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DNA sequences necessary for packaging of bacteriophage λ DNA

(cosmid/*in vivo* packaging/*in vitro* packaging of restriction fragments)

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ABSTRACT The extent of DNA flanking the "cohered cohesive end" site of bacteriophage λ DNA, which is required for packaging, was determined by using defined DNA fragments and a cosmid *in vivo* packaging assay. From the right end of λ DNA a 20- to 36-base-pair stretch extending from the center of the cohered cohesive ends is shown to be required, whereas the packaging efficiency of cosmids extending to 70 base pairs into the left λ arm is reduced to 10% (compared to a fragment extending until about 80 base pairs). A 60-base-pair stretch of the left arm leaves an efficiency of only 1%. The segment thus delineated, by the nature of the assay, is both necessary and sufficient for the binding of packaging proteins to the DNA, the packaging of DNA itself, the DNA cleavage, and successful injection of the DNA into a bacterial host. By contrast, *in vitro* packaging of restriction fragments of mature λ DNA directly demonstrated the selectivity of the packaging proteins for the fragment originating from the left end of the DNA. The results of the two complementary experiments are discussed in terms of the various steps before, during, and after packaging for which different sequences flanking and including the cohered cohesive ends might be required.

The condensation of DNA is necessary for the organized availability of the genetic information as a replicating and expressing entity. In the case of viruses the existence of a compact and nuclease-resistant form of the DNA is a condition for the transport of the genetic information from one host cell to another.

The DNA inside complex bacteriophages such as λ is especially highly condensed. The virus has evolved a sophisticated process that achieves this condensation (1): the packaging precursor form of λ DNA, the multimeric concatenated DNA, binds the product of genes *A* and *Nu1* (termed pA/p*Nu1* for simplicity) and the bacteriophage prehead. With the help of phage packaging proteins and host factors DNA gets condensed inside the phage head, which at the same time expands by 20% in diameter. λ terminase (pA/*Nu1*) then introduces staggered nicks into the "cohered cohesive end" site, producing the 12-base-long cohesive ends (Fig. 1).

The *cos* site (cohesive end site) was defined as the target for λ "terminase" (3, 4). It is the subject of this paper to define and localize sequences of *cos* in addition to the cohered cohesive ends (termed *cce* in this communication), which are required for the specificity of packaging. Only a relatively short stretch of λ DNA around the *cos* site is expected to be required for packaging: phages in which most of the right or the left half of λ DNA has been replaced by DNA of another phage or even *Escherichia coli* DNA are packaged as efficiently as λ DNA (5); plasmids containing only short stretches of λ DNA are efficient cosmids (6, 7). Several observations point to the extreme left end of λ DNA as being important in determining the packaging specificity: packaging is polar from the left to the right end (3); hybrids of λ and phage 21 are packaged in a λ *in vitro* or *in vivo* packaging system only when the left end originates from λ (8, 9); phage 21 is a lambdoid bacteriophage containing the same cohesive ends as λ but nevertheless is not accepted by the λ packaging system (5). Finally, a λ mutant with the symmetrical *cce* sequence deleted that still can bind packaging proteins as measured by a competition assay has been described (10).

In this study the minimal sequences flanking the *cce* site that are necessary for packaging are determined: a biological assay measures the sum of physical packaging, DNA cleavage, and injection; complementary experiments using restriction enzyme fragments of λ DNA allow the determination of sequences necessary for physical packaging only.

Table 1. Clones of defined restriction fragments of λ

Plasmid	No. in Figs. 2 and 3	Vector (restriction site)	Fragment of λ DNA cloned
pcos 0	—	pUC7 (*Hin*cII)	*Hin*cII (−210) to *Hin*cII (+193)
pcos 1	1	pUC7 (*Hin*cII)	*Hae* III (−83) to *Hae* III (+141)
pcos 2	2	pUC7 (*Hin*cII)	*Hae* III (−83) to *Aha* III (+86)
pcos 7	7	pUC7 (*Bam*HI)	*Sau*34 (−20) to *Bam*HI (+203)*
pcos 16	16	pBR322 (*Cla* I)	*Hpa* II (−25) to *Hpa* II (+37)

Coordinates of restriction sites were deduced from the sequence (2), the base to the right of the center of the recognition sequence being chosen as the exact coordinate. 0 is the base to the right of the center of the λ terminase cleavage substrate (see Fig. 1).

