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Construction of Bacteriophage φX174 Mutants with Maximum Genome Sizes

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The bacteriophage φX174 strain *ins*6 constructed previously was used to investigate the maximum genome size that could be packaged into the icosahedral phage without concomitant loss of phage viability. The J-F intercistronic region of *ins*6, which already contains an insert of 117 base pairs with a unique *Pvu*II site, was enlarged further by insertion of *Hae*III restriction fragments of the plasmid pBR322 into that *Pvu*II site. By using a biochemical approach for the site-specific mutagenesis as well as selection of mutant genomes, a series of mutants was isolated with genomes of up to 5,730 nucleotides, 6.4% larger than that of the wild-type DNA. Phages with genomes larger than 5,550 nucleotides were highly unstable and were rapidly outgrown by spontaneously occurring deletion mutants. The data predict that genomes of at least 6,090 nucleotides could be constructed and, most likely, packaged, but the resulting phages would not grow well. We speculate that the volume of the phage capsid is not the limiting factor of genome size or is not the only limiting factor.

The genome of bacteriophage φX174 codes for 10 genes and four transcribed but untranslated intercistronic regions with a total of 5,386 nucleotides. Three of the genes are overlapping (11), several give rise to more than one gene product through internal start sites (13), and more than one regulatory role has been identified or suggested for each of the three large intercistronic regions (3, 5, 10, 14). It appears, therefore, that conserving the genome size was a crucial factor in the evolution of phage φX174 and other closely related single-stranded bacteriophages. There are several reasons that, individually or in combination, may have served as the evolutionary driving force, the most likely of which is a size restriction imposed by the icosahedral capsid. Recently, methodologies have become available to package into the φX174 capsid in vitro (1) or in vivo (15) plasmid DNAs with cloned φX174 fragments containing the origin of single-stranded-DNA synthesis. Plasmids varying in length between 3,300 and 5,700 base pairs (bp) were tested for their ability to be packaged in single-stranded form into phage capsids, and the maximum size for a plasmid with this ability was reported to be between 5,580 and 5,700 nucleotides (15). Müller and Wells (7, 8) have previously reported that 70% of the intercistronic region between genes *J* and *F* is nonessential for growth under laboratory conditions and could be either deleted or disrupted by insertion of extraneous DNA. The largest insert obtained in those experiments was 163 bases, thus demonstrating that the phage genome could be enlarged to at least 5,549 nucleotides without severe effects on phage growth.

Since these data suggest that the φX174 capsid can package more DNA than that contained in the wild-type (wt) genome, we wondered whether the unusual genome arrangement of the single-stranded DNA phages was a result of spacial constraints or due to other factors. Herein, we report on the maximum amount of DNA that can be added to the φX174 genome without loss of phage viability. By inserting additional restriction fragments of the plasmid pBR322 into the J-F intercistronic region of one of the previously constructed φX174 insertion mutants, we constructed viable, although very growth-inefficient, phage particles with genome sizes of up to 5,730 nucleotides, ca. 6.4% more than the wt phage. Phages with larger genomes were also constructed but could not be grown to sufficiently high titers for biochemical examination.

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MATERIALS AND METHODS

Bacterial and phage strains. Bacteriophage φX174 strain *am*3 is referred to herein as wt. Strains *del*3 (φX174 J-F *del*3), *ins*6 (φX174 J-F *ins*6), and *ins*11 (φX174 J-F *ins*11) also contain the *am*3 mutation and were described in detail previously (7, 8). Strain *ins*6 contains an insertion of 117 nucleotides (pBR322 *Alu*I fragments 13 plus 16 plus 12) into the *Hae*III site of its J-F intercistronic region, which was confirmed by DNA sequencing (Müller, Cobourn, and Turnage, unpublished data). The *Pvu*II site between pBR322 *Alu*I fragments 16 and 13 served as the insertion site in this work, and strain *ins*6 is referred to as the parental strain. *E. coli* HF4714 was the permissive host (2), and *E. coli* C was the nonpermissive host.

