J. Mol. Biol. (1988) **204**, 663–674

Variable Electrostatic Interaction Between DNA and Coat Protein in Filamentous Bacteriophage Assembly

David H. Rowitch, Gary J. Hunter and Richard N. Perham

Department of Biochemistry
University of Cambridge
Tennis Court Road, Cambridge CB2 1QW, England

(Received 12 May 1988, and in revised form 27 July 1988)

A restriction fragment carrying the major coat protein gene (gene VIII) was excised from the DNA of the class I filamentous bacteriophage fd, which infects *Escherichia coli*. This fragment was cloned into the expression plasmid pKK223-3, where it came under the control of the *tac* promoter, generating plasmid pKf8P. Bacteriophage fd gene VIII was similarly cloned into the plasmid pEMBL9+, enabling it to be subjected to site-directed mutagenesis. By this means the positively charged lysine residue at position 48, one of four positively charged residues near the C terminus of the protein, was turned into a negatively charged glutamic acid residue. The mutated fd gene VIII was cloned back from the pEMBL plasmid into the expression plasmid pKK223-3, creating plasmid pKE48.

In the presence of the inducer isopropyl-β-D-thiogalactoside, the wild-type and mutated coat protein genes were strongly expressed in *E. coli* TG1 cells transformed with plasmids pKf8P and pKE48, respectively, and the product procoat proteins underwent processing and insertion into the *E. coli* cell inner membrane. A net positive charge of only 2 on the side-chains in the C-terminal region is evidently sufficient for this initial stage of the virus assembly process. However, the mutated coat protein could not encapsidate the DNA of bacteriophage R252, an fd bacteriophage carrying an amber mutation in its own gene VIII, when tested on non-suppressor strains of *E. coli*. On the other hand, elongated hybrid bacteriophage particles could be generated whose capsids contained mixtures of wild-type (K48) and mutant (E48) subunits. This suggests that the defect in assembly may occur at the initiation rather than the elongation step(s) in virus assembly. Other mutations of lysine-48 that removed or reversed the positive charge at this position in the C-terminal region of the coat protein were also found to lead to the production of commensurately longer bacteriophage particles. Taken together, these results indicate direct electrostatic interaction between the DNA and the coat protein in the capsid and support a model of non-specific binding between DNA and coat protein subunits with a stoicheiometry that can be varied during assembly.

1. Introduction

Bacteriophage fd is a class I filamentous virus (others are M13 and f1) that comprises a circular, single-stranded DNA molecule enclosed in a cylindrical protein sheath to form a flexible particle ~890 nm long and 7 nm in diameter (for reviews, see Webster & Lopez, 1985; Makowski, 1985). The protein sheath consists of about 2700 major coat protein subunits (Day & Wiseman, 1978) in a shingled helical array, the symmetry of which is defined by a 5-fold rotational axis combined with a 2-fold screw axis of pitch 3·2 nm (Marvin, 1978; Banner *et al.*, 1981; Makowski & Caspar, 1981). The viral DNA contains 6408 nucleotides (Beck *et al.*, 1978; van Wezenbeek *et al.*, 1980; Hill & Petersen, 1982), incorporating ten genes and extending throughout the length of the particle, but it is not base-paired and has a symmetry different from that of the protein helix. The major coat protein subunit contains 50 amino acid residues and, in the virus particle, adopts a largely α-helical conformation, with the long axis of the helix aligned close to the long axis of the filament (Marvin, 1978; Banner *et al.*, 1981; Makowski & Caspar, 1981; Cross *et al.*, 1983). The negatively charged N-terminal region of the protein is located on the outside of the filament whereas the positively charged C-terminal region is on the inside abutting the DNA (Marvin, 1978; Boeke *et al.*, 1980; Armstrong *et al.*, 1983).

The viral DNA is specifically oriented within the bacteriophage particle to permit a 78-nucleotide hairpin loop to be accommodated at one end of the

0022-2836/88/230663-12 $03.00/0

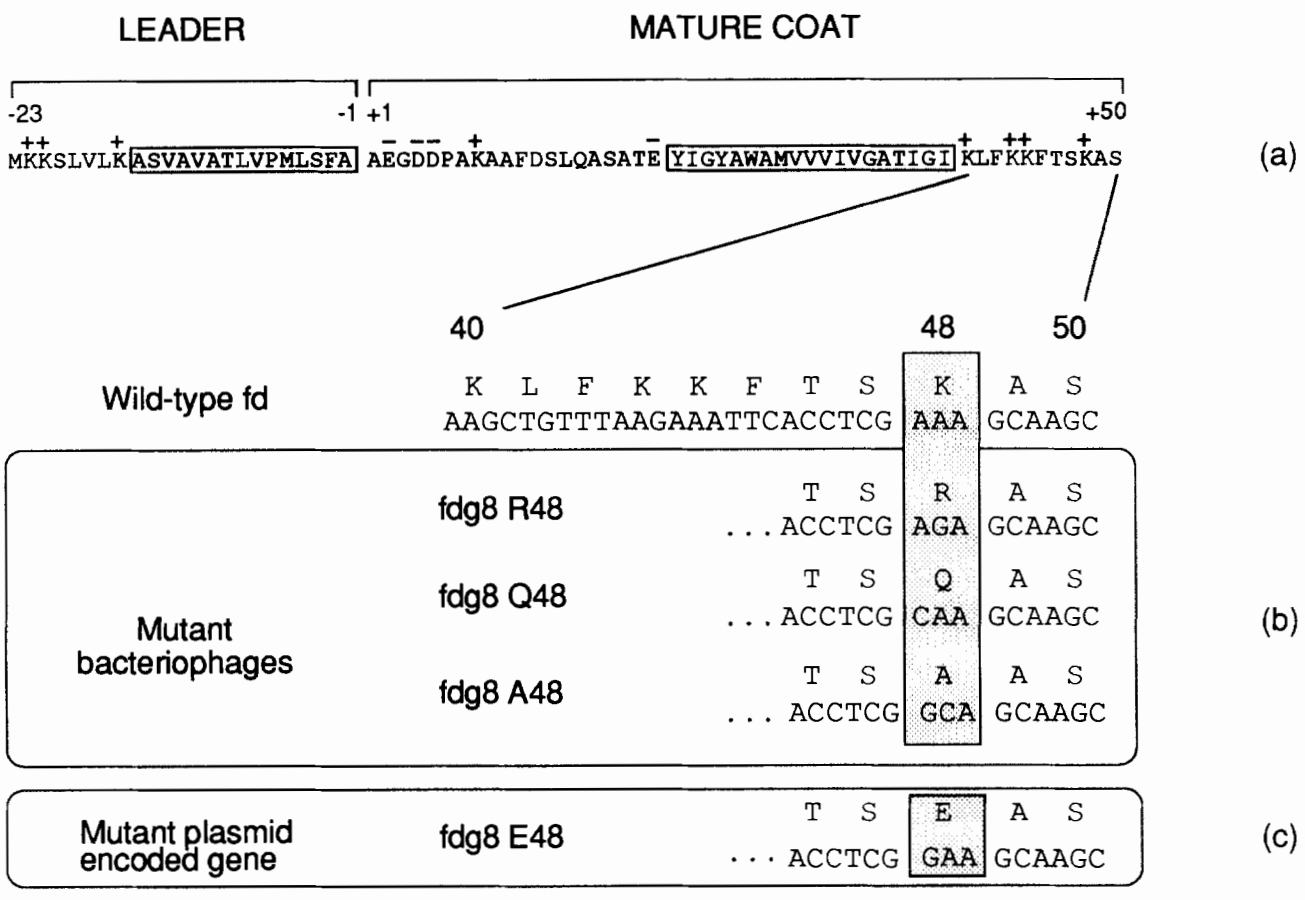

Figure 1. Primary structures of procoats of wild-type and mutant bacteriophage fd. Putative membrane-spanning regions are indicated by a box. Positively and negatively charged side-chains are indicated by + and −, respectively. (a) Procoat of bacteriophage fd. (b) C-Terminal regions of the major coat proteins of bacteriophage fd and bacteriophage mutants fdg8R48, fdg8Q48 and fdg8A48 (Hunter *et al.*, 1987). The altered codons are boxed. (c) C-Terminal region of the plasmid-encoded mutant coat protein fdg8E48. The altered codon is boxed. For further details, see the text.

virion (Ikoku & Hearst, 1981). This end of the particle also carries approximately five copies of each of two minor coat proteins, gene VII protein (gVIIp) and gene IX protein (gIXp), and at the other end there are approximately five copies of each of two other minor coat proteins, gene III protein (gIIIp) and gene VI protein (gVIp) (Grant *et al.*, 1981; Simons *et al.*, 1981; Webster *et al.*, 1981). The major coat protein (gVIIIp) is synthesized as a procoat molecule containing an N-terminal signal peptide of 23 amino acids (Fig. 1(a)). This is processed by signal peptidase and the mature coat protein is left spanning the bacterial inner membrane with its N terminus exposed on the outer face and its C terminus in the cytoplasm (Wickner, 1975; Ohkawa & Webster, 1981). The progeny single-stranded DNA (ssDNA†) is sequestered as a pre-assembly complex by association with the product (gVp) of viral gene V (Webster & Cashman, 1973; Pratt *et al.*, 1974). During assembly of the virion, which occurs at the bacterial inner membrane, the gVp is displaced from this complex and gVIIIp is drawn from the membrane and added to the elongating particle that is being extruded. The assembly is a vectorial process, beginning at the gVIIp-gIXp end of the filament (Armstrong *et al.*, 1983; Lopez & Webster, 1983), and is probably initiated by interactions of these and other proteins in the membrane with the hairpin loop of DNA found at that end of the finished particle (Webster & Lopez, 1985). Assembly is completed by the addition of the two other minor coat proteins, gIIIp and gVIp, to the opposite end of the filament as it leaves the membrane.