* The *Bam*HI site of the λ DNA stems from the site in the linker in pcos 0, which was used for the construction of pcos 7.

MATERIALS AND METHODS

***In Vivo* Packaging.** Strain BHB 3169 [= W3110 (λ*imm*434 *c*Its *red*3 *b*2 Sam7)] was used as transformation recipient. Standard procedures were used for transformation, and plating was done on ampicillin-containing plates at 30°C. For *in vivo* packaging a transformant or, as in the case of *Bal*31 trimming experiments, pools of transformants were grown in L broth plus ampicillin at 30°C to an OD_{600} of 0.3. Induction was achieved by incubating the cultures for 15 min at 45°C; phage and cosmid packaging occurred during a 3-hr period of rapid shaking at 37°C. The cultures were lysed with chloroform and titered for phage on BHB 2600 [= 803 *supE supF* r_k^- m_k^+ (N. Murray)] and for cosmid on BHB 3225 [= W3110 (λ*imm*434)], both at 37°C.

Construction of Plasmids with Defined Restriction Fragments Spanning the λ *cos* Region. Table 1 indicates the λ fragments cloned. Cloning was done by using pUC7 (11) or pBR322 as vectors. The published λ sequence (2) was used for the choice of the restriction enzymes. The source of the λ *Hin*cII fragment yielding clone pcos 0 was λcIts857 Sam7. The λ *Hpa* II fragment used to construct pcos 16 was recovered from a 10% acrylamide gel of *Hpa* II-cleaved λ *cos Hinc* II fragment.

Standard procedures were used for restriction, ligation, and

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Abbreviations: bp, base pair(s); kb, kilobase pair(s).

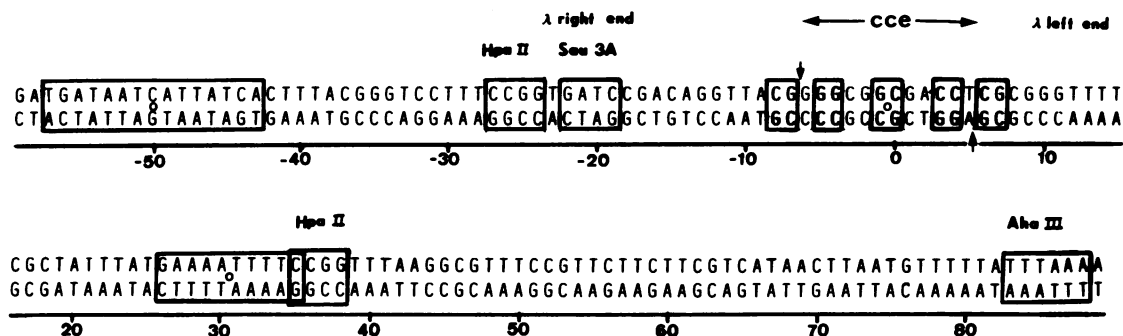

FIG. 1. Sequence of λ DNA around the *cos* site (2). Symmetrical areas are boxed, the centers of symmetry marked with a small circle. Arrows mark the points of *cos* cleavage. The coordinates of enzyme cuts are defined as the bases to the right of the center of the recognition sequence. Coordinate 0 is thus the base to the right of the center of the "cohered cohesive end" (*cce*) symmetry region.

bacterial transformation. Restriction and ligation enzymes were purchased from New England BioLabs, Boehringer Mannheim, and P & S Biochemicals (Liverpool, UK) (*Aha* III).

Construction of Plasmids with *Bal*31-Trimmed Ends. The *Hae* III/*Hae* III or the *Hae* III/*Aha* III fragment was recovered from pcos 1 and pcos 2 by *Eco*RI digestion. Different molecular weight ranges of *Bal*31- (New England BioLabs) trimmed fragments were eluted from 10% acrylamide gels and cloned in *Hinc*II-cut pUC7. Strain BHB 3169 was used for transformation and pools of transformants were induced.

***In Vitro* Packaging of ^{32}P-Labeled λ DNA.** λ*c*Its857 Sam7 DNA was nick-translated and packaged *in vitro* either as such or after *Eco*RI or *Bam*HI digestion. Unpackaged DNA was degraded with DNase and the supernatant of a centrifugation in an Eppendorf centrifuge was freed from lower molecular weight radioactive material by passing it over a small Sepharose 4B (Pharmacia) column. The high molecular weight material in the excluded fractions was banded in CsCl equilibrium gradients and localized by its radioactivity.

