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Ff Coliphages: Structural and Functional Relationships

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INTRODUCTION

Filamentous bacteriophages are a group of related viruses which only infect gram-negative bacteria and specifically adsorb to the tip of pili.They are long and thin particles, threadlike, with no organization into head or spikes. Their genetic material consists of a single-stranded, closed circular deoxyribonucleic acid (DNA) molecule. For an excellent review classifying the several types of bacteriophages and describing them in detail, published in 1969, see Marvin and Hohn (187).

In this article, we will deal exclusively with the most thoroughly investigated Ff phages, fd, f1, and M13, which infect *Escherichia coli*. Ff denominates those filamentous phages which require F pili as the host receptor. Accordingly, they are male specific (androphage) and would not use other pili also found on the surface of certain *E. coli* cells. f1 was first isolated at Rockefeller University in 1960 and described by Loeb (171) and Zinder et al. (356); M13 was discovered in Munich (121) and fd was discovered in Heidelberg (185, 186). They resemble each other so closely that it is legitimate to consider them as slight mutations of basically the same phage. Except for a small number of base changes their DNA sequences are identical. The same holds true for the composition of their coats as visualized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Throughout this review, we will therefore generally make no distinction between research done with one or the other phage.

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MORPHOLOGY AND STRUCTURE

The general appearance of bacteriophage fd (f1, M13) is that of a flexible filament, about 900 nm long and 6 to 10 nm thick, with a molecular weight of 12×10^6 (17, 120, 186, 220). Its coat consists of 2,700 to 3,000 copies of a major protein, the product of gene 8 (g8p) (220, 247), and four minor components, the products of genes 3, 6, 7, and 9 (g3p, g6p, g7p, g9p), present in ca. five copies each. It contains no lipid or carbohydrate. The coat envelopes one molecule of DNA that extends the total length of the virus and constitutes ca. 12% of its weight (16, 220). The DNA is single stranded, closed circular, with no Watson-Crick base pairing except for a few specific regions (120, 186). It is about 6,400 nucleotides long, codes for 10 proteins, and has a molecular weight of 2×10^6 (64, 220).

The phage filamentous structure is highly sensitive to mechanical shearing and ultrasonic treatment. Also, in contrast to λ-like phages, it is sensitive to detergents and organic solvents, especially chloroform. On the other hand, the virus is rather resistant to proteolytic and nucleolytic digestion, with the sole exception of subtilisin, which partially digests one of the minor coat proteins, the gene 3 product (187).

By using X-ray diffraction techniques, the molecular architecture of Ff has been extensively investigated, and the following structural features have been discussed (9, 188, 190): the filament is a hollow protein cylinder with an outer

diameter of 6 nm and an inner diameter of 2 nm. Other data concerning the inner diameter (4 nm) have also been reported (320). The DNA molecule is embedded in the cylinder along its longitudinal axis. Two alternative models for the coat protein arrangement around the DNA have been discussed (188–190, 320). The so-called one-strand model proposes that the two antiparallel chains of the DNA molecule extend along the central axial hole of the cylinder. In the alternative "two-strand model" (105), the "up" and "down" strands of the circular genome are separately wrapped with coat protein and then twisted around each other.

The compiled data, however, definitely support the "one-strand model" (320). The DNA packaged in native Ff virus is folded back on itself with no protein inside; the molecule is dense due to the extensively stacked nucleotide bases and has a fixed orientation (65, 282): a major hairpin structure (hairpin A; see section, "Genetics"), which is located near the origins of replication, is invariably found at one end of the phage filament, no matter how long the DNA is (135, 327). The DNA forms a helix with a radius of 0.85 nm and a pitch of about 2.67 nm; the rise per nucleotide is 0.272 nm (9).

The major coat protein subunits form a helix of their own. On the basis of their X-ray diffraction pattern, filamentous bacteriophages can be separated into two classes, I and II. The class II pattern (190), displayed for example by phage Pf1 (infecting *Pseudomonas aeruginosa*) or XF (infecting *Xanthomonas oryzae*), can be interpreted as a simple one-start helix of 1.5 nm pitch with 4.4 (190) or 5.4 (180) U per turn.

fd, f1, and M13, together with phage If1 and Ike, which all infect *E. coli*, belong to class I (188). Their molecular architecture is a perturbed version of the simpler class II helix, yet the two patterns are not interconvertible. The symmetry of class I particles is a five-start helix of protein subunits with an approximately twofold screw axis, 4.5 U per turn, and 1.5-nm pitch (65, 180, 188). The helix is left-handed and has repeated perturbations along its axis.

The major coat protein subunits appear as flexible, α-helical rods (9), "elongated in an axial direction" and sloping radially, "so as to overlap each other and give an arrangement of molecules reminiscent of scales on a fish" (188). They are tilted by about 20° to the main helix axis and firmly interdigitate, each subunit helix fitting into the gap between two molecules of the next turn. More details are contained in references 16, 17, 65, 188–190, 220, and 282.

The current X-ray-derived phage structure is fully consistent with the results gained by solid-state nuclear magnetic resonance at much higher resolution. The latter technique, applied to whole phage as well as to its major coat protein in solution (25, 52, 53, 239, 276), gives information about individual atomic sites. According to the results of Opella and co-workers (239), the DNA-protein interactions substantially immobilize the DNA packaged in phage fd. The coat protein subunits are held rigidly in the structure and display limited mobility except for some aliphatic side chains with rapid rotations. There is no evidence of chemical interactions between DNA and coat protein. The g8p in the phage is almost completely and continuously α-helical, with segments that vary somewhat in their orientation relative to the filament axis (53, 276).

The native architecture of the coat seems to be primarily maintained by hydrophobic interactions between the individual major coat protein molecules (134, 218, 257, 264). The g8p amino acid sequence displays three distinct domains: acidic amino acids are accumulated in the N-terminal part which is exposed on the virus surface and determines the electrophoretic mobility of whole phage in gels (23); near the C terminus basic amino acids predominate and supposedly interact with the negatively charged DNA phosphate backbone (5); and finally, hydrophobic residues are concentrated in the central section of the sequence (336). This hydrophobic "core" is in contact with the corresponding domains of neighboring subunits, leading to the tight packing of molecules in the coat seen in X-ray and nuclear magnetic resonance analyses. The detailed structure of the major coat protein will be dealt with in a later section.

Chaotropic agents such as guanidinium hydrochloride, SDS, or cholate dissociate the phage coat into the major protein monomers. This dissociation is highly cooperative (all-or-none mechanism) (134). The same is true for alkali-induced disassembly (264), whereas heat inactivation of the phage induces intermediate states (262, 263).

One common structural feature of filamentous phages is the existence of still another complex of viral DNA and viral protein during their "life cycle." The protein in this case is the product of gene 5, which has a decisive role in the synthesis of progeny single-stranded DNA and may be regarded as scaffolding protein in the early stage of phage assembly (see below).

In contrast to the major coat protein, which is uniformly distributed along the phage envelope, the minor coat proteins are located exclusively at the ends of the particle. Unfortunately, X-ray analysis of phage fibers provides no information about the exact arrangement of these minor components. This is especially true for the products of genes 7 and 9. They are found together at one end of the filament, while the products of genes 3 and 6 are located at the other (96, 98, 288, 289, 345).

The "adsorption protein" g3p is anchored to the virus coat via the C-terminal part of the polypeptide chain, whereas the N terminus is exposed and mediates the attachment of the virus to the tip of a host F pilus (6). The electron microscope reveals a "knob-on-stem" structure at one virus end which represents the viral adsorption complex (102): the N-terminal part of the protein sequence is folded into the "knob" and is susceptible to subtilisin digestion, thereby diminishing phage infectivity, while the "stem" consists of the C-terminal part or g6p or both.

Most interestingly, in wild-type phages containing unit-length genomes, the sequence of the DNA encoding gene 3 is located at the end bearing g3p, whereas the other end harboring g7p and g9p always contains the major hairpin A at the junction of gene 4 and the intergenic region (IR) (327). The number and distribution of the minor coat proteins in any phage type irrespective of length is always the same (98).

During infection ("eclipse"), a structural transition in the phage coat must take place as the DNA is ejected. Using a chloroform-water interface, Griffith and co-workers have tried to mimic these early events of the infection process in vitro. They found that, in their model system, the long filament contracts in an ordered fashion to an intermediate structure (I-form) of 250-nm length. One of its ends, where the g3p is located, ruptures to an open, flared structure, and upon further contraction two-thirds of the DNA are released through this opening. The process stabilizes after formation of an open hollow spheroid of 40-nm diameter. The part of the DNA remaining in the spheroid is the large IR between genes 4 and 2. This DNA region seems to be thoroughly associated with the phage coat, for example, linked to the minor coat proteins g7p and g9p (107, 172, 182).

The contraction is supposedly brought about by a sequence of small, localized changes in the filament structure, independent of DNA or any of the minor proteins. It is accompanied by a drastic conformational change in the major coat protein, the helical content of which drops from almost 100 to about 85% in the I-forms and to 50% in the spheroids (183).