As with other helically symmetrical rod-like viruses, nucleic acid–protein interactions are likely to be a major determinant of particle structure. The positively charged C-terminal region of the major coat protein (Fig. 1(a)) has been predicted to play an important part in neutralizing the negatively charged phosphodiester links in the encapsidated DNA (Marvin, 1978) and direct evidence for a crucial role of this region in DNA-packaging was recently obtained (Hunter *et al.*, 1987). Thus, site-directed mutagenesis of the lysine residue at position 48 in the sequence of the coat protein (Fig. 1(b)) demonstrated that a positive charge in this position is not essential for bacteriophage

† Abbreviations used: ssDNA, single-stranded DNA; bp, base-pairs; RF, replicative form; IPTG, isopropyl-β-D-thiogalactopyranoside.

assembly, but that loss of the positive charge leads to an elongated particle being produced in which commensurately fewer nucleotides are packaged per protein subunit (Hunter *et al.*, 1987).

We have now taken this study further by attempting to generate *in vivo* bacteriophage particles whose capsids are composed of two types of major coat protein, termed hybrid phage. This has been achieved by cloning the major coat protein gene (gVIII) of wild-type bacteriophage fd into a suitable plasmid expression vector, essentially as described for the major coat protein gene of bacteriophage Pf1 (Rowitch & Perham, 1987). Thus, in a plasmid-transformed cell, production of wild-type fd coat protein can be induced from the plasmid-encoded gene at the same time as an infecting mutant bacteriophage contributes mutant coat protein encoded by its own gene VIII. This permits the two sorts of coat protein to compete in the assembly process in the same cell. We also describe a system whereby mutations in bacteriophage fd gene VIII can be made and studied in suitable plasmid vectors, without the requirement to produce viable bacteriophage particles inherent in the previous mutagenesis protocol (Zoller & Smith, 1983; Carter *et al.*, 1985; Hunter *et al.*, 1987). This has enabled us to identify a mutation (Lys48 → Glu48) in the coat protein (Fig. 1(c)) that allows correct processing and membrane insertion but does not support the initiation of virus assembly. On the other hand, the mutant coat protein can participate in the subsequent elongation process, again with an interpretable effect on the mode of DNA packaging.

2. Materials and Methods

(a) *Materials*

Components of media were obtained from Difco Laboratories. Restriction endonucleases and cloning enzymes were from New England Biolabs and Boehringer-Mannheim; 3,3′-diaminobenzidene, 5-bromo-4-chloro-3-indoyl-β-galactoside (BCIG) and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Sigma. Deoxy- and dideoxyribonucleoside 5′-triphosphates used in mutagenesis and sequencing experiments were purchased from P-L Biochemicals. The oligodeoxyribonucleotides E48 (d(AGCTTGCTTCCGAGGTGAA)), SELP (d(AATAAAGCCACAGAGCATA)) and sequencing primers were synthesized by the method of Mathes *et al.* (1984), with materials purchased from Cruachem. Marvel (Cadbury's) was from J. Sainsbury plc. Goat anti-rabbit IgG–peroxidase conjugate was purchased from Tago, Inc., Burlingame, CA. Silica gel thin-layer plates (Polygram SIL G) were obtained from Macherey-Nagel, Düren, West Germany.

(b) *Bacterial strains, bacteriophages and plasmids*

Escherichia coli Hfr strain K37 (*sup*1) (Lyons & Zinder, 1972) and the gene VIII amber mutant bacteriophage R252 (Moses & Horiuchi, 1982) were supplied by Dr K. Horiuchi (Rockefeller University, NY). *E. coli* JM101 (F′, *sup*2, *lacI*^{q}) (Messing *et al.*, 1981) and bacteriophage M13mp8 were gifts from Dr J. Messing (University of Minnesota, St Paul, MN), and *E. coli* HB2151 (F′, Su$^-$, *lacI*^{q}) and HB2155 (F′, Su$^-$ *mut*L : : Tn*10*) (Carter *et al.*, 1985) were kindly provided by Dr G. Winter (MRC Laboratory of Molecular Biology, Cambridge). *E. coli* TG1 (F′, *sup*E, *lacI*^{q}) was a gift from Dr A. Gibson (MRC Laboratory of Molecular Biology, Cambridge). Wild-type bacteriophage fd was originally obtained from Dr I. Molineux (Imperial Cancer Research Fund, London). The plasmid pEMBL9+ was a gift from Dr J. Armstrong (Imperial Cancer Research Fund, London) and the expression plasmid pKK223-3 was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

(c) *DNA manipulation and sequencing*

Digestion with restriction endonucleases and incubations with bacteriophage T4 DNA ligase and calf intestinal alkaline phosphatase were carried out according to the manufacturer's instructions. DNA was electrophoresed on 0·7% (w/v) agarose gel and bands were visualized by staining with ethidium bromide (Maniatis *et al.*, 1982). Specific DNA fragments were isolated by electrophoretic transfer onto Whatman DE-81 paper (Drelzen *et al.*, 1981). DNA sequencing was carried out by the dideoxy chain termination method (Sanger *et al.*, 1980; Biggin *et al.*, 1983) using the synthetic oligonucleotide d(CACGTTGAAAATCTCCA) as a primer for sequencing bacteriophage fd ssDNA directly. pEMBL ssDNA was prepared as described by Dente *et al.* (1983), and sequenced using "universal" primer, in the presence of helper bacteriophage (fd) ssDNA.

(d) *Cloning of bacteriophage fd gene VIII into an* E. coli *expression vector*

An 829 bp fragment containing bacteriophage fd gene VIII and its immediate upstream promoter was excised from RF DNA by digestion with the restriction endonuclease *Hpa*II (Fig. 2(a)) (Moses *et al.*, 1980; Moses & Horiuchi, 1982) and ligated into M13mp8 vector DNA that had been cleaved by digestion with *Acc*I and treated with alkaline phosphatase to limit self-closure. The products of ligation were transformed into *E. coli* JM101 and grown on minimal agar plates containing BCIG (1 μg/ml) and IPTG (25 μg/ml). Sequence analysis identified a clone, designated mp8f8P (Fig. 2(b)), containing the desired insert oriented 5′ to 3′ with respect to the *lac* promoter of the vector (results not shown). Bacteriophage fd gene VIII was excised from bacteriophage mp8f8P as part of a 756 bp fragment by double digestion with the restriction endonucleases *Sna*B1 and *Hin*dIII, which ensured that the endogenous promoter sequence present in the previously isolated *Hpa*II fragment (Fig. 2(a)) would not be included in the construct to follow. The expression vector pKK223-3, a pBR322-derived plasmid that carries the controllable *tac* promoter (de Boer *et al.*, 1983) and *rrn*B transcription terminators, was cleaved with the endonucleases *Sma*I and *Hin*dIII and treated with alkaline phosphatase. Ligation of the 756 bp fragment and vector, followed by transformation into *E. coli* TG1 plated out on ampicillin-containing medium, permitted the directional cloning of the fragment (Fig. 2(b)). The identity of the fd gene VIII-containing fragment in the plasmid, designated pKf8P, was confirmed by restriction analysis (results not shown).