Chemicals and enzymes. Buffers and media were as previously described (7). All enzymes were purchased from Bethesda Research Laboratories, and restriction and ligation buffers were either supplied by the manufacturer (core buffer) or prepared by Bethesda Research Laboratories specifications.

Isolation and purification of restriction fragments. pBR322 DNA (1.5 mg) was purified by the method of Hardies et al (4) and digested to completion with restriction endonuclease *Hae*III. The fragments were isolated either by high-pressure liquid chromatography fractionation with RPC-5 analog as resin (16) or by electrophoresis on discontinuous polyacrylamide slab gels. After the gels were stained with ethidium bromide, the DNA bands were visualized under UV light, cut out of the gel, placed into dialysis bags with 1 ml of Peacock buffer per band (9), submerged in the same buffer, and electroeluted for several hours at 150 V. After electroelution, DNA fragments were extracted with phenol and ether and dialyzed against water.

Insertion of *Hae*III fragments into the *Pvu*II site of φX174 *ins*6 RF DNA. Ligation reactions generally contained 0.5 to 1 μg of *Pvu*II-linearized, replicative-form (RF) DNA and an approximate molar excess of 40-fold of *Hae*III ends over *Pvu*II ends, unless otherwise stated. The reactions were carried out in ligase buffer in a total volume of 20 μl at 14°C for 16 h with ca. 2 U of T4 DNA ligase per μg of RF DNA. The extent of ligation was checked by agarose gel electrophoresis. The ligation mixture was then subjected to the enzymatic screen by adding 4 volumes of restriction buffer and excessive amounts of restriction endonucleases *Pvu*II and *Pst*I or *Sst*II (each 20 U/μg of RF DNA). For double digestion with *Pvu*II and *Pst*I, the *Pvu*II restriction buffer was used; *Pvu*II and *Sst*II double digests were in core buffer (Bethesda Research Laboratories). Digests were for 6 h at 37°C.

Production of miniprep lysates and miniprep RF DNA. Plaques obtained after transfection of *E. coli* HF4714 cells were propagated to 6-ml starter lysates as described previously (6). *E. coli* HF4714 (15 ml; A_{550} = 0.35) was added, and the cultures were incubated at 37°C until lysis. A 10-ml sample was removed and stored over chloroform (miniprep lysates). An *E. coli* C culture (30 ml; A_{550} = 0.5) was added to the remaining 11 ml of lysate for the preparation of miniprep RF DNA as described previously (6).

Other methods. Transfection of $CaCl_2$-shocked *E. coli* HF4714 cells and gel electrophoresis were as previously described (4).

RESULTS

Enzymatic construction and selection of mutants. A series of φX174 mutants with deletions of or insertions into the J-F intercistronic region was constructed previously (7, 8). One of these mutants, φX174 *ins*6, contains an insert of 117 bp and was the parental strain for the insertion experiments described herein. This strain was chosen because it contains a unique *Pvu*II restriction site in the insert sequence and because its total genome size is 46 nucleotides shorter than that of strain φX174 *ins*11 (insert size, 163 bp), the largest viable strain previously constructed. Consequently, insertions of up to 40 bp into the *Pvu*II site of strain *ins*6 should be possible, and fragments of that length could be used as controls in insertion experiments with larger fragments.

The rapid enzymatic procedure used to construct and select for insertion mutants was essentially as described previously (6). The general approach consisted of 4 steps: (i) treatment of the circular double-stranded φX174 *ins*6 RF DNA with *Pvu*II, (ii) ligation in the presence of one or more *Hae*III fragments of pBR322 DNA, (iii) treatment of the ligation products with *Pvu*II and *Pst*I, and (iv) transfection of $CaCl_2$-shocked *E. coli* cells. In step i, linear blunt-ended parental genomes were produced, which could be recircularized in step ii, whereby the *Pvu*II site was regenerated. If blunt-ended restriction fragments were present in the ligation reaction and became inserted into the phage genome, the *Pvu*II site was lost. This fact was used in step iii to specifically inactivate parental genomes which had one site each for *Pvu*II and *Pst*I, whereas mutant genomes were cleaved only once, at the *Pst*I site. The linear but genetically intact mutant genomes gave rise to plaques after transfection in step iv, whereas the fragmented parental genomes were noninfective. Depending on the completeness of the enzymatic screen in step iii and for reasons discussed later, the percentage of mutants among the plaques obtained varied from 70 to 100%.