For analysis of the packaged DNA the radioactive fractions were extracted with phenol, and the DNA in the aqueous phases was concentrated by precipitation with ethanol and subjected to agarose gel electrophoresis. The dried gel was radioautographed.

RESULTS

In vivo packaging of plasmids containing minimal functional sequences surrounding the *cos* sequence of λ DNA

The general strategy to assay *cos* in functional terms was to in-

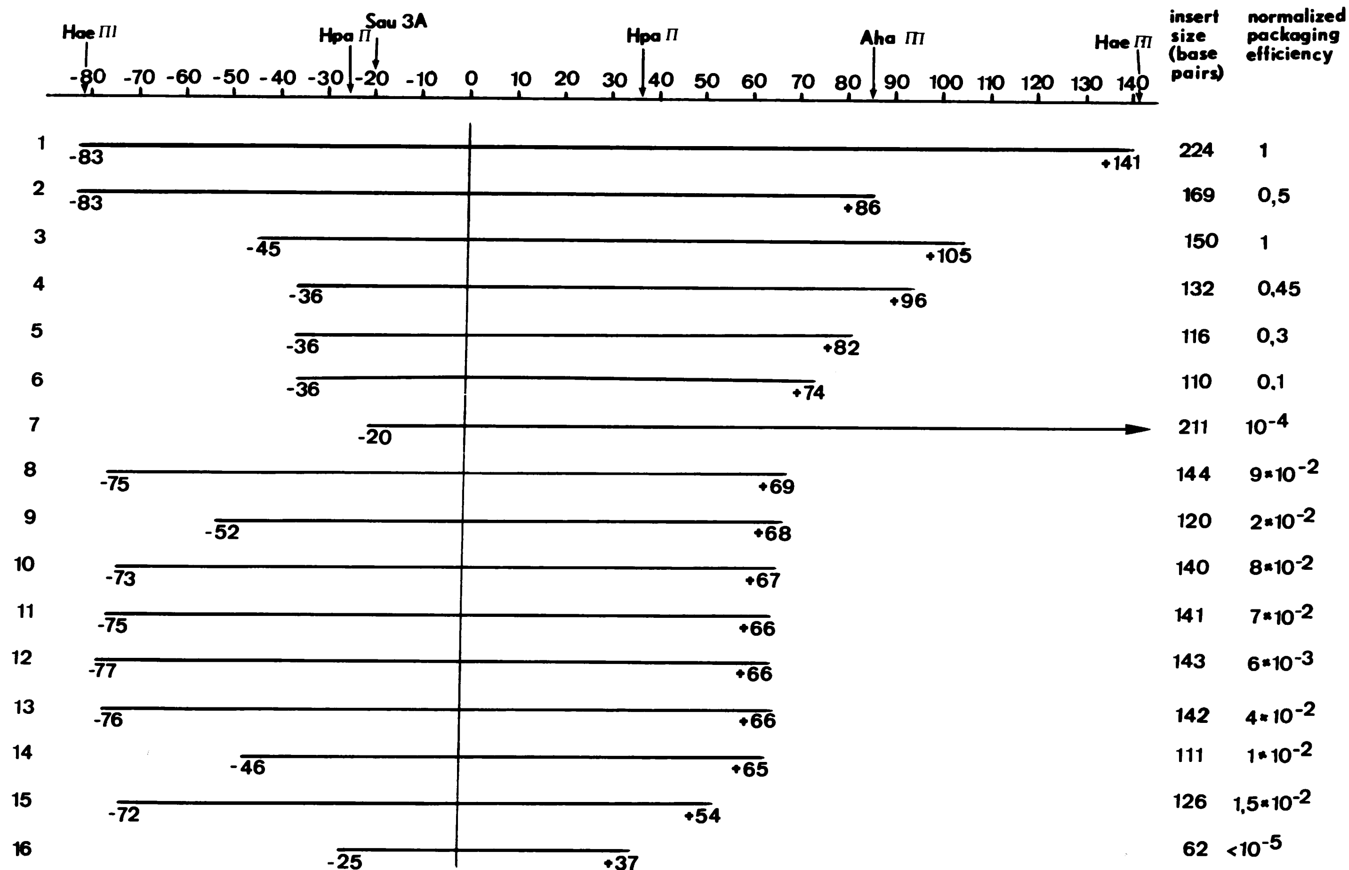

FIG. 2. Restriction enzyme- and *Bal*31-generated *cos*-containing fragments, their precise endpoint mapping, size, and packaging efficiency, normalized to the efficiency of the *Hae* III/*Hae* III fragment.