(e) *Construction of a plasmid for fd gene VIII mutagenesis*

Bacteriophage fd RF DNA was digested to completion with the restriction endonucleases *Rsa*I and *Bam*HI

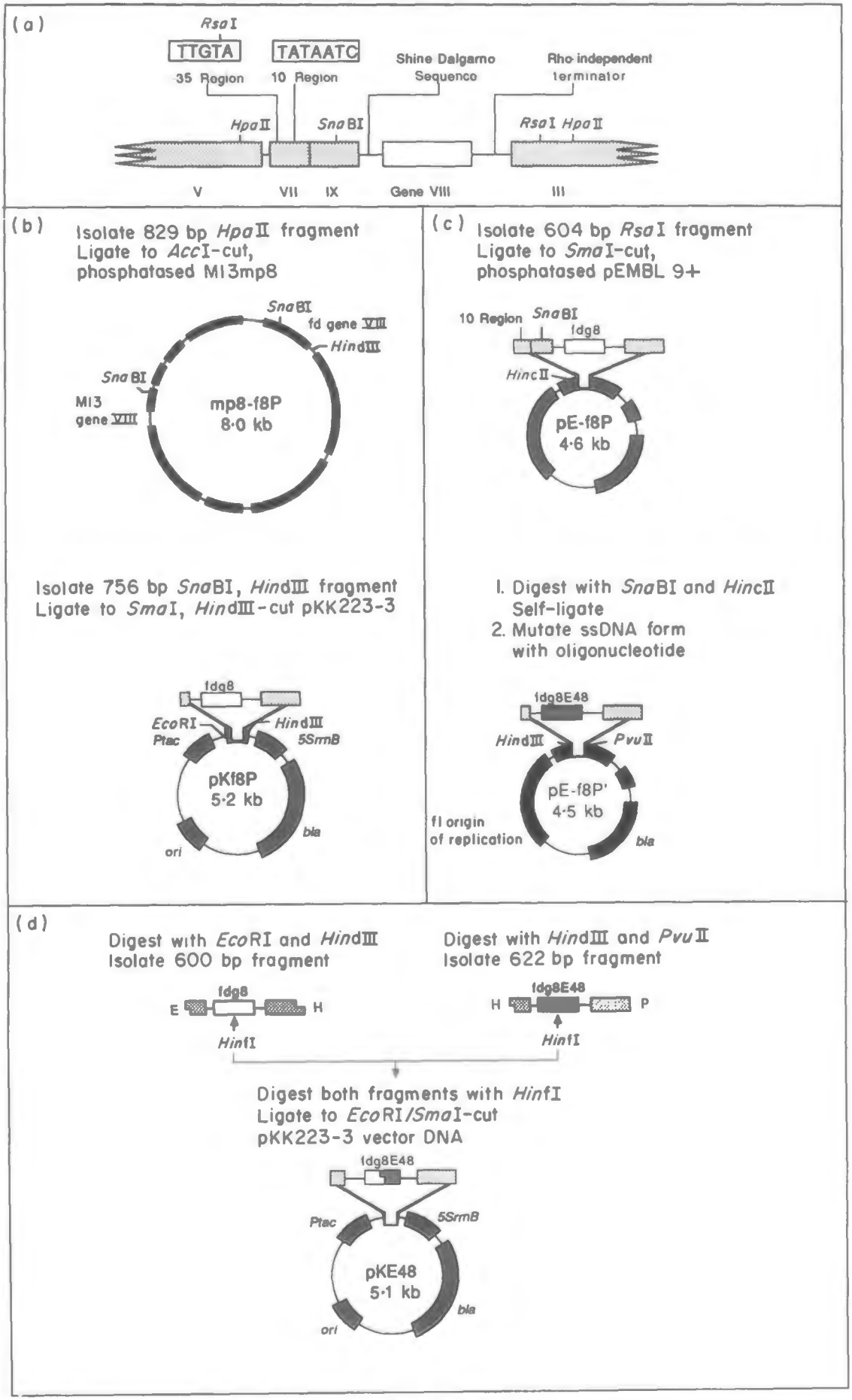
(a)
RsaI
TTGTA
TATAATC
35 Region
10 Region
Shine Dalgarno Sequence
Rho-independent terminator
HpaII
SnaBI
RsaI HpaII
V
VII
IX
Gene VIII
III
(b)
Isolate 829 bp HpaII fragment
Ligate to AccI-cut,
phosphatased M13mp8
SnaBI
fd gene VIII
HindIII
SnaBI
M13 gene VIII
mp8-f8P
8·0 kb
Isolate 756 bp SnaBI, HindIII fragment
Ligate to SmaI, HindIII-cut pKK223-3
f1dg8
EcoRI
Ptac
HindIII
5SrnB
pKf8P
5·2 kb
bla
ori
(c)
Isolate 604 bp RsaI fragment
Ligate to SmaI-cut,
phosphatased pEMBL 9+
10 Region
SnaBI
f1dg8
HincII
pE-f8P
4·6 kb
1. Digest with SnaBI and HincII
Self-ligate
2. Mutate ssDNA form
with oligonucleotide
f1dg8E48
HindIII
PvuII
pE-f8P'
4·5 kb
f1 origin of replication
bla
(d)
Digest with EcoRI and HindIII
Isolate 600 bp fragment
f1dg8
E
H
HinfI
Digest with HindIII and PvuII
Isolate 622 bp fragment
f1dg8E48
H
P
HinfI
Digest both fragments with HinfI
Ligate to EcoRI/SmaI-cut
pKK223-3 vector DNA
f1dg8E48
Ptac
5SrnB
pKE48
5·1 kb
bla
ori

Fig. 2.

(*Bam*HI cuts a contaminant fragment of about the same size) and a 604 bp blunt-ended fragment (Fig. 2(a)) was cloned into *Sma*I-cut, alkaline phosphatase-treated pEMBL9+ and transformed into *E. coli* JM101. One ampicillin-resistant transformant colony was chosen whose plasmid DNA migrated on agarose gel electrophoresis with a mobility consistent with a 610 bp insert. pEMBL phage-like particles were generated from cultures of *E. coli* JM101 transformed with this plasmid (Dente *et al.*, 1983). Dideoxy sequence analysis of ssDNA from these samples using "universal" primer (which binds to pEMBL9 + ssDNA but not to fd ssDNA) confirmed that the construct contained the 604 bp *Rsa*I fragment (Fig. 2(a)) oriented 5′ to 3′ with respect to the pEMBL9+-encoded *lac* promoter (results not shown). This construct was designated pEf8P (Fig. 2(c)).

(f) *Site-directed mutagenesis of bacteriophage fd gene VIII in pEf8P*

The ssDNA prepared from plasmid pEf8P-containing cells was used as the template for oligonucleotide-directed mutagenesis. Initial results revealed a very low efficiency of transformation following mutagenesis, possibly because of expression in transformed cells of the coat protein gene from pEf8P, which contains part of the endogenous promoter for fd gene VIII (Fig. 2(a)). This remaining promoter sequence was therefore removed by double digestion of pEf8P with the endonucleases *Sna*BI and *Hin*cII followed by self-ligation (Fig. 2(c)). The ssDNA form of this plasmid, obtained after infection of transformed cells with bacteriophage fd, was annealed without further purification to the synthetic oligonucleotides E48 and SELP (see above) and second-strand synthesis was carried out by standard techniques (Zoller & Smith, 1983; Carter *et al.*, 1985). The oligonucleotide SELP was designed to mutate the *Eco*B site present in the vector pEMBL9+ sequence so as to afford some biological selection of the mutated strand when transformed into *Eco*B$^+$ cells, in a manner analogous to the *Eco*B/*Eco*K selection system of Carter *et al.* (1985). Before transformation of the heteroduplex DNA into *E. coli* HB2155 (*mut*L, *Eco*B$^+$) cells, contaminating fd heteroduplex DNA (also formed during second-strand synthesis) was rendered ineffective by digestion with the endonucleases *Bam*HI and *Hin*cII, the plasmid DNA being resistant to this treatment (Fig. 2(c)). Mutated clones were identified by colony hybridization with radiolabelled oligonucleotide E48 (Carter *et al.*, 1985). These clones were infected with bacteriophage fd and ssDNA from the progeny phage-like particles were checked by dideoxy sequence analysis, to confirm the presence of codon GAA (glutamic acid) at position 48. The rest of the gene was unchanged. The progeny from a confirmed mutant were used to infect *E. coli* TG1 cells, which were plated out on YT medium containing 25 μg ampicillin/ml. This enabled the plasmid form of the construct, designated pEf8P′, to be stably maintained (*E. coli* TG1 is not *mut*L).