Shotgun insertion of pBR322 fragments. The feasibility of inserting additional DNA into φX174 strain *ins*6 was tested by ligating *Pvu*II-linearized parental RF DNA in the presence of all 21 *Hae*III fragments of pBR322 DNA, which vary in length from 7 to 587 bp (12). Insertion of fragment 21, 20, 19, or 18 would increase the genome size of strain *ins*6 to not more than 5,524 nucleotides, which is still smaller than that of strain φX174 *ins*11 (5,549 nucleotides) (8). Insertion of any of the 17 larger *Hae*III fragments would exceed the genome size of strain *ins*11. After screening and transfection, 55 plaques were picked at random, and phage lysates and RF DNA were prepared by the miniprep procedure as described above. Double digestion of these DNAs with *Pst*I and *Pvu*II revealed that 48 of the 55 isolates were mutants as defined by absence of the *Pvu*II site (data not shown). Further restriction analysis with restriction endonucleases *Hae*III, *Hha*I, and *Hpa*II clearly identified two of these as insertion mutants, most likely containing pBR322 *Hae*III fragment 19 as the inserted fragment in both cases (data not shown). More surprisingly, 31 of the remaining 46 strains were clearly identified as deletion mutants, whereas the other isolates appeared to be mixtures of two or more insertion mutants, deletion mutants, or both (discussed below).

Determination of the sizes of the deletion mutants revealed that only one had retained part of the original parental insert sequence, whereas in all other strains the entire parental insert plus nucleotides from the adjoining J-F intercistronic region had been deleted. In 29 cases, these deletion mutants appeared to be identical in size to the previously constructed strain φX174 *del*3, which lacks all 27 nucleotides between the stop codon of gene *J* and the ribosome-binding site of gene *F* (8). There are basically two mechanisms by which the deletion mutants could have been generated and selected for in this experiment. The first possibility is a contamination of the reaction mixture in step i or ii with exonucleases which nibbled off the *Pvu*II ends of the linear parental RF DNA. Such molecules, once recircularized in step ii, would have been equally selected for in step iii. The other possibility is that, in fact, all mutant plaques originated from cells transfected with an insertion mutant. In most cases, the insertions may have resulted in a very growth-inefficient strain which was rapidly outgrown by spontaneous deletion mutants during the many phage generations required for growth from a single transfected cell to a plaque and from there to a high-titer phage lysate. The following experiments (summarized in Table 1) present strong evidence for the second mechanism.

Stepwise enlargement of the phage genome. Since no mutants with genome sizes exceeding that of strain *ins*11 were isolated in the preceding experiment, purified *Hae*III fragments or pools of purified fragments were used in three successive experiments to test the upper insertion limit. A summary of the results is shown in Table 1. In our experience, the yield of plaques by transfection of $CaCl_2$-shocked *E. coli* cells with linear φX174 RF DNA is generally ca. 10^4 plaques per μg of DNA (6), independent of the type of restriction enzyme used for linearization of the circular genome. We do not see a deviation from this with *Pvu*II-linearized strain *ins*6 RF DNA, which suggests that the DNA is relatively undamaged. This is further supported by religating this DNA into the circular form in the absence of any *Hae*III fragments and selecting for mutants with damaged *Pvu*II sites with a *Pvu*II and *Pst*I double digest. Generally, at least 20% of the DNA was recircularized during the ligation reaction as judged by agarose gel electrophoresis (data not shown). Since circular RF DNA is 100-fold more active in transfection than linear DNA, over 99% of the religated

TABLE 1. Summary of insertion experiments for screened, ligated RF DNA[a]