corporate small restriction fragments containing the λ *cos* region in a plasmid, to introduce it in an *in vivo* packaging strain, and to quantitatively compare the efficiency of packaging/transduction. In a second round the functional sequences were narrowed down by digestion of functional fragments with *Bal*31.

Assay of Defined Restriction Fragments. As a starting and reference cosmid, the 403-base-pair (bp) *Hin*cII fragment of λ DNA surrounding the *cos* sequence was inserted into the *Hin*cII site of pUC7, yielding plasmid–*Eco*RI–*Bam*HI–insert–*Bam*HI–*Eco*RI–plasmid arrangements. Subcloning of the *Hin*cII fragment yielded cosmids with the numbers 1, 2, 7, and 16 (Table 1, Figs. 2 and 3). In the two figures the delineations of the tested insert fragments are listed/plotted against the packaging-transduction efficiency, normalized to the *Hae* III/*Hae* III fragment. The absolute efficiency for this cosmid was 5×10^8 transducing particles and 3×10^9 plaque-forming particles per ml.

In interpreting the *in vivo* packaging data several points have to be considered. (*i*) Since *in vivo* packaging of small cosmids depends on *recA*- (or λ *gam*)-mediated polymerization (unpublished experiments; refs. 12 and 13), the monomer size might affect the degree of this polymerization. For instance, pUC7, which contains the 403-bp *cos Hin*cII fragment (−210 to +193, pcos 0), has a molecular length of about 3.1 kilobase pairs (kb), a dodecamer of which would fit the 37-kb minimal packaging size (14); the corresponding *Hae* III clone (244-bp insert extending from −83 to +141, pcos 1) needs to be polymerized about 13-fold to reach the same size range. The efficiencies of these multimerizations are not known. The difference in packaging efficiency between the *Hin*cII and the *Hae* III cosmid is a factor of 2 in favor of the *Hin*cII fragment. To avoid complications resulting from possible size effects, efficiencies were normalized to the *Hae* III/*Hae* III cosmid, as stated above. (*ii*) Artificial sequence arrangements at the junctions between λ and plasmid DNA could contribute, in a positive or negative way, to the efficiencies with which the cosmids are packaged. (*iii*) Cosmids sometimes rearrange by recombining with intracellular phage DNA. This in some cases resulted in a much too high packaging efficiency. Careful checking of the structure of the packaged versus original cosmid/noncosmid therefore was necessary. (*iv*) Mutations cannot be excluded.

pcos 1 and 2 were clearly positive, and pcos 7 and 16 were clearly negative, although with a reproducibly measurable difference. When the *Sau*3A cloning was repeated, except that the resulting clone was selected as a packageable cosmid by direct transformation and induction of the *in vivo* packaging strain, a stretch of λ DNA extending from the *Sau*3A site located at −135 on the *cce* = 0 map to the *Bam*HI site located in the pUC linker was recovered. This constitutes a positive control for the packaging test of the −20 to pUC linker *Sau*3A fragment.

Cloning and Assay of *Bal*31 Shortened Fragments. Two series of experiments were performed: *Bal*31 digestion of the *Hae* III/*Hae* III and *Hae* III/*Aha* III fragments, each isolated as *Eco*RI fragments by using the *Eco*RI sites located in the plasmid linker. Certain size ranges of trimmed fragments were extracted from acrylamide gels and ligated to *Hin*cII-cleaved pUC7. Transformation was carried out into the *in vivo* packaging strain and packageable plasmids were obtained upon induction of pools of transformants. By this procedure only packaging-positive clones were isolated, but since different size range fragments were used in separate experiments there was not a strong bias towards the bigger and better packageable plasmids. The fact that plasmids with very different packaging efficiencies were recovered justifies the approach.

Mapping was done by using *Sau*3A digestion of the pUC7 hybrids followed by acrylamide gel analysis. The two new fragments stemming from the linker *Bam*HI site to the *Sau*3A site at position −20 and from there to the other linker *Bam*HI site were measured with an accuracy of ±1 bp or better.