(g) *Cloning of mutated fd gene VIII into an* E. coli *expression vector*

In order to subclone the mutant fd gene VIII (fdg8E48) directionally from pEf8P′ into the expression plasmid pKK223-3, it was necessary to reconstitute appropriate gene VIII fragments as outlined in Fig. 2(d). This was because the orientation of the polylinker in pEf8P′ was the opposite of that in pKK223-3. Two DNA fragments were gel-isolated, one after digestion of pEf8P′ DNA with the endonucleases *Hin*dIII and *Pvu*II (622 bp fragment), and the other after digestion of pKf8P DNA with the endonucleases *Eco*RI and *Hin*dIII (600 bp fragment) (Fig. 2(d)). A mixture of the 2 fragments was digested with the endonuclease *Hin*fI, which cleaves within the fd gene VIII sequence to leave non-palindromic "sticky" ends, and the products were ligated into *Eco*RI–*Sma*I-cut pKK223-3 vector DNA (Fig. 2(d)). After transformation of *E. coli* TG1, a colony that carried the desired construct was identified by restriction analysis of the plasmid DNA (results not shown). This was designated pKE48 (Fig. 2(d)).

(h) *Polyacrylamide gel electrophoresis and immunoblotting*

Phage-encoded and bacterial proteins were submitted to electrophoresis on 15% polyacrylamide/0·4% (w/v) bisacrylamide/8 M-urea/SDS gels (Simons *et al.*, 1979) and were silver-stained by the method of Morrissey (1981). For immunoblotting, proteins were electrophoretically transferred to nitrocellulose filters (Towbin *et al.*, 1979) using a Biorad transblot apparatus. The filter was then incubated in phosphate-buffered saline (pH 7·3) containing 5% (w/v) Marvel (Johnson *et al.*, 1984) and rabbit antiserum raised against bacteriophage fd (according to Hudson & Hay, 1976). Antigen–antibody complexes were detected by treating the filter with goat anti-rabbit IgG peroxidase conjugate and subsequent development with hydrogen peroxide and 3,3′-diaminobenzidene.

(i) *Expression of fd gene VIII* in vivo *and production and purification of hybrid bacteriophage particles*

E. coli TG1 cells transformed with plasmid pKf8P or pKE48 were grown up at 37 °C in YT medium (10 g Bactotryptone/l, 5 g yeast extract/l, 5 g NaCl/l) supplemented with 50 μg ampicillin/ml to an A_{600} value of approximately 0·5, and then induced with IPTG (final concentration 1 mM). At various times after induction, samples of whole cells were removed, centrifuged, washed and analysed by means of urea/SDS/polyacrylamide gel electrophoresis.

To obtain simultaneous expression of wild-type and mutant coat protein genes in the production of hybrid bacteriophage particles, *E. coli* HB2151 cells harbouring the plasmids pKf8P or pKE48 were grown up on TY medium (10 g Bactotryptone/l, 10 g yeast extract/l, 5 g NaCl/l), supplemented with 50 μg ampicillin/ml, at 37 °C with vigorous shaking. At an A_{600} value of 0·3, bacteriophage fd was added at an m.o.i. (multiplicity of infection) of 25 and the culture incubated for a further 15 min. IPTG was then added as a sterile solution (25 mg/ml) to a final concentration of 1 mM (unless otherwise stated) and growth permitted to continue for about 12 to 16 h. Bacterial cells were then removed by centrifugation and bacteriophages recovered from the supernatant by precipitation with polyethylene glycol

Figure 2. Construction of the expression plasmids pKf8P (for bacteriophage fd wild-type coat protein) and pKE48 (for mutant fdg8E48 coat protein). (a) Genes in bacteriophage fd DNA in the region of the coat protein gene (gene VIII) showing the promoter sequences (−10, −35 regions) and relevant restriction endonuclease recognition sites. (b) Scheme for the construction of the expression plasmid pKf8P. (c) Scheme for the construction of the mutagenesis plasmid pEf8P and the subsequent modifications used to produce pEf8P′. (d) Construction of the expression plasmid pKE48. For further details, see the text. kb, 10^3 bases.

and purified by CsCl density-gradient centrifugation (Yamamoto *et al.*, 1970), followed by extensive dialysis against TE buffer (10 mM-Tris·HCl, 1 mM-EDTA, pH 8·0).

(j) *Agarose gel electrophoresis of bacteriophage particles*

Intact virions (15 μl, approx. 10^{11} particles) were submitted to non-denaturing electrophoresis in 2% agarose gels in 0·37 M-Tris–glycine buffer (pH 9·5), at 8 V/cm gel bed for 12 to 16 h (Moses *et al.*, 1980). The virions were then denatured by placing the gel in 0·2 M-NaOH for 50 min and then 0·5 M-Tris·HCl (pH 8·0), for 60 min, after which the bacteriophage DNA was visualized by staining in ethidium bromide (Nelson *et al.*, 1981).

(k) *Peptide mapping of bacteriophage coat protein*

A sample of bacteriophage was dialysed extensively against 0·5% (w/v) ammonium bicarbonate, heated to 100 °C in 1% (w/v) SDS, and then the dialysis was repeated. Digestion with chymotrypsin (1/50, w/w) was performed at 37 °C for 4 h, and the sample was lyophilized. A portion (80 μg) of the digest was analysed on thin-layer silica plates by chromatography (butan-1-ol : acetic acid : water : pyridine, 15 : 3 : 12 : 10, by vol.) and electrophoresis at pH 6·5 (Perham, 1978; Armstrong *et al.*, 1983). Peptides were visualized by using a ninhydrin–cadmium stain (Perham, 1978).

(l) *Electron microscopy and bacteriophage length measurements*

Virions were spread on copper grids in 50 mM-sodium acetate (pH 5·5), and negatively stained with 2% uranyl acetate. Electron micrographs were taken at 40,000× magnification on an AEI 801S electron microscope operated at 60 kV and the contour lengths of individual virions were determined using a Graf/Bar sonic digitizer (Hunter *et al.*, 1987).

3. Results

The construction of the plasmids pKf8P and pKE48 (see Materials and Methods) provided a system for the controlled expression of bacteriophage fd gene VIII, similar to that of Kuhn & Wickner (1985), but more effective for our purpose. It permitted a systematic analysis of the effects of mutation of the coat protein on its ability to undergo membrane processing and insertion and to participate in virus assembly.

(a) *Synthesis of wild-type fd and fdg8E48 mutant coat proteins* in vivo

To test expression of the cloned wild-type fdg8 and the mutant fdg8E48 genes, *E. coli* TG1 cells transformed with either pKf8P or pKE48 plasmid DNA, respectively, were grown in the presence or absence of the inducer, IPTG (1 mM), and samples were removed and analysed by urea/SDS/polyacrylamide gel electrophoresis. As shown in Figure 3(a), transformed cells, 40 minutes after induction, contained a protein with electrophoretic mobility similar to that of authentic fd coat protein, which was not present in uninduced cells. The identities of the pKf8P- and pKE48-encoded products were further confirmed by means of an immunoblot employing antibody raised against bacteriophage fd (Fig. 3(b)). The level of expression of the mutated gene VIII from plasmid pKE48 was apparently similar to that of the wild-type gene from plasmid pKf8P. It was also noted during the course of these studies that the growth rates (measured spectrophotometrically) of cells expressing protein from either plasmid were slightly retarded 20 minutes after induction with IPTG whereas the growth of vector (pKK223-3)-transformed cells was unaffected by the inducing agent. The same effect was observed previously when bacteriophage Pf1 gene VIII was over-expressed from the same plasmid in *E. coli* JM101 (Rowitch & Perham, 1987).

(b) *Assembly of plasmid-encoded coat protein into bacteriophage virions*

The ability of plasmid-encoded coat protein to participate in virus assembly was investigated by measuring the plaque-forming ability of bacteriophage R252 on induced and uninduced cultures of pKf8P- or pKE48-transformed *E. coli* HB2151. Bacteriophage R252 (Moses & Horiuchi, 1982) is an engineered class I (fd) bacteriophage containing the *am8H1* amber mutation at position 2 of the mature coat protein encoded by gene VIII; as such it can be propagated on the permissive *E. coli* strain K37 (*supI*) but fails to grow on non-suppressor strains, e.g. *E. coli* HB2151 (su^-). The results were compared with the growth of bacteriophage R252 on pKK223-3 vector-transformed *E. coli* HB2151, on untransformed *E. coli* HB2151 and on *E. coli* K37 cells.