It is interesting to note Rossomando's results of heating phage f1 in this context (262, 263), since in a sense the intermediate configurations he got are comparable to Griffith's structures. When the virus is heated to 65 to 85°C and investigated by electron microscopy, a short bar perpendicular to the long axis of the filament appears at one of its ends. The rest of the phage remains structurally intact. Further heating increases the branched region at the expense of the intact part (263). These alterations start at the g3p end, since the phage is inactivated, its adsorption protein is altered, and a terminal DNA segment gets solvent exposed (262). Of course, there is no evidence that similar structural transitions take place during infection.

The earliest descriptions of filamentous phages fd, f1, and M13 report the occurrence of miniphage and polyphage in stock solutions (120). Essentially, the length of a phage particle is determined by the length of its DNA, as cloning experiments in the last few years have shown.

The poly- and miniphage content of wild-type phage stocks is around 1%. This amount tends to increase in certain mutants. Mutations in any of the minor coat protein genes 3, 6, 7, or 9 cause the accumulation of polyphage with several times unit length (13, 173, 247). These particles contain unit-length DNA molecules and give rise to normal progeny (with the usual content of "abnormals").

Miniphage or defective interfering particles, on the other hand, do not contain unit-length genomes. However, they always contain the IR which bears the origins of replication and is also required for phage assembly (see section, "Genetics"). The DNA remains circular. Its length may vary between 0.2 and 0.5 times the normal length, reflected in a particle size of also 0.2 to 0.5 times unit length. Miniphage interfere in infection with normal phage but depend on the presence of helper phage for efficient propagation (81, 83, 106). Sometimes they contain partial duplications of the IR. This confers a marked selective advantage as compared with unit-length virions (81, 125).

Serial passages of phage f1 at high multiplicities of infection in addition lead to the appearance of maxiphage (125). They are longer than standard length and harbor the same tandem reiteration of DNA in the region of the replication origins as the miniphage. In the presence of miniphage, maxiphage grow better than wild type.

Comparison with Other Filamentous Viruses

There are some other filamentous viruses not belonging to the Ff group and in part with differing host specificities that have been investigated in some detail. The struture of the DNA is basically the same for phages fd, If1, Ike, and Xf; the circular DNA strand is wound into two antiparallel, right-handed helices, with the bases near the center of the structural axis and the phosphates directed outward, but there are differences in packing details (33, 34). Phages Pf1 and Pf3, however, are supposed to have a DNA structure completely different from that of the four phages mentioned above, namely, an inverted structure (I-helix) with the bases directed outward and the phosphates inside (33, 34, 65, 250, 342).

The major coat protein of phage fd is closely related to those of phages ZJ/2, Ike, and If1 (216, 257).

In a recent thorough investigation comparing fd, Ike, If1, Pf1, and Xf, Thomas et al. (309) have shown that in all viruses examined the predominant secondary structural element of the major coat protein is the α-helix, but the extent varies considerably, ranging from 100% for Pf1 and fd to ~50% for Xf. Pf1, Pf3, and Xf major coat proteins are able to undergo reversible transitions from α-helix to β-sheet while still associated with the DNA, yet fd, Ike, and If1 major coat proteins are not (119, 218).

In phage Pf3 the genome organization, especially in the part coding for the major coat protein and the single-stranded DNA-binding protein (g5p analog), is similar to that of the Ff phages, but there are differences imposed on these proteins by the presumed inverted DNA structure (I-helix) of Pf3 (176, 250).

As has been pointed out recently by Peeters et al. (242, 243), phage Ike has most of its properties in common with fd (M13). Probably these two viruses are derived from a common ancestor as is reflected, for example, in an overall DNA sequence homology of 55%.

GENE PRODUCTS

Survey

The genome of filamentous bacteriophage fd codes for 10 proteins, which differ widely in molecular weight and copy number (15, 114, 272). One of them, the gene 4 protein, has not yet been detected in vivo and its existence is only inferred from the genome organization, in vitro synthesis (152, 210), and the availability of amber mutants.

We first give a short characterization of each of these proteins, as far as data are available (15, 152, 210, 272, 317), and then describe some of them in detail.

Gene 1: The product of gene 1 is permanently needed for virus assembly (286). Recently, it has been shown that g1p interacts with the host's gene *fip* product during phage assembly (166, 269, 270). Gene *fip* of *E. coli* is identical to gene *trxA* encoding bacterial thioredoxin (166, 270). Probably g1p is present in only a few, membrane-bound copies per cell. Its molecular weight ranges from 35,000 (protein band in gels) to 39,500 (as deduced from DNA sequence analysis). Its synthesis is apparently down-regulated, since artificial overproduction of g1p in a plasmid system leads to rapid cell death (123).

Gene 2: The g2p of gene 2 has a highly strand- and site-specific endonuclease and topoisomerase activity, responsible for cleaving ("nicking") the viral strand (VS) of the replicative form (RF) DNA and thus enabling RF replication to take place. In its absence, no DNA synthesis beyond the parental complementary strand (CS) formation can occur. The protein also closes linear viral DNA strands to circles. It is probably membrane bound and has a molecular weight of ca. 46,000. It is synthesized in ca. 10^3 copies per cell and its gene expression seems to be under the control of g5p (209, 349).

Gene X: Gene X is completely contained within gene 2 and transcribed in phase with it. The protein consists of the C-terminal, 111-amino-acid-long portion of g2p, corresponding to a molecular weight of 12,600 (317). It cross-reacts with antibody against g2p, yet it is not produced by proteolysis of the latter, since its N-terminal amino acid (methionine) is N formylated (348).

The protein has been identified in vitro as well as in vivo. Its function is not yet fully understood, but evidently it is

required for normal phage DNA synthesis. In the absence of gene X, VS synthesis especially is drastically impaired (88). gXp is synthesized to a maximum of 500 molecules per cell (209, 349).

Gene 3: The g3p of gene 3 is the attachment or adsorption protein of the phage (247), localized in five copies at exclusively one end of the particle. It is also responsible for maintenance of the virus' structural integrity and for correct morphogenesis to unit-length phage (51, 114, 173, 247, 262–264). It is synthesized as a precursor containing 18 additional amino acids at its N terminus (152). Probably due to the remarkable glycine/serine clusters in its sequence, it migrates abnormally in polyacrylamide gels and, hence, the difference between its apparent molecular weight and the one calculated for the mature protein from the DNA sequence is unusually large (56,000 to 70,000 and 42,000, respectively).

Gene 4: The product of gene 4, with a molecular weight of 48,000 to 50,000 as observed experimentally, is probably bound to the inner membrane and responsible for virus assembly in a way similar to g1p.

Gene 5: The g5p of gene 5 consists of 87 amino acids of known sequence and has a molecular weight of approximately 10,000. It binds specifically and cooperatively to single-stranded DNA (1) and has a regulatory role in the process of phage DNA synthesis: it initiates the "switch" from RF replication to progeny VS synthesis (271), and, as it controls expression of gene 2 (349), it indirectly influences the level of RF replication also (209). It protects the single-stranded viral DNA in the host cytoplasm and is replaced by g8p during phage assembly.

g5p is the most abundant of all of the phage-encoded proteins, being present in more than 10^5 copies per cell (20 to 30 min postinfection) (1, 349). Its structure has been extensively studied. It is a highly economical protein which serves a maximum of physiological requirements with a minimum of structure.

Gene 6: g6p of gene 6 is one of the minor coat proteins, located in about five copies at the same end of the phage as g3p. Probably it also constitutes part of the "adsorption complex." It is involved in virus morphogenesis, but little is known about its exact biological function. The protein is 112 amino acids long.

Genes 7/9: The products of genes 7/9 have almost identical molecular weights (3,580 and 3,650) and were originally regarded as one protein. After establishment of the phage DNA sequence, they were identified as two different gene products. They are minor coat proteins, present in about five copies each at the end of the phage filament opposite to the other minor coat proteins g3p and g6p. Their precise function is still unknown.

Gene 8: Due to its natural abundance as major coat protein (2,700 to 3,000 copies per virion), the gene 8 product has been subject to detailed analysis (7, 247): it is 50 amino acids long in its mature form (molecular weight, 5,200) (217) and synthesized as a precursor with a 23-amino-acid-long "signal peptide" at the N terminus (305). It displays remarkable structural features and is able to adopt several distinct conformations depending on its environment. g8p in its host membrane integrated form has become a favorite model for investigations of membrane protein translocation.

Replication Proteins

Gene 2 protein. In the early stages of research on Ff phages, two observations pointed to the function of the gene 2 product: no RF DNA replication was possible when this gene product was missing (114, 245), and amber mutations in gene 2 were not lethal to the host cells (122), in contrast to infections with phage mutated in any other gene.

g2p is the key enzyme for viral DNA replication; in its absence, the state of the cell resembles that prior to infection. It is absolutely required for replication of the double-stranded RF DNA as well as for synthesis of progeny single strands (167, 191, 245).

g2p is a soluble, globular monomer, heat labile at temperatures above 42°C (in vivo as well as in vitro) and possibly membrane attached (204). It may be extracted from infected cells as part of a dense complex which seems to be associated with the inner membrane. Its copy number may be increased under certain conditions, for example, elevated temperature or inactivation of gene 5, which lead to g2p accumulation (168, 328).

g2p has been successfully synthesized in in vitro coupled transcription-translation systems with isolated RF DNA as template (152, 210). The protein is a site- and strand-specific endonuclease: it specifically cleaves ("nicks") the VS of RFI DNA, thus converting it into RFII and enabling VS synthesis, and hence RF replication, to take place. Only the parental RFI DNA is found in amber 2-infected *E. coli* and solely RFI DNA, when gene 2 is blocked later in the infection cycle (167). This "nickase" only uses supercoiled RFI DNA of filamentous phages; it does not cleave single-stranded DNA, relaxed RF, or other supercoiled DNAs (204, 205). The only cofactor needed is Mg^{2+}; the enzyme is inactive in the absence of divalent cations (205). The cleavage site which corresponds to the origin of VS formation has been precisely mapped in the phage genome: it is located in the IR between nucleotides 5781 and 5782, near the origin of CS synthesis (208). Its immediate structural environment is a region of rotational symmetry, which suggests that the enzyme recognizes a hairpin in the DNA.

g2p does not bind covalently to the cleaved VS and accordingly "cannot conserve energy like ΦX gene A protein" (111, 205).