Insert size (bp)[b]	Yield of plaques (%)	No. of insertion mutants	No. of deletion mutants	No. of parental strains	No. of mixtures
	100				
0	1–3	0	2	5	0
18, 21	20	7	0	0	0
51–124	10	8	1	0	0
51–213	3.5	14	3	6	2
234	0.3	0	6	5	0
267	0.2	0	5	3	0
51–213, 234	0.5	3[c]	5	3	2[c]
51–213, 267	0.6	2[c]	6	15	1[c]
434	0.1	0	0	1	0
458	0.3	1[c]	5	3	0
504	0.3	1[c]	5	3	0
587	0.3	1[c]	7	3	0

[a] The total number of plaques analyzed in each experiment equaled the sum of mutants, parental strains, and mixtures. The yield of plaques for *Pvu*II-cleaved parental RF DNA was 100%. DNA was screened by treatment with *Pvu*II and *Pst*I as described in the text.

[b] Insert size refers to the length of the fragment(s) added to the linear RF DNA in the ligation reaction.

[c] Inserts were smaller than 230 nucleotides.

DNAs must have contained intact *Pvu*II sites, as they were inactivated by the subsequent enzymatic screen.

The 1 to 3% yield of plaques remaining after the enzymatic screen was mostly due to RF DNA molecules that had escaped one of the screening enzymes (5 of 7 randomly picked plaques were wt). The presence of pBR322 *Hae*III fragments during the ligation reaction increased or decreased the recovery of infective genomes in this procedure, depending on the size of the fragment(s) used for insertion. Since it had been shown in the first experiment that insertion of *Hae*III fragment 18 into φX174 *ins*6 RF DNA could be tolerated, the number of plaques obtained with the screened ligation mixture containing fragments 18 and 21 served as a control for the efficiency of construction of viable insertion mutants in the ligation reaction (20%). Further increases in the insert size resulted in concomitant decreases in the yield of viable plaques. Up to an insert size of 213 nucleotides, only a fivefold reduction was observed, whereas any size fragment of 234 bp or larger resulted in an overall 70-fold reduction. The 10-fold reduction between the sizes of 213 and 234 bp, as well as the following lower plateau, indicated a narrow range for the maximum sized genome that could be constructed and isolated as a viable phage. It is important that this lower plateau was below the yield of plaques which was generally obtained with the control sample (no added fragments). This indicated that inserts of 234 bp and larger result in phage genomes that are either nonviable or grow so poorly that they are rapidly outgrown by spontaneously occurring deletion mutants.

A restriction analysis of RF DNA obtained from randomly picked plaques supported this conclusion. Insertions of fragments up to 213 bp yielded phages with increased genome sizes (Table 1). Beyond this size, mutants containing the complete fragment could not be isolated. On the other hand, an increased fraction of deletion mutants was obtained concomitant with the increase in insertion fragment size.

The best demonstration for an insertion limit of ca. 234 bp was obtained by adding to the ligation reaction the successfully used fragments ranging between 51 and 234 bp (each in twofold molar excess over RF DNA) and, in addition, a 234- or 267-bp fragment (both in 50-fold molar excess over RF DNA). In both cases the yield of plaques was two to three times higher than with either of the two larger fragments alone. However, the insertion mutants that were obtained contained inserts of below 230 bp, although insertion of the larger fragments was statistically favored and biochemically feasible.

Isolation and restriction mapping of the largest insertion mutants. In the foregoing experiments we observed an increase in the percentage of smaller plaques parallel to the increase of fragment size used in the ligation. To isolate the largest mutant possible, the 23 smallest plaques, which were obtained when fragments ranging from 51 to 213 bp were used for insertion, were picked. Restriction analysis of miniprep RF DNAs with *Hae*III revealed that 20 of the RF DNAs stemmed from insertion mutants, and 3 were from deletion mutants. Some of these digests are shown in Fig. 1. Only 9 of the 11 typical *Hae*III fragments of φX174 wt are shared by all of these strains. The *Hae*III site between fragments 5 and 8 was used for insertion (*ins*6 and *ins*11) or deletion (*del*3) of DNA in the original construction (7), and the resulting fusion fragment in the parental strain *ins*6 contained the *Pvu*II site. Thus, the size of the 5-×-8 fusion fragment (the × indicates additional DNA inserted or deleted) was characteristic for each mutant strain and could be determined graphically on such gels with an accuracy of ±5 bp. The four new isolates (Fig. 1) (and 7 others not shown) had insert sizes exceeding that of strain *ins*11. Strain *ins*6003 (Fig. 1, lane a) contained a single insert of ca. 64 bp (possibly *Hae*III fragment 15) and had a total genome size of 5,567 nucleotides. Strains *ins*6004 (lane b), *ins*6006 (lane c), and *ins*6007 (lane d) had insertions of two restriction fragments with a regenerated *Hae*III site between them. Their 5-×-8 fusion fragment was therefore cleaved in this *Hae*III digest, and two subfragments appeared. Their size was used for a