Induction of pKf8P-transformed cells enhanced the plaque-forming ability of the amber mutant bacteriophage R252 by six orders of magnitude compared with untransformed cells; in the uninduced state, a tenfold increase in plaque-forming ability was still noted (Table 1). These results provide strong evidence that the plasmid-encoded fd coat protein followed the pathway of biosynthesis normally used during infection and imply that correct membrane insertion and processing had taken place. Moreover, the failure of the virions productively to infect *E. coli* HB2151 cells (results not shown) confirmed that the original R252 amber mutation had been maintained. Analysis of the intact bacteriophage particles by electrophoresis on 2% agarose gels demonstrated, as expected, that the mobility was identical to that of wild-type fd and not that of R252 (Boeke *et al.*, 1980). In contrast, growth of bacteriophage R252 on pKE48-transformed *E. coli* HB2151 was unimproved, even by induction with IPTG (Table 1), indicating that the mutant coat protein produced by plasmid pKE48 was not capable of encapsidating R252 viral DNA. It is important to note that, in the case of both pKf8P- and pKE48-transformed cells, induction of coat protein with excess IPTG (100 μM)

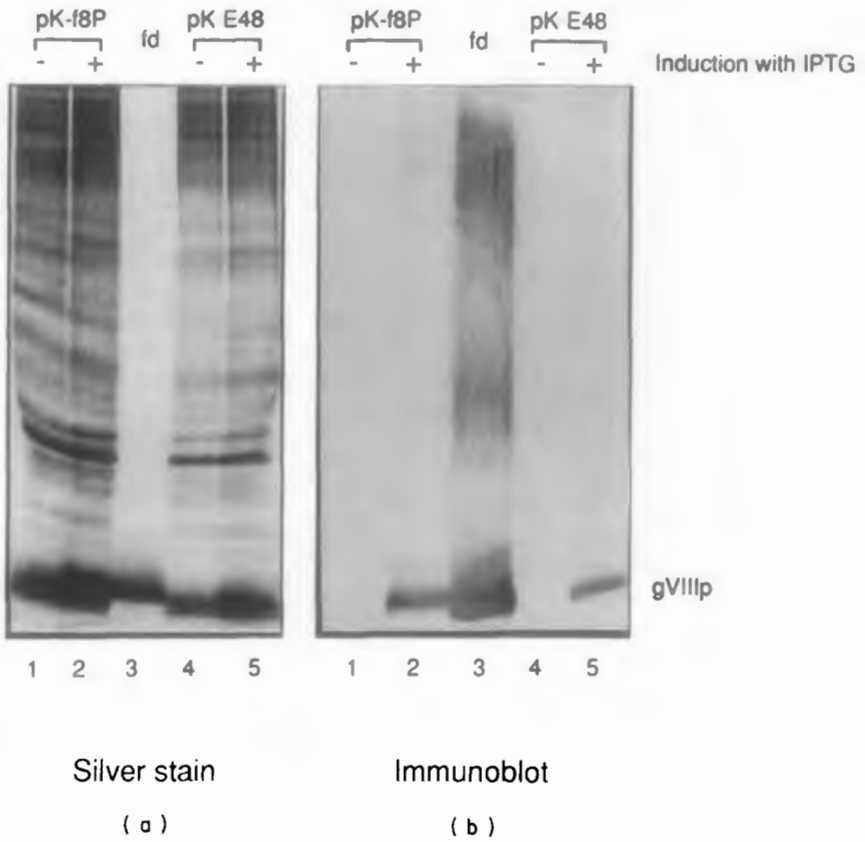

Figure 3. Expression of wild-type bacteriophage fd and fdg8E48 mutant coat protein genes in *E. coli* HB2151 cells transformed with plasmids pKf8P and pKE48, respectively. (a) Samples of whole cells were submitted to urea/SDS/polyacrylamide gel electrophoresis and the products were visualized by silver staining. Lane 1, HB2151 cells transformed with plasmid pKf8P, not induced with IPTG; lane 2, pKf8P-transformed cells, 40 min after induction with IPTG; lane 3, approx. 1 μg bacteriophage fd; lane 4, pKE48-transformed cells not induced with IPTG; lane 5, pKE48-transformed cells, 40 min after induction with IPTG. (b) Immunoblot of a urea/SDS/polyacrylamide gel employing rabbit antiserum raised against bacteriophage fd. The lanes are as described for (a). For further details, see the text.

resulted in failure of the cells to be grown as a lawn, presumably owing to high levels of gene expression.

Although the mutant coat protein produced by induction of plasmid pKE48 did not support the growth of bacteriophage R252, it did not appear to inhibit the assembly of class I viruses, for the plaque-forming ability of wild-type fd was virtually identical on pKE48-transformed *E. coli* HB2151 in the presence or absence of IPTG (results not shown). Similarly, infection of pKf8P-transformed *E. coli* HB2151 cells with the mutant class I bacteriophages fdg8R48, fdg8Q48 and fdg8A48 (Hunter *et al.*, 1987) (see Fig. 1) was also unchanged. Bacteriophages produced from induced cultures of either pKf8P- or pKE48-transformed cells infected with various class I mutant bacteriophages (Hunter *et al.*, 1987) were therefore analysed by agarose gel electrophoresis, peptide mapping, and electron microscopy to test for the production and properties of hybrid bacteriophage particles.

Table 1

Infectivity of bacteriophage R252 on pKf8P (wild-type gene VIII)- and pKE48 (mutant gene VIII)-transformed E. coli *HB2151*

Bacterial strain	Concentration of IPTG	R252 (p.f.u./ml)
E. coli HB2151 (*su*$^-$)	0	2×10^8
	1 mM	2×10^8
pKK223-3-transformed *E. coli* HB2151	0	2×10^8
	1 mM	3×10^8
pKf8P-transformed *E. coli* HB2151	0	3×10^9
	10 μM	2×10^{14}
pKE48-transformed *E. coli* HB2151	0	2×10^8
	10 μM	2×10^8
E. coli K37 (*supI*)	0	1×10^{14}
	1 mM	1×10^{14}

p.f.u., plaque-forming units.

(c) *Agarose gel electrophoresis of hybrid bacteriophages*

The bacteriophage mutants fdg8R48 and fdg8Q48 migrate more slowly than wild-type fd during electrophoresis in agarose gels, whereas mutant fdg8A48 (Fig. 1) migrates more rapidly (Hunter *et al.*, 1987). When the same mutant bacteriophages were grown on induced cultures of pKf8P-transformed cells, differences in electrophoretic mobility of progeny virions were found

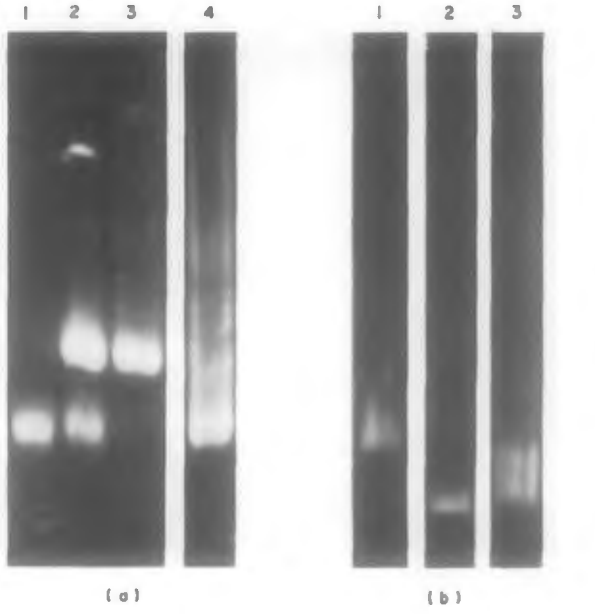

Figure 4. Agarose gel electrophoresis of hybrid bacteriophage particles. Electrophoresis of intact virions was carried out in 2% agarose gels in 0·37 M-Tris–glycine buffer (pH 9·5), and the positions of the virions were revealed by staining the viral DNA with ethidium bromide. (a) fdg8Q48 hybrid virion preparations. Lane 1, bacteriophage fd; lane 2, bacteriophages fd and fdg8Q48; lane 3, bacteriophage fdg8Q48; lane 4, bacteriophage fdg8Q48 grown on pKf8P-transformed *E. coli* HB2151 cells, induced with IPTG (10 μM). (b) fdg8A48 hybrid virion preparations. Lane 1, bacteriophage fd; lane 2, bacteriophage fdg8A48; lane 3, bacteriophage fdg8A48 grown on pKf8P-transformed *E. coli* HB2151 cells, induced with IPTG (10 μM).

that are consistent with the incorporation of some wild-type fd gene VIII protein into the capsids. Thus, the mobility of hybrid fdg8Q48 particles was increased and approached that of bacteriophage fd. On the other hand, the mobility of hybrid fdg8A48 particles was reduced, again as might be expected for the incorporation of wild-type coat protein subunits. Similar results were obtained with mutant fdg8R48. Virions from such preparations migrated as a "smear" between the extremes characteristic of wild-type and mutant bacteriophage mobility whereas mixtures of bacteriophages applied to the gel resolved into discrete bands characteristic of the virions of which they were composed (Fig. 4). The ssDNA was the same size in all cases, as judged by agarose gel electrophoresis, and dideoxy sequence analysis confirmed the presence of the appropriate mutation in gene VIII, eliminating any possibility of contamination. This evidence strongly favours the formation of hybrid particles with different coat protein subunits assembled into the same capsid and argues against the alternative of two distinct wild-type and mutant phage morphologies being elaborated independently.