In addition to its endonuclease function, the enzyme has a second activity: it generates unit-length viral DNA strands after each round of replication and seals them to the closed circular form (111, 202, 205). This "sealing activity" depends on the Mg^{2+} concentration and the presence of other divalent cations.

In vitro, the enzyme has to be present during the synthesis of progeny DNA, as it cannot exert its function on multiple-length single strands (202).

The cellular level of g2p correlates with the content of RFI DNA (204), which is physiologically reasonable, and is regulated by g5p (209, 349). In the presence of a functional g2p, the amount of cellular RF DNA increases. Since transcription and translation probably take place from every one of these DNA molecules (191), the amounts of g2p and g5p also increase. Finally, g5p inhibits synthesis of g2p, probably by blocking translation of its mRNA. In this stage, approximately 20 to 30 min postinfection, g2p is no longer needed in very large quantities, since a "pool" of RF molecules is established and DNA synthesis switches to production of progeny viral single strands. In the absence of functional g2p, no DNA replication takes place and almost no g5p is formed. On the other hand, when g5p is defective, g2p accumulates and RF DNA replication and protein synthesis proceed, but no virus assembly is possible and the cell finally dies. g5p in this way indirectly regulates the level of expression of the phage's genome.

Two remarkable aspects of gene 2 expression should be mentioned. First, there are two in-phase initiation codons for the synthesis of g2p and both are used (15, 203, 317). Accordingly, two versions of the enzyme exist. Both start with methionine which is found nonformylated in the mature protein; the minor species (10 to 13% of all synthesized g2p) is three amino acids shorter than the bulk protein. The physiological significance of this phenomenon is unclear; it might have regulatory functions or some properties of the protein may be altered.

Second, the nucleotide sequence for g2p codes for still another protein, namely, X protein (348). Gene X is completely contained within and transcribed in phase with the C-terminal 27% of g2p; protein synthesis is due to an internal start codon (15, 317) and is also under the regulatory control of g5p.

Recently, gene 2 has been cloned into expression plasmids (141, 206). These systems enable a large-scale production and isolation of biologically active g2p.

Gene 5 protein. g5p, a DNA-binding protein, is the most numerous of the phage-encoded proteins in the infected cell (1, 233). It is present in 1×10^5 to 2×10^5 copies, an amount which already points to a structural rather than an enzymatic role for this protein in the replication of viral DNA (257). Due to its natural abundance, it can be easily prepared in large quantities, and its purification and characterization are facilitated through its DNA-binding properties. The protein has also been synthesized in vitro in biologically active form in a coupled transcription-translation system with phage RF DNA (151, 153, 154).

g5p is a primary translation product and does not undergo further modification; it is globular and soluble. The monomer has a molecular weight of 9,800 and consists of 87 amino acids of known sequence (54, 215). It contains no tryptophan. Almost all of the hydrophobic residues are clustered in the middle of the polypeptide chain, while basic residues are located at both ends of the protein. The protein has been crystallized in active conformation (194) and subjected to X-ray analysis (27, 28, 193, 196). The results of these investigations supported earlier conclusions about the structure of g5p found by spectrophotometric analysis (49, 63): the protein contains no α-helical secondary structure and very little random coiling; the main structural element is the β-conformation. The polypeptide chain is folded into a three-stranded antiparallel sheet, comprising the first two-thirds of the sequence, a two-stranded β-ribbon, and a broad, flexible connecting loop (27). The protein may be visualized as a central hydrophobic core with three functional β-loops extending from it: a DNA-binding loop, consisting of amino acids 15 to 32; a complex formation loop (residues 33 to 49); and a dyad loop (residues 62 to 82). The connecting band is made up of amino acids 51 to 61; the DNA-binding and the complex loop together form the three-stranded β-sheet, while the dyad loop structurally corresponds to the two-stranded β-ribbon. The C and N termini are located close to each other. As a whole, the g5p structure is highly compact and dense. Two monomers unite to an even more compact, globular dimer, which is stabilized by hydrophobic interactions and constitutes the active species in DNA binding. Under physiological conditions, the protein is present predominantly as a dimer (39, 233, 249, 251). The presence of DNA or oligonucleotides (8 bases or more) triggers an association of several g5p dimers to larger aggregates, with lysine residues 7 and 69 being mainly involved in contact formation between the species (12, 251).

g5p binds tightly and cooperatively (39) to single-stranded DNA but not to native double-stranded DNA (1, 233). In the absence of Mg^{2+} it denatures ("unwinds") double-stranded structures (1). Binding to DNA is not affected by pH (in the range of 2.5 to 10.3) (76), yet it is sensitive to salt, especially divalent cations; they dissociate the complex (4). The dissociation constant K_D for the DNA-g5p complex is $<5 \times 10^{-8}$ M (4, 50).

Binding to single-stranded (4) and double-stranded ribonucleic acid (RNA) (103) has also been reported. In the latter case, the RNA molecule is not denatured by g5p, and the bases remain paired and stacked. Evidently, the binding mode of g5p for double-stranded RNA and the structure of the resulting complex are completely different from the complexation of single-stranded DNA which occurs normally in the infected cell.

The protein has a much higher affinity for DNA than for short oligonucleotides (4 to 8 bases) (4, 76). In fact, it probably has two different DNA-binding modes, one specific for oligonucleotides and another one specific for polynucleotides. The two modes differ with respect to stoichiometry as well (three and four nucleotides covered per polypeptide chain, respectively) (2; N. Alma-Zeestraten, Ph.D. thesis, University of Nijmegen, Nijmegen, The Netherlands, 1982). This may at least partially be a consequence of the protein's cooperative binding character: if one dimer has associated with a longer DNA chain, binding of all of the next ones side by side to the preceding molecule(s) is facilitated through protein-protein interactions. In the case of short oligonucleotides, however, each protein unit has to start binding anew, with no initiating species present on the same molecule.

Binding is independent of the nucleotide sequence (233), although there are variations in affinity towards homopolydeoxynucleotides of the four bases: most pronounced is a preference for adenine (49, 50).

The precise number of nucleotides covered by each g5p monomer is still a matter of controversy. Originally and most often, a ratio of four nucleotides per protein monomer was found (1, 4, 63). The in vivo isolated g5p-DNA complex differs structurally from that reconstituted from DNA and protein in vitro (165, 246, 249). The value of four nucleotides seems to be typical for the in vitro complex, while in vivo, five nucleotides per protein monomer are more likely to hold true (corresponding to 1,600 and 1,300 g5p monomers per DNA of 6,400 bases, respectively) (246, 249, 257, 310). Alma et al. (2) found three nucleotides covered by one g5p monomer in vitro when using an excess of medium-sized oligonucleotide (chain length, 25 to 30). When the complex was shifted to excess protein, the binding mode changed to four nucleotides.

Mainly aromatic and basic amino acid residues of g5p are involved in the DNA-binding process. According to the current model, derived from investigations using several techniques (circular dichroism, nuclear magnetic resonance, fluorescence, X-ray, cross-linking), binding proceeds via the following mechanism (4, 27, 28, 49, 193, 196, 249; Alma-Zeestraten, Ph.D. thesis): the negatively charged phosphate backbone of the DNA is first recognized by the protein and drawn and fixed in the interior of the binding channel by electrostatic interactions with lysine and arginine residues. Then the protein undergoes small conformational rearrangements to bring aromatic side chains down to stack upon the bases of the associated DNA. The DNA itself is completely unstacked and fully stretched in the complex, whereas binding does not induce large changes in the protein's overall shape (28, 63). Three of the five tyrosines of g5p (no. 26, 34,

and 41) are accessible to modifying reagents in the uncomplexed protein; the other two are buried. In the complex with DNA all tyrosine residues are inaccessible (4). The fluorescence of tyrosines 26, 34, and 41 is quenched upon binding. Hence these three "surface tyrosines" are the most likely candidates to stack upon the DNA bases.

Lysines are exposed on the surface in free protein as well as in the DNA complexed form. One to two lysine and two to three arginine residues seem to participate in binding (Alma-Zeestraten, Ph.D. thesis); acetylation of all six lysines of g5p prevents complex formation (4).

The single cysteine residue of g5p is unlikely to play an active role in DNA complexation (4, 27, 196, 241).

To sum up, the amino acids directly involved in DNA binding are tyrosines 26, 34, and 41, phenylalanine 73, lysine 46, and arginines 16, 21, and 80.