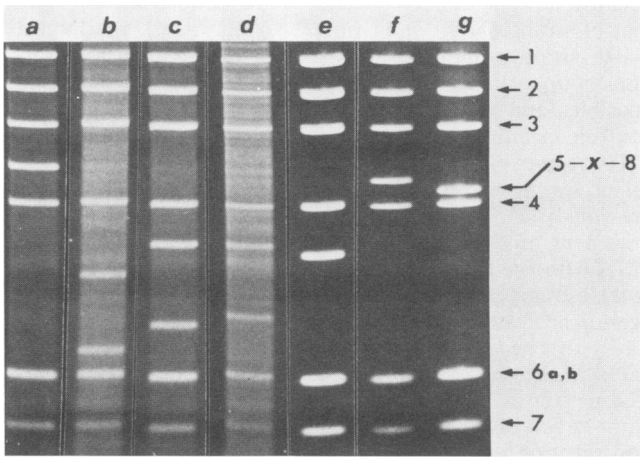

FIG. 1. *Hae*III digest of mutant RF DNAs. CsCl-purified RF DNA of φX174 strains *del*3 (lane e), *ins*11 (lane f), and *ins*6 (lane g) was digested with restriction endonuclease *Hae*III and electrophoresed on 4% polyacrylamide gels. With the exception of fragments 5 and 8, all *Hae*III fragments were identical to those in a *Hae*III digest of φX174 wt (Fig. 2) (bands 9 and 10 could not be observed in this gel). The *Hae*III site between fragments 5 and 8 was destroyed by deletion (*del*3) or insertion (*ins*6, *ins*11) of DNA, resulting in a fusion fragment of variable size (5-×-8). The position of the 5-×-8 fragment band of strain *ins*6 is indicated by the arrow. The digests of miniprep RF DNAs of four new mutants are shown in lanes a (*ins*6003), b (*ins*6004), c (*ins*6006), and d (*ins*6007).