Progeny bacteriophage were also prepared after infection of IPTG-induced pKE48-transformed cells with bacteriophage fd and with mutant bacteriophages fdg8R48, fdg8Q48 and fdg8A48, and these were submitted to agarose gel electrophoresis. With bacteriophage fd, some virions were observed that migrated slightly ahead of authentic fd. With the mutant bacteriophages, there appeared to be an effect on virion mobility similar to that just described for the incorporation of wild-type coat protein, suggesting the incorporation of some fdg8E48 coat protein subunits (expressed from the pKE48 plasmid) into hybrid bacteriophage particles. However, the results of electrophoresis were less clear-cut than those obtained for the other hybrid bacteriophages described above.

(d) *Peptide mapping of hybrid bacteriophages*

Peptide mapping was also undertaken to examine the C-terminal regions of the coat proteins present in the putative hybrid bacteriophage particles. Previous studies have shown that three soluble peptides are liberated from the fd wild-type major coat protein by digestion with chymotrypsin and that these can be resolved by thin-layer chromatography followed by electrophoresis at pH 6·5 (Armstrong *et al.*, 1983). The chymotryptic peptides termed C1, C2 and C3 are derived from coat protein residues 1 to 11, 43 to 45 and 46 to 50, respectively. The peptides C1 (ADGDDPAKAAF) and C2 (KKF) stain red with ninhydrin–cadmium and are to be expected in chymotryptic digests of all the coat proteins studied. However, the properties of peptide C3 (TSXAS, where X is residue 48), which has an N-terminal threonine residue and stains a yellow colour with ninhydrin–cadmium, are dependent upon the character of the residue at position 48, and are therefore changed in the mutant coat proteins. In bacteriophages fd (where position 48 is K) or fdg8R48 (where position 48 is R), peptide C3 carries a net positive charge and migrates towards the cathode. In mutants fdg8Q48 and fdg8A48, peptide C3 is neutral (and remains near the origin of electrophoresis), whereas for mutant fdg8E48 peptide C3 will carry a net negative charge and migrate toward the anode. The different mobilities of the C3 peptides made it relatively easy to detect the presence of, and to identify, two different coat proteins (each of which generated different C3 peptides) in the various hybrid phage preparations (Table 2).

(e) *Electron microscopy of bacteriophage particles*

Conclusive evidence of change in the length of hybrid bacteriophage virions was obtained by electron microscopy (Figs 5 and 6). The lengths of viable mutant bacteriophages from a previous study (Hunter *et al.*, 1987), which were used as parental bacteriophages in the production of hybrid virions, are given in Table 3A. Table 3B summarizes contour length data for various hybrid bacteriophages grown in pKf8P-transformed cells in the presence of varying amounts of the inducer.

Table 2
Peptide mapping of chymotryptic digests of bacteriophage coat proteins

Bacteriophage	Peptide†	Peptide mobility: Chromatography‡	Peptide mobility: Electrophoresis§
fd wild-type and fdg8R48	C1	0·20	−0·33
	C2	0·32	+0·80
	C3	0·24	+0·37
fdg8Q48 and fdg8A48	C3	0·31	0·0
fdgE48	C3	0·21	−0·43

† Chymotryptic peptides C1 and C2 are present in all bacteriophage samples.
‡ Relative to solvent front.
§ Positive values indicate migration towards the cathode, measured relative to marker lysine; negative values indicate migration towards the anode, measured relative to marker aspartic acid.

Table 3
Lengths of bacteriophage particles

	Bacteriophage	Concentration of IPTG	Contour length (nm) ±S.D. (*n*)	Relative length
A.	fd (wild-type)		866± 37 (59)	1·00
	fdg8R48		891± 32 (34)	1·03
	fdg8Q48		1153± 55 (34)	1·33
	fdg8A48		1178± 57 (34)	1·36
B.	fdg8R48/pKf8P	1 mM	911± 56 (27)	1·05
	fdg8A48/pKf8P	0	1132± 90 (25)	1·31
	fdg8A48/pKf8P	10 μM	1060± 74 (26)	1·22
	fdg8A48/pKf8P	1 mM	988± 78 (38)	1·14
	fdg8Q48/pKf8P	1 mM	1015± 92 (33)	1·17
C.	fd/pKE48	1 mM	1080±147 (60)	1·25
	fdg8R48/pKE48	1 mM	1041±133 (26)	1·20
	fdg8Q48/pKE48	1 mM	1313±199 (53)	1·52
	fdg8A48/pKE48	1 mM	1270±130 (53)	1·47

n, number of bacteriophage particles measured.
A, Results taken from Hunter *et al.* (1987); B and C, results obtained in the present work.

IPTG. As expected, the fdg8R48-hybrid bacteriophage particles were the same length as the fdg8R48 parent, essentially the same as wild-type fd. The average length of the other hybrid virions correlated with the concentration of inducer and the upper and lower limits to particle length appeared to have been set by the two types of coat protein present. For example, in the fdg8A48 hybrid virions no particles as short as wild-type fd or as long as fdg8A48 were observed. There was no obvious preference for any intermediate length.

The growth of wild-type and mutant bacteriophages on pKE48-transformed *E. coli* HB2151 resulted, in each case, in a mean increase in length of the progeny virions (Table 3C) compared with the parental bacteriophages. In some 10% of bacteriophage fdg8Q48-derived virions, the contour length was a little over twice that of wild-type fd. These results all indicate that the mutant fdg8E48 coat protein is able to assemble into a class I viral

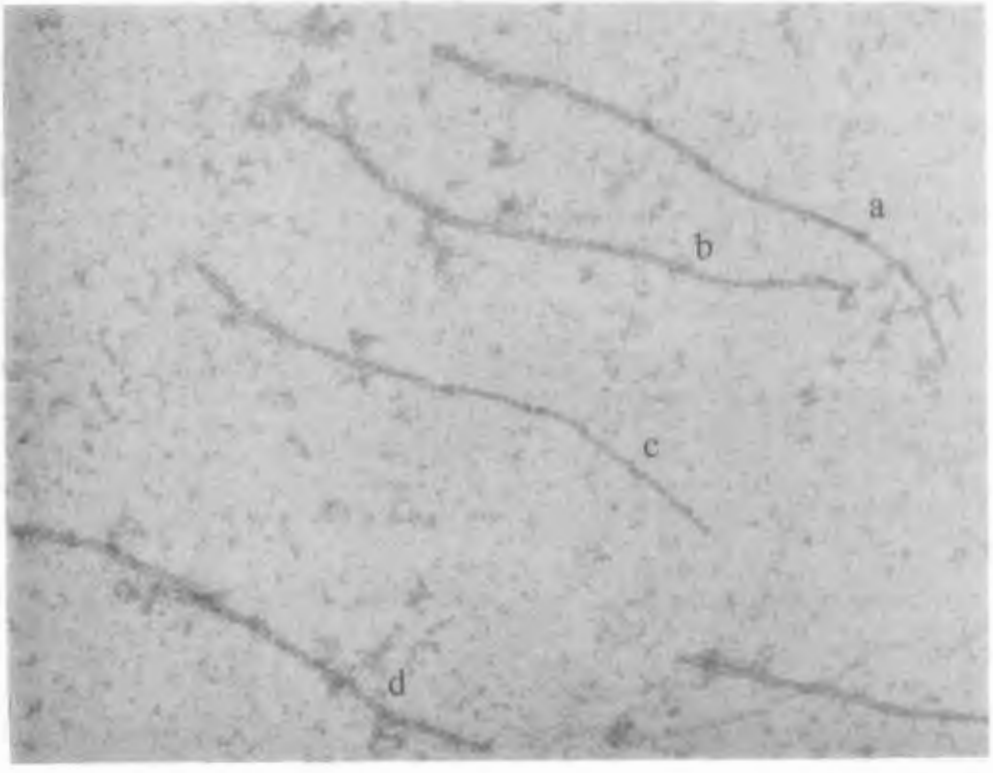

Figure 5. Electron microscopy of hybrid fdg8Q48/pKf8P bacteriophage particles. Bacteriophage fdg8Q48 was grown on *E. coli* HB2151 cells transformed with plasmid pKf8P in the presence of the inducer, IPTG. Four virions (a to d) can be seen, with the following contour lengths a, 1165 nm; b, 1080 nm; c, 995 nm; and d, 945 nm. For further details, see the text.