The g5p-DNA complex at first sight appears morphologically similar to the structure of the assembled phage: it is also filamentous, a very flexible rod; somewhat longer (1.1 μm) and significantly thicker (16 nm) than the virus (246). Nevertheless, there are distinct differences between the complex and the phage. Whereas the major coat protein subunits are α-helices and the DNA in the virus displays a highly stacked arrangement, g5p has mainly β-conformation and the DNA bases are unstacked in the complex (63). The g5p-DNA complex is more readily dissociated by destabilizing agents than virions. Some amino acid side chains of g5p are sufficiently close to the DNA to allow ultraviolet irradiation induced cross-linking between g5p and the phage DNA (3). Covalent adducts were formed upon irradiation of the in vivo as well as in vitro complex (3, 165, 240, 241), yet the portion of g5p susceptible for cross-linking varied in different studies. In vitro, an association of the cysteine residue with a thymine was demonstrated (241), while in vivo, the link occurred near the C terminus, between amino acids 70 and 77, pointing perhaps to a participation of serine 75 (165). Paradiso and Konigsberg recently presented data showing the involvement of cysteine in the photochemical cross-link for the complex isolated from infected cells (240).

The DNA-binding unit of g5p in vivo is the dimer. Since the DNA is circular but extended in the complex, it must be folded into two oppositely oriented strands. Therefore, it is reasonable to assume that each protein dimer binds to both the up and down running DNA strands in a way similar to a ladder rung. This model still has to be proven. Also it is not yet clear whether the DNA is on the outside of the complex, wound in an open helix on the backbone of protein dimers, or whether it is located in the interior and shielded by, for example, the very flexible g5p-binding loop(s) (27, 28, 193, 196, 310).

In crystalline complexes with short oligonucleotides, the basic g5p unit is composed of six dimers; in the in vitro reconstituted complex of DNA and g5p, six dimers are spaced along each turn of the helix (195).

Brayer and McPherson (29) recently presented a structural model for the intracellular g5p-DNA complex which is in excellent agreement with the experimental data gathered so far. Surprisingly, the computer analysis yielded only one satisfactory mode of association of DNA and g5p dimers. That is, the DNA is wound on the outside of the helical potein band which constitutes the core of the complex with a ration of five nucleotides per protein monomer. The DNA strands are deeply embedded in the continuous binding channels of associated dimers. Amino acids 38 to 42 of g5p change their conformation upon binding and act as a stereochemical "switch" allowing or prohibiting the dimer-dimer association depending on the presence or absence of DNA. The full complex contains 637 dimers. The model does not, however, answer the question of what the ends of the complex may look like, where the circular DNA "turns."

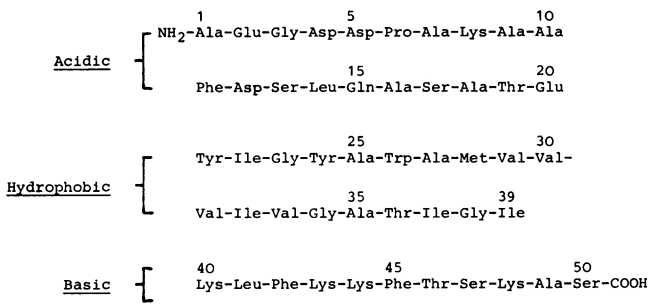

FIG. 1. Amino acid sequence of gene 8 protein (7, 217).

Due to the morphological similarity between the g5p-DNA complex and mature phage and to the "precursor" role the former has for assembly of the latter, efforts were made to detect minor protein constituents of the g5p-DNA complex. Very interestingly, purified in vivo complex was found to always contain three specific proteins of molecular weights 60,000, 50,000, and 25,000 in addition to g5p, which account for about 5% of the total weight (240). Their nature is unknown; the 60-kilodalton polypeptide in any case is not the viral gene 3 product. Grant and Webster (100) also proved that no other phage-encoded protein except for g5p is present in the complex, especially none of the minor coat proteins, but one to three molecules of *E. coli* single-stranded DNA-binding protein seem to be firmly associated with it.

The physiological role of g5p is to bind to newly formed VS DNA, protect it from host nucleases, and prevent synthesis of the CS (271). Thus g5p initiates the last stage of the viral DNA replication, the production of exclusively viral single strands which are then packaged into new phage particles (see later sections).

Finally, a regulation of g2p and gXp formation by g5p has been detected in vitro and in vivo (209, 349), indicating that this protein is a main control element for the synthesis of any type of phage DNA. The repression of g2p messenger RNA (mRNA) translation is very specific and suggests a sequence recognition by g5p (87). In addition, the single-stranded DNA-binding capacity of this protein is perhaps independent of its regulatory function, since a point mutation in gene 5 which leads to a vast overproduction of g2p and gXp seems not to affect g5p itself (73).

Coat Proteins

Major coat protein. Gene 8 codes for the major coat protein (109, 247). The mature protein is 50 amino acids long, with a corresponding molecular weight of 5,200. The sequence (7, 217) is given in Fig. 1.

The protein lacks histidine, arginine, and cysteine. It is highly hydrophobic and aggregates in aqueous solution. A remarkable feature of its primary structure is the segregation of amino acids with similar functional side chains in three domains which are indicated in Fig. 1: acidic amino acids predominate at the N terminus (residues 1 to 20), 4 of the 11 C-terminal amino acids are lysine residues, and the central part (positions 21 to 39) is hydrophobic. This amino acid distribution clearly corresponds to the functions of g8p as

the major phage coat component: the acidic N-terminal part is in contact with the solvent, while the opposite end of the protein probably interacts via its basic residues with the negatively charged DNA phophate backbone (5, 187). The protein only binds to single-stranded, not to double-stranded DNA (149).

g8p is initially synthesized as a precursor with an N-terminal signal peptide of 23 amino acids (305). This extension conforms to the specific character defined for signal sequences by Inouye and Halegoua (136). To conform with usage common in the eucaryotic world, we will use the term "precoat" for the precursor form (instead of Wickner's "procoat"). Precoat is probably generated in soluble form in the cytoplasm on free polysomes (138). Processing to mature coat protein, i.e., cleavage of the signal peptide by a specific peptidase, takes place in or at the membrane and requires no other phage-specific proteins (181, 285, 286).

Newly synthesized g8p molecules are always found associated with or integrated into the host's inner membrane, and it is generally assumed that they are "stored" there until progeny phage is assembled (293, 326).

Recognition and cleavage of precoat by its signal peptidase is an absolute requirement for the viability of mutants defective in gene 8 (156, 157). A g8p species altered in such a way that it keeps the enzyme from processing is lethal to the phage and its host. Especially mutations in the leader peptide affecting the highly conserved amino acid residues −6, −3, and −1 (relative to the cleavage site) are deleterious. The mutated precoat proteins are synthesized in normal amounts and correctly inserted in the membrane, yet they are not recognized by leader peptidase and accumulate in the membrane in their precursor form (157).

Up to now, the "permitted" mutations seemed to be restricted to the N-terminal 11 codons of the mature protein (23). Only one conditional lethal mutant has been isolated: the M13-derived strain am8H1 (114). Its amber mutation changes the GAG-triplett coding for Glu-2 in mature coat protein and is suppressible by insertion of serine at this position (20). The mutated precoat protein thus produced allows growth of the phage at almost normal rates.

Plaque formation by M13am8H1 can be supported by g8p cloned into a plasmid (156). This complementation assay has been used to search for mutants in the cloned gene 8 which fail to produce a fully functional protein. The mutants detected fall into three classes: they synthesize no precoat at all, or the precoat cannot be processed to mature coat, or the coat protein is not assembled into infectious virus (156).

Phenotypic mixing of two different types of g8p in the same coat during phage assembly is possible (211, 212, 247).

g8p has a fascinating characteristic. It is capable of adopting two distinct conformations in response to its respective environment: an almost 100% α-helical secondary structure when the protein is integrated in the phage coat and the "50% α-helix" state present in membranes, phospholipid vesicles, deoxycholate micelles, or certain detergents (19, 232). In the latter conformation, the ends of the protein account for the α-helix. The hydrophobic core (~30% of the polypeptide chain) assumes β-structure and the remaining 20% of the chain is random. The protein is dimeric in this state. Nozaki et al. (231, 232) thoroughly investigated the different conformations under several experimental conditions. They detected some other states which are only present in vitro and may not be relevant for in vivo functions (341), but may yield some valuable information about the factors stabilizing the protein's native structures. A most interesting finding is that the transition from the 100% α-helical conformation to any other form is essentially irreversible. This means that, whenever a mature, processed g8p molecule dissolves in a membranous structure, it will hardly be able to (re)gain its "phage coat conformation." Hence, it is difficult to explain how parental protein deposited in the host membrane during infection can be reused for progeny assembly (see next section).

The 50% α-helix form was observed in lipid environments and in ionic detergents, whereas in nonionic detergents the protein aggregates irreversibly to a complex "β-polymer." Concentrations of ≧7.35 M guanidinium hydrochloride dissociate g8p into a random coil state, which is interconvertible with the 50% α-helical form. The 50% α-helical conformation is stable even in SDS. In 10 mM deoxycholate, the protein is present as a stable dimer; upon removal of the detergent it undergoes aggregation to complex filamentous structures (38, 179).