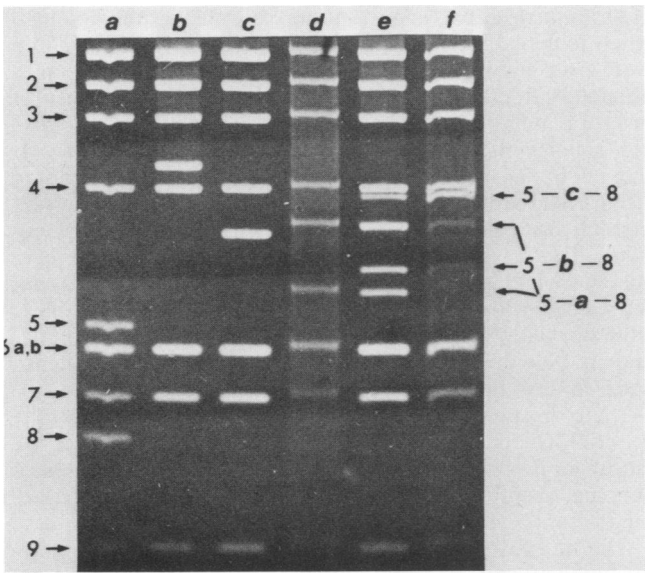

FIG. 2. *Hae*III digest of different preparations of ϕX174 *ins*6007 RF DNA. CsCl-purified RF DNAs of ϕX174 wt (lane a), *ins*11 (lane b), and *del*3 (lane c) were digested with restriction endonuclease *Hae*III and run as controls on a 4% polyacrylamide gel. Three different RF DNA preparations of strain *ins*6007 were run in lanes d, e, and f. The original miniprep RF DNA was digested with *Hae*III and run in lane d. The original miniprep lysate (1 ml) was added to 5 ml of *E. coli* HF4714 (tryptone-yeast extract; A_{550} = 0.3), and 15 ml and then 80 ml of host culture (A_{550} = 0.5) were each added after lysis. The final phage lysate was used for the growth and preparation of CsCl-purified RF DNA, which, after *Hae*III digestion, was run in lane e. Phage from an individual plaque was grown to a miniprep lysate, 1 ml of which was used for the preparation of a second miniprep lysate and miniprep RF DNA (lane f). For identification of bands, see the legend to Fig. 1. Fragment 5-×-8 of the original *ins*6007 isolate is referred to as fragment 5-a-8 and contained a *Hae*III site and was cleaved into two fragments, both migrating between fragments 4 and 6a, b (lane d). Loss of DNA in the smaller of these fragments led to fusion fragment 5-b-8, which still had an internal *Hae*III site (lane e) and was therefore cleaved into two fragments (the two weak bands in lane f). Further loss of DNA included the internal *Hae*III site and led to fusion fragment 5-c-8 (lanes e and f).

preliminary identification of the origin of the insert. For strain *ins*6007, this appeared to be *Hae*III fragments 12 and 11a or 11b. The identity of *Hae*III fragment 12 was confirmed by the presence of the unique *Bam*HI site of pBR322 (not shown). Strain *ins*6007 had a genome size of 5,730 ± 5 nucleotides and together with strain *ins*6008 was the largest mutant isolated in this work (*ins*6008 had the same *Hae*III restriction pattern as *ins*6007). These data suggest that the insertion limit for strain *ins*6 is between 226 and 234 bp; i.e., the maximum amount of DNA that can be stably packaged into the ϕX174 capsid appeared to be between 5,725 and 5,735 nucleotides.

Instability of insertion mutants. During further work with our newly constructed phage strains, we noted repeated difficulties in maintaining pure phage cultures upon subculturing of the original isolates. This problem was further investigated by preparing two separate phage subcultures of strain *ins*6007, isolating RF DNA, and analyzing its composition by restriction digests (Fig. 2). *Hae*III-digested RF DNA produced from the original miniprep *ins*6007 lysate is shown in Fig. 2, lane d. This lysate (1 ml) was used to produce a 100-ml phage stock in three successsive growth cycles (see the legend to Fig. 2). In each step, clearing of the culture occurred within 30 min. Assuming a phage generation time of 15 to 20 min (8), this phage culture had undergone three to six additional generations. *Hae*III-digested RF DNA from this lysate (Fig. 2, lane e) showed the appearance of two additional bands which were clearly the result of the two 5-×-8 fusion fragments. The RF DNA from a regular miniprep procedure that was started with a single plaque obtained after titrating the original miniprep lysate is shown in Fig. 2, lane f. Assuming that this plaque had originated from a single phage, the number of phage generations between plating this phage and producing the miniprep RF DNA should be similar to the original miniprep RF DNA of *ins*6007 (Fig. 2, lane d). Yet, the characteristic fusion fragment (Fig. 2, lane d) (5-a-8) was almost completely replaced by the shorter fragment 5-c-8 (Fig. 2, lane f). The previous digest (Fig. 2, lane e) showed the 5-c-8 band also, but it was the least prominent fusion fragment in this lane. A new intermediate-sized fusion band (5-b-8), which appeared to be present in similar concentration as fragment 5-a-8, could be observed here. We interpret these findings as indications of an evolution of phage strain *ins*6007 from the large genome size of 5,730 nucleotides to an intermediate size of ca. 5,640 nucleotides and then to a more stable size of 5,460 nucleotides. The appearance of these smaller genomes most likely reflects outgrowth of the original *ins*6007 strain by more growth-efficient spontaneous deletion mutants.