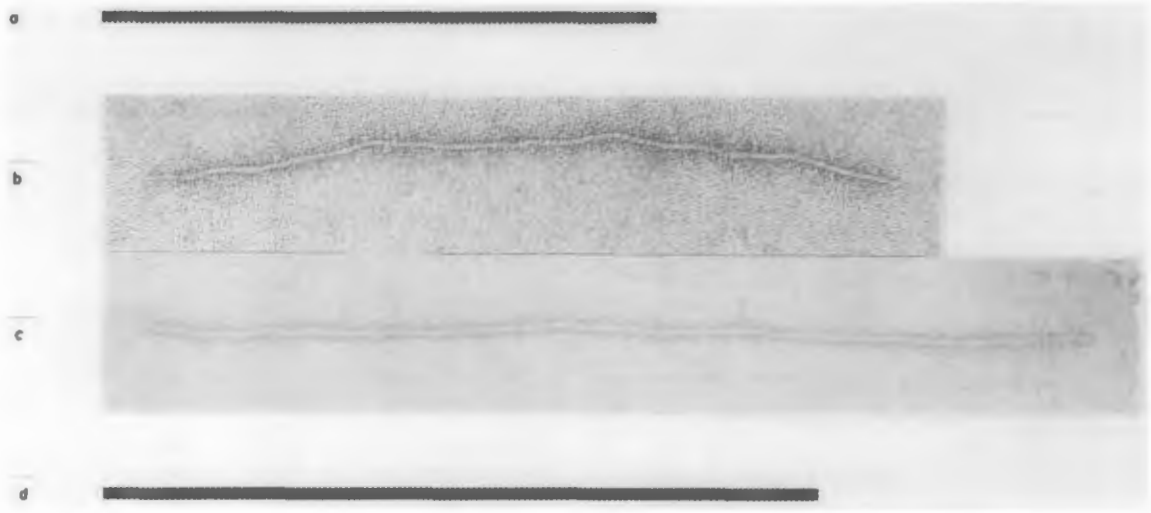

Figure 6. Electron microscopy of fdg8E48 hybrid bacteriophage particles. Particle a, a bar indicating the contour length (880 nm) of wild-type bacteriophage fd. Particle b, a typical virion produced by pKE48-transformed *E. coli* HB2151 cells infected with bacteriophage fd in the presence of the inducer, IPTG. Particle c, a typical virion produced by pKE48-transformed *E. coli* HB2151 cells infected with bacteriophage fdg8Q48 in the presence of the inducer, IPTG. Particle d, a bar indicating the contour length (1153 nm) of the mutant bacteriophage fdg8Q48. For further details, see the text.

particle in the presence of another fd-derived coat protein, and that its presence causes the formation of a more extended virion.

4. Discussion

Bacteriophage fd and similar filamentous viruses have been the subjects of extensive genetic and structural studies (Denhardt *et al.*, 1978; Webster & Lopez, 1985). Early in these investigations many conditional lethal mutants were isolated (Pratt *et al.*, 1966), but none was found to map within gene VIII, which encodes the major coat protein. Special techniques allowed for the selection of one amber gene VIII mutant (Pratt *et al.*, 1969) and several pseudo-revertants of this have been characterized (Boeke *et al.*, 1980). The difficulty in isolating mutants in gene VIII presumably reflects the stringent constraints imposed on procoat primary structure to comply with the requirements of biosynthesis and assembly. These include insertion and processing of the procoat at the inner membrane, protein–protein interactions important for nucleation and other steps of viral assembly, as well as DNA–protein interactions, which play a crucial part in determining virus structure. Genetic reconstruction experiments have suggested that the cluster of positive charges found in the C-terminal region of the coat protein (Fig. 1(a)) is of major importance for correct membrane insertion and processing (Kuhn *et al.*, 1986). The successful conversion of the lysine residue at position 48 (Fig. 1(a)) into uncharged alanine, glutamine or threonine residues (Hunter *et al.*, 1987) (see Fig. 1(b)) and, in the present experiments, into the negatively charged residue glutamic acid (Fig. 1(c)), all generating mutant coat proteins still capable of membrane insertion and processing (Fig. 3), demonstrates that positive charge at lysine 48 is not essential. Indeed, this part of the assembly process can evidently occur with a net positive charge of only two on the side-chains in the C-terminal region of the protein. In this context, it is perhaps relevant that the major coat protein of bacteriophage Pf1 (a class II bacteriophage infecting *Pseudomonas aeruginosa*), which carries only two positively charged side-chains in the C-terminal region, is also capable of membrane insertion and processing in *E. coli* (Rowitch & Perham, 1987).

Although it underwent correct membrane insertion and processing, mutant coat protein fdg8E48 was not capable of packaging DNA from bacteriophage R252, an fd-derived bacteriophage that carries an amber mutation in its own gene VIII (Table 1). This may reflect some special requirement for positive-charge density in the C-terminal region of the major coat protein during the nucleation phase of morphogenesis when the subunits may be interacting directly with the double-stranded DNA hairpin found at one end of the particle (Makowski, 1985) or with other proteins of the putative pre-initiation complex at or in the bacterial inner membrane (Webster & Lopez, 1985). The result was not entirely unexpected, as repeated attempts at oligonucleotide-directed mutagenesis in which viral fd DNA was used as template (Hunter *et al.*, 1987) failed to produce fdg8E48 mutant bacteriophage (G. J. Hunter, D. H. Rowitch & R. N. Perham, unpublished results). The method we have now developed for generating and testing such deleterious mutations in gene VIII has enabled the defective step in assembly to be identified and opens the way to further systematic studies of this kind.

We have previously shown that positive-charge density in the C-terminal region of the fd major coat protein has a direct part to play in DNA-packaging and in determining the length of the

finished virus particle (Hunter *et al.*, 1987). Thus, conversion of lysine-48 to arginine-48 retained four positively charged side-chains in this region (Fig. 1) and was without effect on particle length, whereas conversion to alanine-48, glutamine-48 or threonine-48 lowered the number of positive charges to three and led to an approximately 35% increase in particle length. If the protein helix is unchanged but each subunit has less C-terminal positive charge, the observed increase in length could be simply explained by a need for more protein subunits to package the same amount of viral DNA and neutralize the negatively charged phosphodiester bonds, thereby necessitating a longer virion (Hunter *et al.*, 1987).

The hybrid bacteriophage particles described in the present study, with wild-type and mutant coat proteins in the same capsid, strongly support this model of non-specific "delocalized" binding between DNA and the protein capsid, and a DNA–protein interaction that can be varied. The existence of the hybrids, as demonstrated by agarose gel electrophoresis (Fig. 4), peptide mapping (Table 2) and electron microscopy (Figs 5 and 6; Table 3) indicates that the wild-type and mutant coat proteins participate in similar if not identical protein–protein interactions in the generation of the capsid. The smaller number of positive charges in the C-terminal regions of the mutant coat proteins again acted to increase the length of the particle required to encapsidate the same length of viral DNA (Table 3B).

This effect was demonstrated most dramatically with the fdg8E48 coat protein (Table 3C). With a net positive charge of only two on the amino acid side-chains in the C-terminal region, half the wild-type fd value, it would be expected that twice as many mutant coat protein subunits would be needed to encapsidate the fd genome, leading to a particle length of approximately 1760 nm. It proved impossible to determine the full effect of fdg8E48 subunits on viral structure because this subunit turned out to be incapable of supporting virus assembly on its own. However, it may be significant that in the population of fdg8Q48/fdg8E48 hybrid bacteriophage, a few of the particles were some 1800 nm long. These might be carrying a relatively high proportion of fdg8E48 subunits, causing the packaged DNA to be stretched out almost to its limit (see Marzec & Day, 1983). A strict correlation between hybrid bacteriophage types is difficult, owing to possible differences in the expression and processing of coat proteins from both plasmid-encoded and bacteriophage-encoded genes within the same cell. Our results do indicate, however, that the hybrid bacteriophage particles have a length that is dependent upon the ratio of the two coat protein types within the capsid (Table 3).

The filamentous bacteriophages are flexible structures (Figs 5 and 6). We envisage the bacteriophage particle as a hollow tube composed of coat protein subunits whose symmetry is governed by protein–protein interactions and within which the viral DNA must find its own spatial arrangement during the elongation phase of viral assembly. Whether this is influenced in any way by the organization of the DNA in the gVp–DNA pre-assembly complex is unknown, but our results unequivocally demonstrate the pivotal importance of direct electrostatic interactions between the DNA and the coat protein in the capsid.