Solid-state nuclear magnetic resonance of g8p solubilized in SDS micelles gave the same results (25, 52, 239). The protein is dimeric in the detergent and has a stable, native folded structure which differs from that of a typical globular protein or the conformation in the virus coat by having a partially flexible backbone and some rapidly rotating aromatic rings (phenylalanine and tyrosine). Its rigid hydrophobic midsection spans the micelle with the hydrophilic termini exposed to solvent.

g8p readily integrates into native or reconstituted membranes, synthetic phospholipid vesicles, and micelles. Its conformation seems to be largely independent of their composition. This ability to behave as a "typical" integral membrane protein has made g8p a favorite object for studying protein insertion into membranes. The consequences such insertions have on the lipid environment, for example, disturbance of the bilayer architecture and composition, and the nature of the lipid-protein interactions have been investigated in detail (35, 38, 40, 41, 109, 110, 130, 148, 179, 231, 295, 344, 346).

In vivo, the orientation of the mature gene 8 product in its host inner membrane is asymmetric: its N-terminal part of about 20 amino acids is exposed on the outside (protrudes into the periplasm), and the C-terminal 11 residues are exposed to the cell cytoplasm (234, 329, 330). Since the N terminus is exposed on the surface of the phage coat too, it constitutes the protein's antigenic site. Antibodies recognize about the first eight amino acid residues (329, 330). The central hydrophobic section (Trp-26 to Lys-40) is the "membrane anchor" which retains the molecule in the bilayer (331). This asymmetric orientation is independent of the insertion mechanism, since it is the same for parental protein (coming from outside) and newly synthesized molecules (inserted from the cell interior) (329). That means that orientation is determined mainly by the protein structure and, as reconstitution experiments have shown, to a lesser extent by the physical state of the bilayer: correct asymmetric incorporation is maximal near the lipid phase transition temperature (331, 358). In particular, the orientation seems to be independent of other (membrane) proteins and the asymmetry of membrane lipids (330).

Since in the natural succession of events the majority of the major coat protein molecules encounter the cell membrane as precoat protein, special attention has focused on synthesis, membrane insertion, and processing of this precursor form. Two main hypotheses have been formulated for the general molecular mechanisms by which newly synthesized secretory or integral membrane proteins are translocated across or inserted into a biological membrane:

the "signal hypothesis" (18) and the "membrane trigger hypothesis" (332). Both are based on the finding that many excreted and some integral membrane proteins in procaryotic as well as eucaryotic cells are initially made as larger precursors with a 15- to 30-residue-long signal sequence at their NH_2 termini.

According to the signal hypothesis (200), this sequence, after emerging from a eucaryotic ribosome, is quickly recognized by the cytoplasmic signal recognition particle (321–324). The signal recognition particle binds to the ribosome, leading to an immediate cessation of translation. The block is removed only when the elongation-arrested complex contacts membrane-bound receptor, called "docking protein" (94, 95, 201). Thereupon translation resumes and translocation commences in a still unknown way, perhaps by formation of a transmembrane proteinaceous pore. The membrane selects signal sequence-bearing nascent chains by means of specific receptors, and transfer includes a transient cessation of protein synthesis until the presence of an "export site" is "guaranteed." Export is assumed to be strictly cotranslational. Removal of the signal sequence is usually concomitant with it and may happen before or after protein synthesis is completed.

The signal hypothesis has been found to fit the situation encountered in procaryotes as well (see, for example, reference 158), and it accounts for many experimental data, for example, for the observation that excreted proteins seem to be primarily, if not exclusively, synthesized by membrane-bound polysomes. It may be adapted to the situation of membrane proteins by postulating more export signals in addition to the signal sequence, for example, a "stop transfer" and an "insertion sequence" (284).

Wickner's "membrane triggered folding hypothesis" (abbreviated to "membrane trigger hypothesis") (332–334, 336), on the other hand, suggests that the main function of the signal peptide is to promote folding of a hydrophobic membrane protein into an initial conformation which is compatible with the aqueous cytoplasmic environment. Membrane insertion then may start either co- or posttranslationally. The target membrane itself triggers refolding of the precursor into a lipid-soluble conformation, the protein penetrates the bilayer, and cleavage of the signal sequence makes the process irreversible.

In the membrane trigger hypothesis, no ribosome-membrane contact is needed for protein translocation nor are membrane-bound or cytoplasmic receptors or a transmembrane protein tunnel necessary.

Wickner and co-workers have compiled many data in support of the validity of their theory for precoat/coat protein membrane integration. In short, they found that (i) precoat protein is synthesized in the cytoplasm on free polysomes as a soluble species (60, 137, 138, 340); (ii) precoat associates spontaneously and posttranslationally with membrane vesicles or liposomes added after protein synthesis is completed (97, 181, 325, 340); (iii) precoat in vitro and in vivo is the precursor of coat protein (138); (iv) "signal peptidase," a processing enzyme newly discovered in the course of these studies (343, 357, 359), which specifically cleaves the signal peptide of precoat (and other preproteins), is able to split its substrate co- or posttranslationally to give processed, mature, correctly membrane-inserted coat protein (181, 236, 285, 325).

Membrane integration and processing of precoat are affected by Mg^{2+} ions and depend on an energized membrane state; when the membrane's electrochemical gradient is destroyed with uncouplers, precoat associates with the membrane inside, but is not integrated (58, 61). Dependence on the membrane potential for translocation has been reported for other secreted proteins also, for example, *E. coli pro-ompA* protein (352).

Signal peptidase and *E. coli* phospholipids are the only components required for correct binding, insertion, and processing of precoat protein in reconstitution experiments. No phage-encoded proteins, no other host-encoded cytoplasmic or membrane proteins, and, in particular, no ribosomes or ongoing protein synthesis are necessary (61, 285, 325, 340). Assembly and processing have to take place if not during synthesis, shortly after the protein is completed, i.e., within a few minutes. Otherwise precoat denatures in some unknown way and loses its competent conformation (97).

For most of these investigations a gene 7 amber phage mutant was used, since its precoat processing is delayed and thus the individual steps of the process are more easily discerned. These so-called amber 7 infections are "pathological," however, because phage maturation is prevented. Since this point has been criticized by others (267), Wickner and co-workers repeated some of their experiments with wild-type phage to demonstrate that the results in either case are the same (60).

Nonetheless, their work is still in dispute. Conflicting evidence has been presented by other authors which, at least partially, is more consistent with the signal hypothesis. Chang et al. (43, 44) found correct precoat cleavage to be strictly cotranslational. In their in vitro coupled transcription-translation system, an inverted vesicle fraction of *E. coli* inner membrane had to be present during precoat protein synthesis if mature coat was to be produced.

Russel and Model (267, 268) were not able to detect the soluble cytoplasmic form of precoat. Using a new method of membrane preparation, they showed the precursor to be an asymmetrical integral membrane component (268). The precoat synthesized in mutant M13am8H1R6 (a gene 8 amber pseudorevertant) is a poor substrate for signal peptidase and thus its processing is impaired (351). Nevertheless, it is found exclusively in the membrane fraction (267).

Other experimental results reported by Wickner and co-workers can also be questioned. For example, precoat and coat protein are both claimed to be found in soluble as well as membrane fractions (137). The explanation given for the occurrence of "cytoplasmic" mature g8p is unsatisfactory in the light of Wickner's membrane trigger hypothesis. In addition, no discrimination is made between membrane binding and membrane insertion of precoat.

The hypothetical reversibility of the mutant M13am8H1R6 precoat membrane insertion is also unreasonable (351). In recent publications, Wickner and co-workers admitted that precursor "cleavage is not necessary to render insertion irreversible" (56, 157).

Minor coat proteins. By standard electrophoretic techniques, g3p appeared to be the only additional protein component of the phage coat besides the major coat protein (114, 247). In 1978–1979, once the viral DNA sequence was established, three additional minor coat proteins were detected, the products of genes 6, 7, and 9, by growing M13 in the presence of radiolabeled amino acids. The minor coat proteins are present in about five copies each per phage particle and are located exclusively at the ends, with the gene 3 and 6 products together at one end of the filament and the g7p/g9p at the opposite end (96, 98, 99, 170, 262, 287–289, 345).

g3p is the adsorption protein of the phage, mediating recognition of and attachment to the tip of an F pilus on the surface of a male *E. coli* cell (114, 247). It is the N-terminal part of the protein which brings about this attachment (219), since this part competes with intact virus for binding to F pili (6, 169). Also, subtilisin treatment of whole phage releases a large fragment of the protein which was shown to be its N-terminal portion; infectivity in this case is lost (6, 98, 102).

The protein's C-terminal part retains it in the virus coat; it is indispensable for phage morphogenesis and stability (51). The same domain anchors g3p to the host membrane where it seems to be stored after synthesis (21). The fine structure of this membrane anchor domain has been thoroughly investigated, using gene deletions of various lengths (62). Like the major coat protein, g3p has a hydrophobic domain and tends to aggregate to oligomers. It is only soluble in detergents, for example, deoxycholate (96, 170, 280, 345). It is synthesized initially in a precursor form, containing 18 extra amino acids at its N terminus (15, 317). The precursor form could be obtained in an in vitro coupled transcription-translation system (152, 210). The molecular weight of the mature, processed g3p as calculated from the nucleotide sequence is approximately 43,000. In polyacrylamide gels, considerable variations in molecular weights were observed. This is probably the consequence of the several glycine/serine clusters in its sequence which may reduce the amount of SDS associated to the protein, thus decreasing its electrophoretic mobility. The apparent values range from 56 to 70 kilodaltons (96, 152, 210, 280, 345).

