*M_B*met thi* Sup^0) to ampicillin resistance. Amp^r transductants were measured by mixing with soft agar and pouring onto plates which contained 150 μg/ml ampicillin.

In experiments measuring bacterial plasmid DNA concentration, 10 ml of bacteria was infected with T4GT7 at a multiplicity of infection of 5 at 30°C and superinfected with the same multiplicity 12 min later. The infected bacteria were pelleted by centrifugation at 5900*g* for 10 min at 60 min after infection. The pellet was resuspended in 1 ml of lysis buffer (50 mM Tris–HCl, pH 7.8, 0.1 M NaCl, 10 mM EDTA, pH 8.0, 0.2% SDS, 100 μg/ml proteinase K, and 100 μg/ml RNase I). After 2 h of incubation at 65°C, the pellet was extracted once with buffer-saturated phenol and once with phenol:chloroform:isoamylalcohol, and the aqueous layer was dialyzed against several changes of TE buffer (10 mM Tris–HCl, pH 7.0, 0.5 mM EDTA).

Purification of phage and phage DNA-transducing λ*imm*^{434} prophage and *E. coli* chromosomal genes

E. coli CES200, CES201, and MC1000 containing λ*imm*^{434} derivatives were grown at 30°C and infected with T4GT7 essentially as above. The T4-transducing particles were purified by low followed by high speed centrifugation as described above, and then in most experiments free λ*imm*^{434} phage were inactivated in 50 mM EDTA, pH 8.0, 10 mM Tris–HCl, pH 8.0. The transduction of λ*imm*^{434} prophage was measured by plating on LE392 (for total phage), on LE392 (λ) for λ*imm*^{434} and on LE392 (λ) /T4 to determine residual free λ*imm*^{434}. An independent and consistent measure of T4 particles transducing λ*imm*^{434} followed incubation of the T4GT7 with λ phage antiserum (free phage λ*imm*^{434} was inactivated to less than 10^{-4} of its original titer under these conditions) followed by infection of LE392(λ). Transduction of wild-type chromosomal genes from CES200 prototrophic for *trpA-trpE, nadA,* and *bioA* operon genes was done by standard techniques (Young and Edlin, 1983). The *E. coli* auxotrophs for these transductions and λ antiserum were gifts of Susan Gargas and Sankar Adhya, NIH. λ*imm*^{434} T4 gene 16 derivatives were prepared from λ*imm*^{434}c$_I$ *ts Sam*7 (a gift from Barbara Hohn, Friedrich Miescher Institute, Basel, Switzerland) by inserting single and double gene 16 *Xho*I–*Eco*RI fragments (0.693 kb) at the λ *Sal*I sites, gifts of V. B. Rao. The MC1000 lysogen of λ*imm*^{434}, a derivative of RS88 (Nancy Kleckner, Harvard), was a gift of Sarbani Banik-Maiti and R. Weisberg, NIH, and was verified by them by PCR assay to be a monolysogen (personal communication). Strain RW01401*supE SA269* Δ[*galT-bioB*]*strA* (deleted for the λ

attachment site) was from R. Weisberg, NIH. Although λ*imm*^{434}*dgal* derivatives were originally constructed to produce multilysogens, in fact, for unknown reasons only multilysogenic strains could be prepared on *E. coli* CES200, CES201, MC1000, and RW01401 from the λ*imm*^{434}c$_I$ ts*Sam7* and its T4 gene 16 insert derivatives as determined by Southern blotting of *Hin*dIII digests of the chromosomal DNA. These blots showed that the ~9-kb *attP* site-containing fragment was intact in all of these lysogens, indicating two or more tandem prophages, unlike the known monolysogen RS88 MC1000 derivative (Fig. 1). Measurement of the relative prophage DNA concentrations in the uninfected and infected bacteria was carried out on DNA prepared as described above for plasmid DNA determinations.

Other techniques

Southern blotting was carried out by the dried agarose gel technique described by Lueders and Fewell (1994). The phage DNA restriction endonuclease digests shown in Figs. 3 and 5 result from digestion of DNA purified from CsCl-centrifuged phage. The plasmids shown in Figs. 2 and 5 were described by Wu and Black (1995) or Lin *et al.,* (1997) or derived from these plasmids by standard techniques. The plasmid derivatives shown were tested by restriction enzyme digestion and by marker rescue tests with 16*am*N87-16*am*N66, 17*am*-NG178, and 16*am*N87 (which lies within the *Xho*I–*Nsi*I deletion) and with 16*am*N66 (which is in the gene 16 5′*Hin*dIII–3′*Bsm*I deletion). These phage mutants also allow for testing by marker rescue of phage genes carried by the B834 amp^r plasmid transductants. For quantitation of DNA fragments in gels, EtBr-stained and Southern-blotted autoradiograms of agarose gels were scanned with a laser densitometer. Other techniques were standard (Sambrook *et al.,* 1989).

Bacteria and phage

Bacteria and phage were described above or standard (Sambrook *et al.,* 1989) except for GBE180 = DH5α *pcnB*::zad*Tn10* (prepared by G. Barcak, University of Maryland Medical School), which reduces pBR322 copy number (He *et al.,* 1993), and the isogenic DH5α. CES200 (F$^-$ *thr-1 ara-14* Δ (*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2 hisG4 rfbD1 rpsL31* (Str^r) *kdgD51 xyl-5 mtl-l argE3 leuB6 hsdR* ($r_k - m_k +$) *recB21 recC22 sbcB15 sbcC* and CES201 = CES200 Δ(*recA-srl*)*306* and MC1000 (F-*araD139* Δ(*araABC-leu*)7679 *galU galK* Δ(*lac*)174 *rpsL thi-1*) were used to construct phage lysogens.

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