The first series of experiments started with the *Hae* III/*Hae* III fragment, which spans the *cos* site asymmetrically. The resulting clones pcos 3, 4, and 5 remain rather asymmetrical, the packaging efficiency decreasing only slightly (or not at all, for

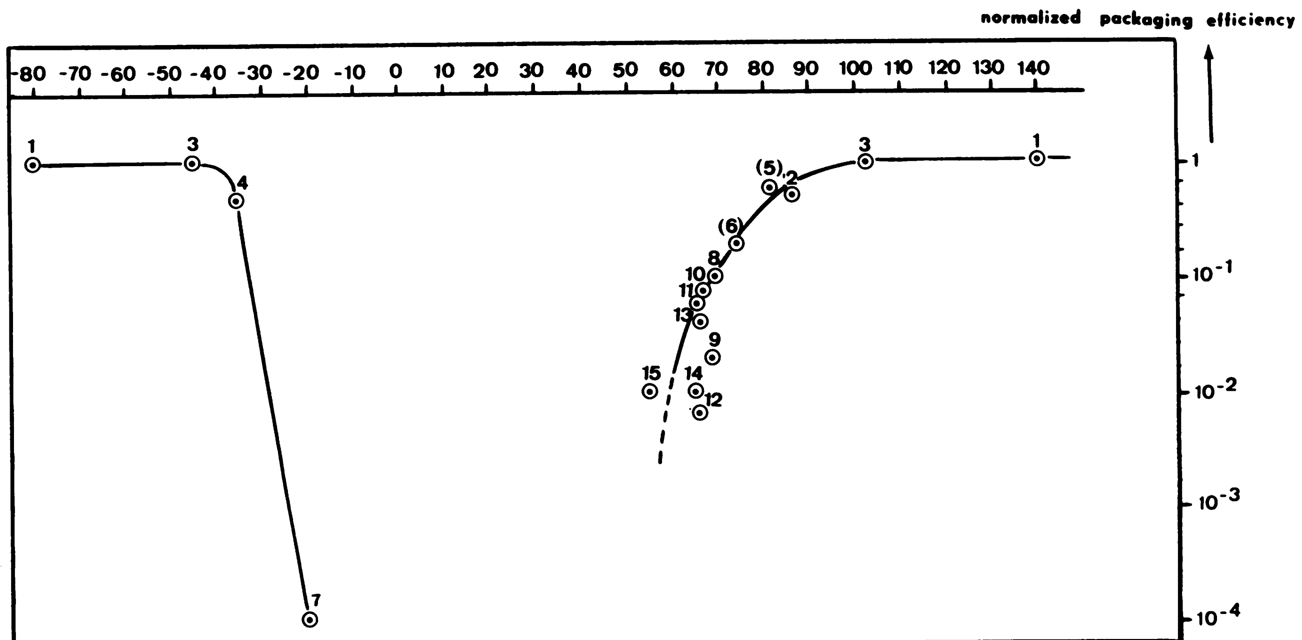

FIG. 3. Plot of the normalized packaging efficiency against the map position of the fragment end(s) determining the efficiency. In most cases missing sequences at one end only could be made responsible for a decrease in efficiency. For clones 5 and 6 a slight correction had to be introduced: the efficiency decrease of clone 4 versus clone 5 was assumed to be due to the −36 rather than −45 end point. The corresponding efficiency reduction was used to "correct" clones 5 and 6 and to attribute their efficiency reduction to a shortening of the right of *cos* area (i.e., left λ end) only. Clone 16, by comparison with clones 7 and 8 to 15, is packaging deficient due to sequences missing on both ends.

Table 2. Densities of particles containing λ DNA segments of various lengths

Restriction enzyme	Length of packaged DNA, kb	Density of particle, g/cm^3
None	48.5	1.50
*Eco*RI	21.2	1.42
*Bam*HI	5.5	1.33
No DNA	—	1.30

pcos 3). In the second set of *Bal*31 digestion products, clones 6 and 8–15 were recovered; in the left end (which is on the right end of the map of mature λ DNA) they remained in the 100% efficiency range (by comparison to clone pcos 3) but they had their endpoints right of *cos* (that is on the left end of the map of mature λ DNA) between +54 (pcos 15) and +74 (pcos 6). The packaging efficiencies of these clones decreased almost in parallel, the lowest amounts being in the order of 1% of the control cosmid. The relatively high efficiency of clone 15 might be due to some specially favorable sequence combination between λ and plasmid sequences or to the removal of some inhibitory sequences. In general the efficiency drops to 10% at position +70 and to 1% at about position +60. Hence the delineation of sequences required for packaging is rather precise. Left of *cce* the limits are around −30 to −36, although fewer clones with specific deletion end points are available. This indicates a strong asymmetry in the packaging-recognition-cleavage substrate, relative to the "cleavage only" substrate.