Gene 6 codes for a protein which may also be part of the knob-on-stem adsorption apparatus of the phage (102). The protein consists of 112 amino acids, has a molecular weight of 12,350, and is highly hydrophobic. It contains no histidine. None of the g6, g7, and g9 proteins is synthesized as a precursor.

g7p and g9p have almost the same size (33 and 32 amino acids, respectively); they lack the amino acids proline, histidine, and lysine and g7p also lacks tryptophan (98, 170, 287–289). Both are extremely hydrophobic (15, 317).

The functions of the gene 6, 7, and 9 products are still not known. Nevertheless, like g3p (51), they seem to be involved in the correct assembly of progeny phage. Amber mutations in any one of the minor coat protein genes result in the production of polyphage of two, three, or several times unit length. Polyphage due to amber mutations in gene 3 or 6 are not infectious. Those in gene 6, in addition, are unstable; they easily dissociate. Polyphage with the g7p or g9p or both missing, however, are infectious (173).

Since g3p and g6p are located at the end of the virus particle leaving the host cell last, they may be mainly responsible for correct "ending" of a nascent phage (98, 173). The instability of gene 6 amber polyphage also suggests a supportive role of g6p for maintenance of the phage's structural integrity. Viability and stability of any phage particle strictly require an intact g3p, since disassembly starts at the g3p end with an inactivation of this protein (262–264).

PHAGE PROPAGATION (PROLIFERATION)

Phage fd adsorbs to the tip of an F pilus on an *E. coli* cell via its "attachment protein" g3p (32). By an as yet unknown mechanism, it is brought near the cell surface, where it releases its DNA directly into the cytoplasm. During this process its major coat protein is deposited in the inner membrane. It is possible that the protein is stored there without degradation and is reused in the assembly of progeny phage (5, 291, 311).

The infecting single-stranded DNA is immediately complemented to a double-stranded replicative form (parental RF formation) by host-specified enzymes. This RF is replicated to build up a pool of double-stranded DNA molecules. Host-encoded enzymes and the viral gene 2 product are involved in this process (92, 167, 299). At the same time, the other viral proteins are synthesized. Late in infection, g5p brings about the switch from RF replication to single-strand synthesis by binding to the newly formed viral strands (192, 271). Inside the cell all the progeny viral DNA strands become complexed with g5p.

Final phage assembly takes place at the inner membrane (or membranous structures) where newly synthesized coat proteins are stored (293, 326, 333). It is not known how the g5p-DNA complex gets access to the membrane and how the g5p is replaced by the major coat protein as the DNA is extruded.

g5p is recycled to wrap new single-stranded DNA molecules (326). In contrast to infection with other single-stranded DNA phages such as φX174 or G4 or double-stranded DNA phages such as T4/T7, there are no ready-made phages in the cytoplasm; assembly is strictly membrane dependent and progeny virus can only be detected in the supernatant of infected *E. coli* cultures.

Infection with wild-type filamentous phage does not lead to lysis or death of the host, but permanent infection is established: the cells apparently remain intact, divide, and constantly release phage particles after a lag phase of approximately 10 to 15 min after infection. Thus the productivity is very high, about 300 virus particles per bacterium per generation. This leads to a titer of 2×10^{12} to 5×10^{12} plaque-forming units per ml, corresponding to a weight of 50 to 150 mg of infectious virus per liter of *E. coli* culture (187, 257).

Although normally an fd-infected *E. coli* cell suffers no damage, characteristic alterations take place in its properties and appearance. The composition of the inner membrane lipids changes; the level of cardiolipin increases, while the level of phosphatidyl ethanolamine decreases. The turnover rate of the membrane lipids remains constant. Concomitantly, some kind of membranous material accumulates in the cytoplasm. The cells become extremely sensitive to detergents such as deoxycholate. These effects are transient in the wild type, but permanent in infections with amber mutant phage (26, 187, 235, 266, 278, 344).

In the outer membrane the lipid content increases by about 25% after infection. The relative concentrations of the phospholipids do not change drastically, yet with the same general tendency as do the inner membrane lipids. Also, the preferred cleavage plane during freeze-fracturing shifts from the inner to the outer membrane (more than 75% cleavage in the latter after fd infection) (11).

Infected bacteria are resistant against penetration of colicins K, E1, E2, and E3 and against superinfection with Ff or male-specific RNA phages. This could at least partially be due to an alteration of their F pili (22, 67, 292, 353). Since the viral major coat protein is inserted in the inner membrane after synthesis, it has most often been cited for causing the alterations observed (344), yet the principles of its possible action are still unclear. At least at low temperatures, it seems to disturb the membrane structure (148). A specific association of this protein with cardiolipin has been suggested (41).

Another possible candidate for causing membrane alterations is the g3p. Mutants in gene 3 cannot confer resistance

to colicins and superinfection (292, 353). Boeke et al. (22) claim that g3p, or its N-terminal fragment, mediates many of the changes displayed by an infected cell, including deoxycholate sensitivity, β-lactamase leakage, resistance to superinfection, and defectiveness of the F pili. Although these are outer membrane effects, they found the protein to be predominantly associated with the inner membrane. Its first glycine-rich portion seems to be essential for its membrane association as well as for the alterations it brings about. These findings were recently confirmed by other authors (51). Dotto et al. (67) describe an effect of g2p or merely its N-terminal part on the host's F pili, leading to specific resistance against superinfection.

The detection of a new bacterial locus termed *fii* raises the possibility that at least a few of the phenotypes observed may be due to an interaction between the *fii* gene product and one of the phage proteins which might be g3p (306). *fii* is required for infection of f1 and Ike. Mutants in this gene exhibit an increased tolerance to colicins E1, E2, and E3 and an increased sensitivity to deoxycholate. These results are rather new and need to be confirmed by further research; they may open a new perspective on the consequences of phage penetration or proliferation or both.

Infections with conditional lethal mutants in any gene except gene 2 kill the host (122). When gene 2 is mutated, no RF replication is possible and the cell behaves as if it were not infected at all. In case of mutated genes other than gene 2 or 8, however, biosynthesis of phage components takes place, but no assembly is possible and g8p accumulates in large amounts in the inner membrane. Preventing the release of progeny phage in any case is lethal for the cell (122, 344). Inhibition of the host involving a rapid decrease in RNA and protein syntheses is also caused by an overproduction of g1p (123). This protein is obviously only tolerable when present in small amounts. The mechanism by which increased expression of g1p stops cell growth is not yet known.

Adsorption and Penetration (Infection)

The receptor of the phage is the F pilus, a protein appendix on the surface of male *E. coli* cells. The phage attaches to the tip of the pilus with its g3p end; thus, adsorption is polar with respect to both infecting particle and receptor (32).

F pili have been intensively investigated and shown to possess properties strikingly similar to those of filamentous viruses (30, 59, 86, 113, 228–230, 347). Brinton (30) even developed the term "epiviruses" for F and similar plasmids and regarded the pilus as the assembled, mature form of this special type of phage, although it contains no nucleic acid.

fd infection mutually interferes with mating, presumably since this process proceeds via the tip of F pili as well. It does not interfere with adsorption of RNA phages (for example, f2), since they use the side of F pili (32, 184, 187).

The precise mode of how the phage DNA crosses the cell membranes is still poorly understood. Originally, DNA conduction via the hollow pilin cylinder was proposed (30), but no DNA inside the F pili could be demonstrated (139), and it is difficult to imagine how a simple protein tube could accommodate three different types of nucleic acids in two different directions (infecting viral RNA and DNA into the cell and, during conjugation, bacterial DNA out of it). The more probable model suggests a retraction of the whole pilus into the cell surface (139). During this process the attached phage might come close to the membrane where it could actually penetrate. Disappearance of F pili has been observed under several conditions, such as energy poisoning of the cells or high temperature (228–230). Especially, F pili seem to be resorbed during the infection process (139). That an adsorption site can be used only once also supports this retraction hypothesis (139, 187).

Adsorption is dependent on the membrane potential, but does not require DNA or protein synthesis (30, 347). Penetration does, however, depend on a bacterial locus named *fii* (306), the product of which seems to be a 24-kilodalton protein associated with the cell envelope. Mutations in *fii* confer resistance to f1 and Ike infection, yet still allow infection of phage f2, φX174, or P1 and conjugation of F plasmid-containing cells. f1 still adsorbs to the tip of the F pilus of *fii*-mutated *E. coli*, but its DNA cannot enter the bacterium.

Phage of any size is infective if it contains a functional adsorption protein (32, 173). Absence or defectiveness of g3p leads to a residual level of infectivity which is $<10^{-8}$ times that of a wild type. This may point to the existence of a second, low-efficiency mode of infection, which is probably pili independent and has only "emergency" function (219). The results of Marco et al. (184) may support the idea that the presence of the F plasmid in the host cell is no absolute prerequisite for infection. These authors reported that F^- *E. coli* cells treated with lysozyme and detergent are as active in binding and reception of M13 phage as F^+ cells.