This mode of DNA–protein interaction is in marked contrast with the RNA–protein interaction in tobacco mosaic virus, the only other helically symmetrical virus for which detailed structural information at this resolution is available. In tobacco mosaic virus, the ssRNA is embedded between the turns of the protein helix and exactly three nucleotides are packaged per protein subunit. This is brought about by a matching interaction between the three negatively charged phosphodiester links and three specific positively charged arginine residues contributed by each protein subunit (Holmes, 1982; Namba & Stubbs, 1986). In the filamentous bacteriophage fd, as we have shown above, the number of nucleotides of the ssDNA packaged per protein subunit is not restricted to any integral value and can indeed be varied from the wild-type value of approximately 2·4 according to the electrostatic charge (positive, zero or negative) contributed by the side-chain at position 48 in the C-terminal region of the major coat protein. Both viruses necessarily demonstrate specificity in the nucleotide sequence of the region of nucleic acid that is assembled first (Holmes, 1982; Butler, 1984; Armstrong *et al.*, 1983; Lopez & Webster, 1983), the ultimate length of the helical particles then being dictated by the length of the nucleic acid encapsidated. In tobacco mosaic virus there is evidence of some variability in the strength of RNA–protein interaction along the virus rod according to the local nucleotide sequence (Perham, 1969; Perham & Wilson, 1978), but in principle any RNA sequence can be accommodated. Similarly, the success of bacteriophage fd (M13) as a cloning vehicle rests on its lack of specificity for the DNA encapsidated during the elongation and termination phases of assembly. Further investigation of the C-terminal region of the major coat protein, particularly of the remaining positively charged residues at positions 40, 43 and 44 (Fig. 1(a)), can be expected to add to our understanding of the processes involved in DNA packaging in the filamentous bacteriophages and in DNA–protein interactions in general.

We thank the Oliver Gatty Fund for the award of a Studentship (to D.H.R.), and the MRC for a research grant (to R.N.P.). We are grateful to Mr J. G. Lay for skilled technical assistance with electron microscopy.

References

Armstrong, J., Hewitt, J. A. & Perham, R. N. (1983). *EMBO J.* 2, 1641–1646.

Banner, D. W., Nave, C. & Marvin, D. A. (1981). *Nature (London)*, **289**, 814–816.
Beck, E., Sommer, R., Auerswald, E. A., Kurz, C., Zink, B., Osterburg, G., Schaller, H., Sugimoto, K., Sugisaki, H., Okamoto, T. & Takanami, M. (1978). *Nucl. Acids Res.* **5**, 4495–4503.
Biggin, M. D., Gibson, T. J. & Hong, G. F. (1983). *Proc. Nat. Acad. Sci., U.S.A.* **80**, 3963–3965.
Boeke, J. D., Russel, M. & Model, P. (1980). *J. Mol. Biol.* **144**, 103–116.
Butler, P. J. G. (1984). *J. Gen. Virol.* **65**, 253–279.
Carter, P., Bedouille, H., Waye, M. M. & Winter, G. (1985). *Oligonucleotide Site-directed Mutagenesis in M-13*. MRC Laboratory of Molecular Biology, Cambridge, U.K.
Cross, T. A., Tsang, P. & Opella, S. J. (1983). *Biochemistry*, **22**, 721–726.
Day, L. A. & Wiseman, R. L. (1978). In *The Single-Stranded DNA Phages* (Denhardt, D. T., Dressler, D. & Ray, D. S., eds), pp. 605–625, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
de Boer, H. A., Comstock, L. J. & Vasser, M. (1983). *Proc. Nat. Acad. Sci., U.S.A.* **80**, 21–25.
Denhardt, D. T., Dressler, D. & Ray, D. S. (1978). Editors of *The Single-Stranded DNA Phages*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Dente, L., Cesareni, G. & Cortese, R. (1983). *Nucl. Acids Res.* **11**, 1645–1655.
Drelzen, G., Bellard, M., Sassone-Corsi, P. & Chambon, P. (1981). *Anal. Biochem.* **112**, 295–298.
Grant, R. A., Lin, T.-C., Konigsberg, W. H. & Webster, R. E. (1981). *J. Biol. Chem.* **256**, 539–546.
Hill, D. F. & Petersen, G. B. (1982). *J. Virol.* **44**, 32–46.
Holmes, K. C. (1982). In *Structural Molecular Biology* (Davies, D. B., Saenger, W. & Danyluk, S. S., eds), pp. 475–505. Plenum, New York.
Hudson, L. & Hay, F. C. (1976). *Practical Immunology*, pp. 8–9. Blackwell Scientific Publications, Oxford, U.K.
Hunter, G. J., Rowitch, D. H. & Perham, R. N. (1987). *Nature (London)*, **327**, 252–254.
Ikoku, A. S. & Hearst, J. E. (1981). *J. Mol. Biol.* **151**, 245–259.
Johnson, D. A., Gautsch, J. W., Sportsman, J. R. & Elder, J. H. (1984). *Gene Anal. Techn.* **1**, 3–8.
Kuhn, A. & Wickner, W. (1985). *J. Biol. Chem.* **260**, 15907–15913.
Kuhn, A., Wickner, W. & Kreil, G. (1986). *Nature (London)*, **322**, 335–339.
Lopez, J. & Webster, R. E. (1983). *Virology*, **127**, 177–193.
Lyons, L. B. & Zinder, N. D. (1972). *Virology*, **49**, 45–60.
Makowski, L. (1985). In *Biological Macromolecules and Assemblies, Virus Structures* (Jurnak, F. A. & McPherson, A., eds), vol. 1, pp. 203–253, Wiley, New York.
Makowski, L. & Caspar, D. L. D. (1981). *J. Mol. Biol.* **145**, 611–617.
Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Marvin, D. A. (1978). In *The Single-Stranded DNA Phages* (Denhardt, D. T., Dressler, D. & Ray, D. S., eds), pp. 583–603, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Marzec, C. J. & Day, L. A. (1983). *Biophys. J.* **42**, 171–180.
Mathes, H. W. D., Zenke, W. M., Grundström, T., Staub, A., Wintzerith, M. & Chambon, P. (1984). *EMBO J.* **3**, 801–805.
Messing, J., Crea, R. & Seeburg, P. H. (1981). *Nucl. Acids Res.* **9**, 309–322.
Morrissey, J. H. (1981). *Anal. Biochem.* **117**, 307–310.
Moses, P. B. & Horiuchi, K. (1982). *Virology*, **119**, 231–244.
Moses, P. B., Boeke, J. D., Horiuchi, K. & Zinder, N. D. (1980). *Virology*, **104**, 267–278.
Namba, K. & Stubbs, G. J. (1986). *Science*, **231**, 1401–1406.
Nelson, K., Friedman, S. & Smith, G. (1981). *Virology*, **108**, 338–350.
Ohkawa, I. & Webster, R. E. (1981). *J. Biol. Chem.* **256**, 9951–9958.
Perham, R. N. (1969). *J. Mol. Biol.* **45**, 439–441.
Perham, R. N. (1978). In *Techniques in Protein and Enzyme Biochemistry* (Kornberg, H. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I. & Tipton, K. F., eds), vol. B110 pp. 1–39, Elsevier, Amsterdam.
Perham, R. N. & Wilson, T. M. A. (1978). *Virology*, **84**, 293–302.
Pratt, D., Tzagoloff, H. & Erdahl, W. D. (1966). *Virology*, **30**, 397–410.
Pratt, D., Tzagoloff, H. & Beaudoin, J. (1969). *Virology*, **39**, 42–53.
Pratt, D., Laws, P. & Griffiths, J. (1974). *J. Mol. Biol.* **82**, 425–439.
Rowitch, D. H. & Perham, R. N. (1987). *J. Mol. Biol.* **195**, 873–884.
Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H. & Roe, B. A. (1980). *J. Mol. Biol.* **143**, 161–178.
Simons, G. F. M., Konings, R. N. H. & Schoenmakers, J. G. G. (1979). *FEBS Letters*, **106**, 8–12.
Simons, G. F. M., Konings, R. N. H. & Schoenmakers, J. G. G. (1981). *Proc. Nat. Acad. Sci., U.S.A.* **78**, 4194–4198.
Towbin, H., Staehelin, T. & Gordon, J. (1979). *Proc. Nat. Acad. Sci., U.S.A.* **76**, 4350–4354.
van Wezenbeek, P. M. G. F., Hulsebos, T. J. M. & Schoenmakers, J. G. G. (1980). *Gene*, **11**, 129–148.
Webster, R. E. & Cashman, J. S. (1973). *Virology*, **55**, 20–38.
Webster, R. E. & Lopez, J. (1985). In *Virus Structure and Assembly* (Casjens, S., ed.), pp. 235–267, Jones & Bartlett Publ. Inc., Boston.
Webster, R. E., Grant, R. A. & Hamilton, L. A. W. (1981). *J. Mol. Biol.* **152**, 357–374.
Wickner, W. (1975). *Proc. Nat. Acad. Sci., U.S.A.* **72**, 4749–4753.
Yamamoto, K. R., Alberts, B. M., Benzinger, R., Lawhorne, L. & Treiber, G. (1970). *Virology*, **40**, 734–744.
Zoller, M. J. & Smith, M. (1983). *Methods Enzymol.* **100**, 468–500.

Edited by A. Klug