This phenomenon could be due to either the insert size or sequence. In the latter case, other mutants with different insert sequences might be more stable or unstable. If this instability is strictly due to the insert size, however, one may expect an inverse relation between insert size and stability of the phage strain. We have tried to demonstrate this by preparing, simultaneously, minipreps of eight phage strains with insertions in the J-F intercistronic region ranging from −27 to +340 nucleotides. The minipreps were started with 1

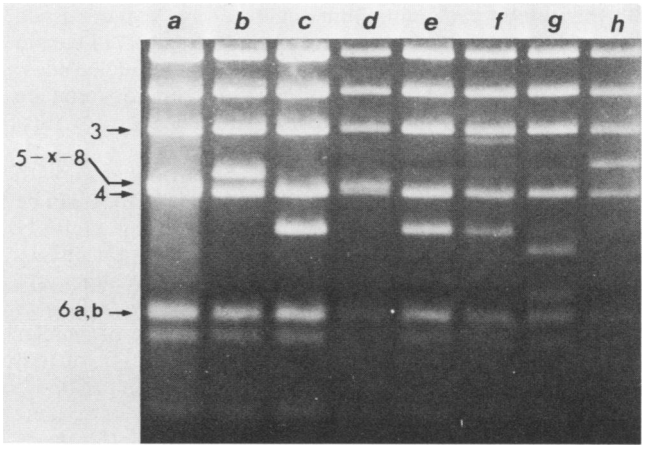

FIG. 3. Analysis of the genome composition of miniprep RF DNAs. All RF DNAs were prepared by the miniprep procedure, digested with restriction endonuclease *Hae*III, and electrophoresed on 1.4% agarose gels. Stock strains *ins*6 (lane a), *ins*11 (lane b), and *del*3 (lane c) showed their characteristic fragment 5-×-8. The other RF DNAs were from minipreps originating from mutants *ins*6008 (lane d), *ins*6006 (lane e), *ins*6005 (lane f), *ins*6004 (lane g), and *ins*6003 (lane h). The fusion fragment in lane d migrated near *Hae*III fragment 4 and was ca. 265 bp shorter than expected from strain *ins*6008. A fragment 5-×-8 identical to the one in lane c appeared alone in lane e and along with the expected fusion fragments in lanes f to h.

ml of lysates (ca. 10^{10} PFU/ml) which had been checked for purity by preparing RF DNA and analyzing *Hae*III digests as before. These starter lysates contained phage which had undergone variable numbers of growth cycles, however. The first three strains (Fig. 3, lanes a, b, and c) were stock cultures from our laboratory which had been subcultured many times. Strains *ins*6003, *ins*6004, and *ins*6006 (Fig. 3, lanes h, g, and e, respectively) were subcultured only once with two growth cycles (two to four generations). The original miniprep lysates were used for strains *ins*6008 (Fig. 3, lane d) and *ins*6005 (Fig. 3, lane f). The composition of each phage culture after this miniprep growth is shown in Fig. 3.

The first three stock strains (lanes a to c) showed their characteristic 5-×-8 fusion fragment only, whereas all other strains showed a displacement of their characteristic 5-×-8 fusion fragment by a new and much smaller fusion fragment; i.e., the original insertion mutant was outgrown to some extent by a deletion mutant. The two largest strains, *ins*6008 (+340 nucleotides) and *ins*6006 (+320 nucleotides), were completely outgrown, the former by a strain which was still ca. 75 nucleotides larger than ϕX174 wt and the latter by one which appeared identical in size to *del*3 (Fig. 3, lane d; 27 nucleotides smaller than ϕX174 wt). It was this strain which also appeared in the *ins*6005, *ins*6004, and *ins*6003 lysates, but with decreasing concentrations. Thus it appears that all mutants with genomes larger than strain *ins*11 (+163) were rapidly outgrown by spontaneously occurring deletion mutants. This instability seems to have been directly proportional to the increase in genome size.