Penetration implies resorption of both viral DNA and proteins into the host cell. The DNA, according to current ideas of the process, is stripped of its major coat protein as it enters the bacterial envelope. The protein monomers are stored in the *E. coli* inner membrane during the whole "life cycle" (5, 187, 291, 311). Since the g3p is also resorbed by the cell, although in a degraded form, and uncoating seemed to be coupled to initiation of DNA replication (155, 257), a multifunctional "pilot protein" role was proposed for this protein a decade ago. It was supposed to function in binding to the receptor, initiating DNA release and directing the viral DNA to the host "replicative machinery" at the inner cell membrane (140, 184). This piloting function, however, is highly unlikely in the face of results gained in the last few years. We will present the evidence against the pilot hypothesis in the section on phage genetics.

Progeny Production (Assembly)

Late in infection several hundred viral DNA strands associated with g5p are present in the cell (326). The DNA-g5p complex is supposed to be bound to the host cytoplasmic membrane (326). Assembly is strictly dependent on this membrane, which constitutes the major morphopoietic factor; phage particles are constructed as the DNA is extruded (187, 337). The cytoplasmic membrane thus is crucial for penetration as well as release of the virus. It is an unresolved question how these opposite events may take place in the very same cellular compartment. Increasing evidence suggests that assembly is restricted to specified domains of the membrane ("assembly sites"). These sites could correspond to the adhesion zones between the inner and outer membranes (11, 175) where perhaps g1p is located (123, 175).

Progeny extrusion as well as infection require an energized membrane state. Blocking respiration, adenosine triphosphate synthesis, or formation of the proton motive force completely shuts off phage assembly (221).

Basically, virus morphogenesis proceeds as follows: the DNA becomes associated with the inner membrane, sheds

off its g5p "envelope," and is wrapped with the phage coat proteins. During this process, about two g8p molecules replace one g5p monomer (249). The main morphogenetic signal is the major hairpin A at the proximal end of the IR (see section, "Genetics") (72, 101, 327). Probably it acts as a "nucleus" or "anchor" for attachment of the minor coat proteins g7p and g9p (173). These proteins are probably also bound to the inner membrane (75) and might serve as receptor sites for the DNA-g5p complex. It is not yet known if binding these proteins proceeds prior to, simultaneously, or after the dissociation of the intracellular DNA-protein complex.

It has been shown that the g7p/g9p end of the phage, associated with hairpin A, is the first to emerge from the cell when progeny phage are extruded (173). Hence, extrusion is as polar as the infection process, and the phage has the same orientation in both cases: the g3p end enters the host first and leaves last.

Correct assembly requires all viral gene functions except for gene 2 and X proteins: in addition to the coat protein genes 8, 3, 6, 7, and 9 and the g5p, the still unidentified products of genes 1 and 4 are indispensable (192, 286, 337).

Up to now, the involvement of host proteins is only poorly investigated. One enzyme clearly required is the signal peptidase which cleaves the precursor forms of g3p and g8p (357, 359). Another protein is encoded by a bacterial gene named *fip* which was supposed to be newly detected (269). Mutants in gene *fip* do not allow virus assembly, although all of the preceding steps (including precursor processing) are possible. A spontaneous mutation in the viral gene 1 renders the phage resistant against the host's *fip* defect. These results suggested an interaction between the host's *fip* gene product and the viral g1p. Very recently, gene *fip* was found to be identical with the known gene *trxA* encoding bacterial thioredoxin (166, 270) which serves as a cofactor in many cellular redox processes. According to Russel and Model (270), phage assembly is dependent on the reduced form of thioredoxin and on the presence of thioredoxin reductase (*trxB* product) also, whereas Lim et al. (166) noted that *trxB* is not required for phage growth.

In contrast to former proposals (270), the redox potential of thioredoxin seems not to be involved in assembly (M. Russel, personal communication). Instead, the reduced form of the enzyme is somehow needed for conformational reasons. Its relationship with g1p thus is not yet understood.

Interestingly, thioredoxin was found to be localized predominanty in an osmotically sensitive cell compartment not corresponding to the periplasm (177). The compartment could be made of the adhesion zones between the inner and outer membranes which probably constitute the assembly sites (11, 175).

Recently, two further *E. coli* loci required for phage morphogenesis have been detected and termed *fipB* and *fipC* (174). In *fibB fipC* double mutants assembly is temperature sensitive (blocked at 41.5°C), while viral DNA replication, gene expression, and protein localization remain unaffected. The mutations have subtle effects on the cell; they seem to cause an alteration of the outer membrane structure or function.

The question remains of how virus maturation is actually brought about. Why does it not interfere with the dissociation events triggered in the membrane during infection?

In this context, we would like to mention some ideas concerning g8p. It has been known for 20 years that parental coat protein molecules are reused for progeny production (5, 291, 311). We believe, however, that this finding has been overemphasized. Recycling of "old" protein might be more or less adventitious and of no biological significance, since its amount is negligible compared to the bulk of newly synthesized g8p (5). We think that more attention should be focused on the properties of the various g8p species. As already described, the protein adopts an almost 100% α-helical conformation in the virus coat, while in lipid bilayers it displays only 50% α-helix; the transition from the first to the second state is almost irreversible (183, 231, 232). Hence it seems reasonable to assume that refolding to the 50% α-helical structure takes place only during infection as the molecule integrates in the membrane. g8p might not have this conformation during assembly. Instead, it participates in the latter process mainly in its precursor form, and the presence of the signal sequence might induce folding of the molecule into an as yet unclassified "in situ membrane conformation" which is different from the 50% α-helix structure. Processing then would trigger the rearrangement to the 100% α-helical state of mature g8p.

These ideas are consistent with the observations of Smilowitz (291) and Armstrong et al. (5) that old g8p is reused for assembly during 1 to 2 h, whereas "new" molecules are detected as phage coat within a few minutes after their production. Since the old protein lacks the signal sequence and has refolded to the 50% α-helical conformation, it may not be built into progeny phage coat as quickly and easily as the newly formed one. Furthermore, as far as we know, the possibility that Wickner's "soluble" g8p might be associated with lipid has never been investigated.

Membranelike structures are always found in the cytoplasm of Ff-infected *E. coli* cells. If one assumes an association of the g5p-DNA complex and precoat protein with these lipid bilayer fragments, this could constitute the first step in assembly. In analogy to other classes of phages, Ff production (in this case the exchange of g5p for g8p on the DNA) would then start in the cytoplasm of the host cell, too, and at the same time would be "membrane mediated." Progeny extrusion could start after fusion of the small lipid fragments (vesicles?) with the inner membrane, perhaps at the defined sites mentioned. Taken together, these hypothetical considerations might be keys to an understanding of why infection and progeny production are both one-way events.

GENETICS

Most of the research devoted to the filamentous bacteriophages has focused on their genetics. The rapid progress that has been made in this field in the past few years is reflected in the many new results that have helped to deepen our understanding of the viral life processes.

In this area, the reader is also referred to three extensive and excellent reviews. One, by Ray (1977) deals with genetics and replication (257). The second, written by Baas in 1985, provides an up-to-date look at the replication processes of filamentous and icosahedral viruses (8). And the third, by Zinder and Horiuchi, also of recent origin, describes the IR and its regulatory functions (355).

The Genome

The Ff phages contain a small and highly organized genome. The DNA includes 6,407 nucleotides in M13 and f1 and 6,408 nucleotides in strain fd. The complete sequences for all three strains are established (14, 15, 117, 272, 317). Except for some discrete regions which are folded into "hairpin" loops, the DNA has no secondary structure (its

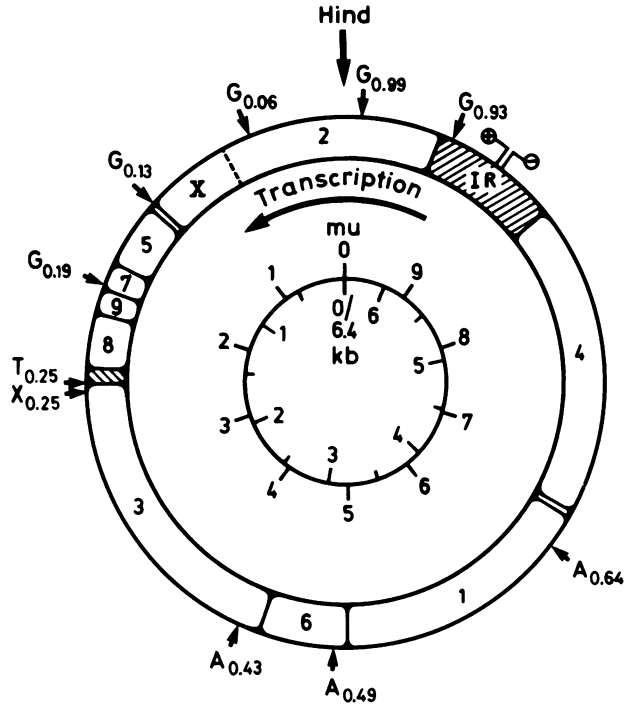

FIG. 2. Genetic and physical maps of bacteriophage fd (adapted from references 37, 115, 212, 297). The outer circle represents the genetic map; the inner circle represents the physical map. Genes are denoted with numerals. Intergenic regions are hatched. The single *Hin*dII cleavage site, which is taken as zero reference point, and the direction of transcription are also indicated. ⊕ and ⊖ represent the origins of viral and complementary strand synthesis, respectively. The regulatory signals are also shown: G, G-start promoters; A, A-start promoters; X, promoter for gene 3; T, central terminator. mu, Map units; kb, kilobases in the physical map.

bases do not undergo Watson-Crick pairing). This is also obvious from the base composition which is as follows: 34.6% thymine (T), 20.2% cytosine (C), 24.6% adenine (A), and 20.6% guanine (G) for fd and M13 (14, 317). A characteristic is the high thymine content, exceeding one-third in all three strains. This allows easy separation of viral and complementary strands in CsCl gradients (255). The thymines are not randomly distributed in the genome, but preferentially occupy the third position of codons (15, 117, 317).