The fragments conferring a high packaging efficiency can be used to construct cosmids for cloning: they can be moved as *Bam*HI or *Eco*RI fragments. A good candidate would be the fragment of plasmid pcos 3, which as a *Bam*HI fragment is 162 bp in length and as an *Eco*RI fragment is 180 bp. In addition, the junctions in pcos 0 between the λ *Hin*cII fragment and pUC7 DNA digested with *Hin*cII consist of reconstituted *Sal* I sites.

***In vitro* packaging of λ restriction fragments**

To test the specificity of packaging independently of cleavage and injection, λ DNA digested with restriction enzymes was used as a packaging substrate and the DNA content of resulting particles was assayed physically. ^{32}P-Labeled λ DNA undigested and digested with *Eco*RI or *Bam*HI was subjected to *in vitro* packaging, resulting particles were purified, and their DNAs were analyzed. The density of the particles (Table 2) decreases with decreasing weight of the packaged DNA as predicted. DNA containing the leftmost restriction fragment was selectively packaged out of the total restriction enzyme digest (Fig. 4). Double digestion with *Eco*RI and *Bam*HI of the labeled DNA prior to packaging confirmed the conclusion that the leftmost *Bam*HI fragment and not the one containing an *Eco*RI site was the exclusively packaged fragment (data not shown).

DISCUSSION

DNA Sequences Necessary for Packaging. The first assay described in this communication, the *in vivo* packaging of plasmids containing sequences flanking the λ *cos* site, measures, since it is a biological assay, the sum of several steps: binding of packaging proteins, packaging itself, DNA cleavage at *cos*, and injection of the DNA. The second approach, the determination of λ restriction fragments that selectively are packaged *in vitro* into particles, measures binding of packaging proteins and physical packaging only. The first assay establishes that sequences between −36 to −20 and about +80 of λ DNA (see Fig. 1) are required. Whereas the efficiency drops rather sharply upon further deletion of λ-specific sequences originating from the right λ end, it decreases only slowly upon deletion of further left end sequences: removal of sequences between +100 and +70 results in a drop of packaging efficiency by one order of magnitude (Figs. 2 and 3); packaging efficiencies are 1% when the cloned fragments extend only to coordinates around +60. An explanation for this difference in the slopes depends on future experiments.

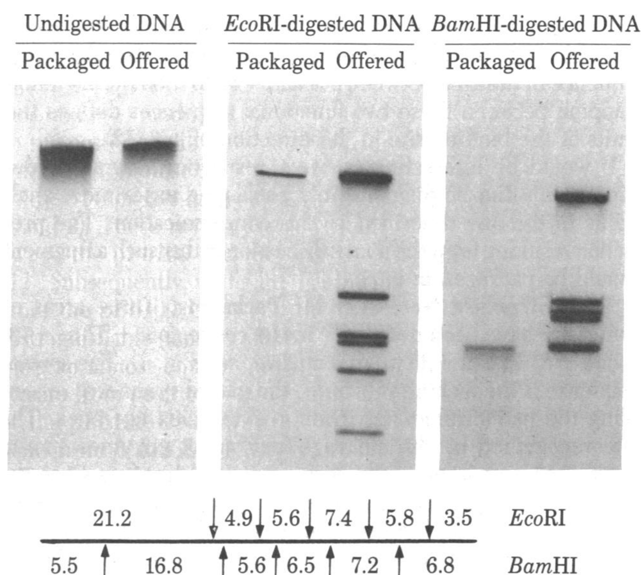

FIG. 4. Radioautogram of λ DNA before and after *in vitro* packaging. Undigested and *Eco*RI- and *Bam*HI-digested radioactive DNA was subjected to *in vitro* packaging and the DNA of the resulting purified particles was analyzed by gel electrophoresis in comparison to the DNA offered. Lengths of restriction fragments are given in kb.