DISCUSSION

We have investigated the largest ϕX174 genome that could be constructed and maintained in a viable form, by further enlarging the J-F intercistronic region of this phage, thus obtaining a series of mutants with genomes of up to 5,730 nucleotides, which is the largest ϕX174 genome available to this date. Although this apparent size limit agrees fairly well with the in vivo packaging limit suggested by Van der Ende et al. (15), who were able to package into the ϕX174 capsid plasmids with 5,580 nucleotides but not 5,700 nucleotides, our data suggest that even larger ϕX174 genomes can be made and packaged into its capsid. The evidence for this, although largely indirect, is as follows.

There is no question that ϕX174 bacteriophages with genomes of up to 5,730 nucleotides are viable, as they can be isolated and their genome size can be determined fairly accurately by restriction digests. It is also clear that phages of that size have some problems, since their plaques are smaller and they cannot be grown into high-titer lysates without the appearance of contaminating phages of smaller size. The most logical explanation is that insertion of that much DNA into the J-F intercistronic region has created a relatively large target size for either spontaneous or specific mutagenesis and that deletion of some or all of the insert DNA apparently generates a more growth-efficient phage. We have shown for strain *ins*6007 that a phage lysate of at least 90% purity was only ca. 50% pure after just three to six more phage generations.

When we attempted to exceed a genome size of 5,730 nucleotides by inserting fragments longer than 225 bp, the yield of viable phage was reduced as compared with yields in reactions in which fragments of smaller acceptable size were offered, and a higher percentage of deletion mutants was obtained. In several instances these deletions were not complete, and the mutants still had a larger genome than that of strain *ins*6. Most significant here are the results obtained when four restriction fragments ranging from 434 to 587 bp were used for insertion into strain *ins*6. Of the 30 plaques obtained after transfection, 3 still showed the presence of insertion mutants with genomes exceeding that of strain *ins*6 but smaller than those obtained if the complete *Hae*III fragments had been retained. This implies that these three strains were deletion mutants originating from insertion mutants with genomes of 6,090, 6,007, and 5,961 nucleotides. Thus, we can not report the upper size limit of a genome which can be packaged into the ϕX174 capsid, but we speculate that it is at least 6,090 nucleotides. It is clear from our data that such phages have only a limited viability and are only reasonably stable once the genome approaches a size of less than 5,735 nucleotides.

Up to this point our data are not surprising and support the generally accepted idea that the physical constraints of the icosahedral phage capsid have limited the size of the phage genome, thereby possibly forcing the evolution of overlapping essential genes.

The outgrowth of larger insertion mutants by spontaneously occurring deletion mutants is also not surprising; however, the type and size of deletions are unexpected. There are two findings that point to a mechanism of specific rather than random mutagenesis. First, we have shown that mutants with genomes larger than 5,549 nucleotides (the size of strain *ins*11) are very unstable, whereas genomes below that size appear as stable as does wt. If the length of the insert was the only determining factor, then strain *ins*11 as well as all of the insertion mutants should be unstable and be outgrown by deletion mutants, and strain *ins*6003 (5,567 nucleotides) should only be 10% more sensitive than *ins*11. Since we have not observed any deletion mutants in our phage stocks in those strains with genomes smaller than 5,550 nucleotides, but relatively rapid occurrence of deletion mutants in those strains with genomes larger than that, there must be a stability plateau for ϕX174 genomes at about 5,550 nucleotides.

Second, it appears that the deletion process is not a random event; otherwise, these deletions should occur in all ϕX174 strains larger than *del*3, and it would be very unlikely that the same type of deletion mutant would occur repeatedly. Instead we found no deletion mutants for genomes smaller than 5,550 nucleotides, and we found defined-size deletions for the larger ones (strains *ins*6007 and *ins*6008; Figs. 2 and 3). Most surprisingly, the deletion mutants obtained in over 90% of all cases were not a reduction of the genome to the original wt size but a further reduction by 27 nucleotides, thus removing all nonessential nucleotides from the J-F intercistronic region (strain *del*3). This implies a nonrandom, progressive mechanism of deleting nucleotides, which seems to be induced by an oversized genome. We speculate that genomes of up to 5,550 nucleotides are adequately protected from nucleases by capsid and scaffolding proteins during morphogenesis and encapsidation but that genomes beyond that size are sensitive to nucleolytic attack, even though they could be packaged.

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