The second experimental approach, the *in vitro* packaging of restriction fragments, showed that only fragments originating from the left end of mature λ DNA are incorporated into phage heads.

A comparison of the two assays and their results suggests that the requirement for sequences on the right end of λ DNA is for the DNA cleavage or injection step or both. Feiss *et al.* (8, 9) found, using the λ–21 system, specificity for *cos* cleavage only at the first of two consecutive *cos* sites; for the nicking of the terminal *cos* site binding of the nicking/packaging enzymes at the initial *cos* site is sufficient. This leaves the injection step [or the correct positioning of the right λ terminus in the tail (15)] as the one requiring the sequences at the right terminus. This would predict the −8 to −36 sequence of phage 21 to be like that in λ. An alternative explanation is that the specificity for nicking the *cos* sequence extends beyond the symmetrical region at least into the right λ arm. Our data on the extent of sequence information required agree with those of Miwa and Matsubara (7).

The necessity for proper packaging of sequences on the left end of λ DNA, outside of the imperfect symmetrical *cce* sequence, was predicted from several lines of argumentation: (*i*) linear mature λ DNA with cohesive ends but lacking the *cos* site is packaged *in vitro* (5); (*ii*) packaging, as opposed to terminase cleavage, is a polar process (3) that necessitates polar binding of the DNA to the packaging proteins and particle precursor; (*iii*) phage 21 DNA, which has cohesive ends identical to λ DNA, is packaged *in vitro* (5) and *in vivo* (8, 9) to only a greatly reduced extent. By examining the λ–21 hybrids Feiss *et al.* could narrow down the specificity to sequences close to the left DNA end. Final proof comes from recent data differentiating by physical separation sequences to be cleaved by ter-

minase and sequences required for the binding of the packaging/cleavage complex (10, 16, 17). Our data localize this latter sequence to the area between *cce* and +60 to +80 bp. Deletion mapping between these two functional sequences defines the limits of the binding site in the direction of *cce* (12).

It would be interesting to use a restriction fragment containing only the binding site in a packaging experiment analogous to the one described in this communication. The prediction resulting from the above discussion is that such a fragment should be packaged *in vitro*.

DNA Structure Necessary for Packaging. Three areas of symmetry have been noted (18) in the *cos* region: −57 to −43, −8 to +7, +26 to +35 (each inclusive, see Fig. 1). The second sequence is the nicking substrate, the partial symmetry resembling the palindromic restriction enzyme cleavage sites. The site recognized by the cleavage/packaging enzymes should consist, at least in part, of nonsymmetrical stretches since the polarity of the packaging process dictates some polar recognition sequence, as discussed above. The role (if any) therefore of the +26 to +35 perfect symmetrical sequences remains obscure. Interestingly, the even longer symmetrical sequence at −57 to −43 lies outside the sequence found necessary for packaging/cleavage at the extreme right end of λ DNA. All three areas of symmetry are conserved without a single base pair change in the lambdoid bacteriophage φ80 (ref. 16; T. Miwa, personal communication).

Packaging Mechanism. The packaging of a restriction fragment originating from the left end of mature λ DNA demonstrates the polarity of packaging in its purest form. The fact that a small fragment of λ DNA is sufficient for packaging it into a λ head also demonstrates that the requirement of a certain minimal size as shown by helper packaging experiments (14) and as used in the cloning of large DNA fragments (19) is for the cleavage stage, not for the packaging step *per se*. Concomitant with packaging, and dependent on it, the λ preheads expand from the prehead size to the size of the mature head (1). This size increase, amounting to as much as a doubling in volume, could theoretically be used to suck the DNA, which already is bound to the head entrance, into the head ["spaghetti model" (20, 21)]. This model would predict that the head expansion directly precedes DNA packaging and that a small DNA fragment also is packaged in a mature-size head. D protein (second major head protein, binds only to enlarged heads) binding assays and electron microscopical analysis of the *Bam*HI-fragment-containing particles, however, have shown that the capsids remain in the prehead size (unpublished data). They are even locked in this structure: no artificial expansion could be demonstrated with chemicals that expand normal preheads (22), which points to the DNA as somehow stabilizing the particle, possibly by neutralizing the charge in the interior of the particle. Particles containing the 21.2-kb *Eco*RI fragment, however, resemble normal phage heads.

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