Figure 2 (37, 297) shows the circular genetic map together with the physical map and the regulatory elements. The single cleavage site of endonuclease *Hin*dII is used as zero reference point.

The arrangement of the 10 viral genes (178, 316, 318) in the order of transcription (counterclockwise) is as follows: 2→X→5→7→9→8→3→6→1→4. They may be classified into three functional groups: proteins needed for replication are encoded by genes 2, X, and 5; coat proteins are encoded by genes 7 through 6; and genes 1 and 4 determine products essential for morphogenesis.

Apart from these coding sequences, the genome contains two noncoding sections of considerable size. The large IR between genes 4 and 2 is 508 nucleotides long. The sites of initiation and termination of both the complementary and viral DNA strand synthesis (replication origins) (81, 128, 303, 313), several strong rho-dependent and -independent transcription terminators (162), and the main morphogenetic signal (72, 101) are located there. The smaller intergenic section between genes 8 and 3 (59 nucleotides) holds the central, rho-independent transcription terminator T at map position 0.25, immediately distal to gene 8 (42, 77).

Except for these two main "gaps," most of the genes are closely spaced, with only a few nucleotides separating the termination signal of one gene from the initiation sequence of the next. Gene 9 overlaps with one nucleotide each at both of its ends with the neighboring genes 7 and 8 (133). Genes 4 and 1 have 23 nucleotides in common. A total of 573 nucleotides are silent in M13 and f1, corresponding to 8.9% of the genome.

Nine promoters have been identified in vitro (42, 78–80, 237, 279, 304). Five start with G (map positions 0.92, 0.99, 0.06, 0.12, 0.19), three start with A (positions 0.44, 0.49, 0.64), and one starts with an as yet unknown base, probably T (position 0.25). This latter promoter, designated $X_{0.25}$, precedes gene 3 and is contained within the termination region following gene 8 (79, 297).

The known promoters are located in front of genes 2 ($G_{0.93}$), X ($G_{0.06}$), 5 ($G_{0.13}$), 3 ($X_{0.25}$) 6 ($A_{0.43}$), 1 ($A_{0.49}$), and 4 ($A_{0.64}$) (78–80, 312). The strongest promoter, $G_{0.19}$, is located within gene 7 and initiates mRNA synthesis for gene 8; gene 2 contains an additional internal promoter ($G_{0.99}$) (78, 80, 261, 304, 318). Genes 7 and 9 have no initiation signals of their own (211).

In addition to terminator $T_{0.25}$, two further important rho-dependent transcription termination signals are active in vivo. They are located distal to genes 4 (162, 213) and 6 (296, 297).

The phage DNA can be cleaved with numerous restriction endonucleases, among which are *Hin*dII, *Hin*H, *Hin*f, *Hae*II, *Hae*III, *Hap*II, *Hpa*II, *Hga*, *Hha*, and *Alu*I (80, 126, 237, 279, 308, 312–316). Some restriction maps are shown in Fig. 3. From a comparison of the cleavage patterns generated by the same restriction enzymes on different phage DNAs, conclusions on the genetic relationship between the individual strains can be made (315).

As part of the intracellular DNA the phage genome is subject to host-specific restriction and modification, for example, in *E. coli* B strains. The DNA of fd and f1 contains two modification sites (SB sites); that of M13 contains only one (126, 178). SB_1 is located in gene 5 or 7; SB_2 is located within gene 2 (126). When grown in the appropriate host, adenine bases at the SB site(s) of the viral DNA are methylated (253). This sensitivity to host-specific modification may be lost through mutations at the SB sites. Restriction does not take place at the modified sites; it is the unmodified double-stranded RF DNA which is a substrate for the host restriction enzyme (126, 127).

DNA Sequencing and Structure Investigations

The sequences of the 6,408 nucleotides of fd DNA (14, 272) and of the 6,407 bases of f1 (15, 117) and M13 DNA (317) have been determined. The deleted nucleotide, at position 3195 in fd, is within the very short intergenic gap between genes 6 and 1. The f1 sequence of Beck and Zink (15) differs in 16 positions from the one published by Hill and Petersen (117).

Some regions of particular interest have been sequenced before the complete DNA sequences were established. Sugimoto et al. (304) sequenced 110 nucleotides of an fd DNA fragment containing a promoter, which can now be located in the integral fd DNA sequence at position 314 to 424 preceding promoter $G_{0.06}$ (gene X).

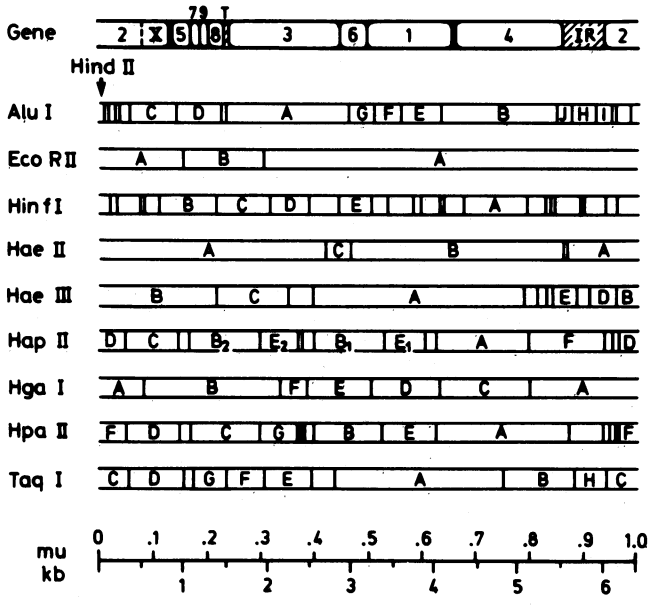

FIG. 3. Restriction enzyme cleavage maps of fd DNA (adapted from references 14, 313, 317). The genetic map is shown at the top, opened at the unique *Hin*dII cleavage site. Genes are designated with numerals; IR and T are the large intergenic region containing the replication origins and the central terminator of transcription, respectively. The physical map is given at the bottom: mu, map units; kb, kilobases. Only the major restriction fragments are represented in the cleavage maps; the names of the enzymes are indicated at the left of each line.

Hulsebos and Schoenmakers (133) sequenced M13 DNA cut from the region of genes 5 to 8. They detected the existence of gene 9 and identified the initiation and termination sites of the genes in this part of the DNA. The reading frame for gene 9 has been confirmed by using an oligonucleotide acting as a site-specific mutagen; in this way, gene 9 amber mutations were created (290).

Ravetch et al. (253) used f1 DNA to determine the recognition sequence of *E. coli* B restriction-modification enzyme (SB sites).

The mRNA for g8p has been sequenced (305) and shown to be identical to the in vitro synthesized species (261). This proved in vivo activity of the strong promoter $G_{0.19}$ and the central terminator $T_{0.25}$ and the synthesis of the major coat protein in a precursor form and confirmed the structural peculiarities of the termination region immediately following gene 8 (see below).

Boeke and Model (20) reported the molecular explanation for the am8H1 lesion in phage M13, the only conditional lethal mutant in gene 8 known so far. They found that a transversion from G to T at position 1373 leads to a TAG amber codon in the GAG codon coding for glutamic acid at position 2 in mature gene 8 protein.

The IR between genes 4 and 2 has been subject to extensive studies. Figure 4 shows this region in its presumptive secondary structure (15, 317; P. van Wezenbeek, Ph.D. thesis, University of Nijmegen, Nijmegen, The Netherlands, 1981).

The presence, in the phage genome, of a nuclease-resistant "core" displaying unusual structural features was detected early (187). The five hairpins in the IR are of great functional and regulatory significance.

Hairpin A (positions 5499 to 5576) immediately following gene 4 is the decisive morphogenetic signal during progeny maturation (72, 101, 327). In addition, it functions as a strong rho-dependent terminator of transcription (162, 163, 213, 296). This terminator also includes hairpin B (positions 5624 to 5678) under certain experimental conditions. Normally, in the presence of rho factor, no readthrough of the IR by RNA polymerase is possible and the replication origins are strictly separated from transcription termination. Without rho or in the in vitro assay, rho-independent termination was observed at several positions near or in hairpins C, D, and even E (162, 213, 296). RNA polymerase covers hairpins B and C during CS initiation (104). Hairpin C (positions 5691 to 5750) constitutes the origin of CS synthesis (144, 252, 273, 274; van Weezenbeek, Ph.D. thesis), while VS replication starts near the tip of hairpin D between nucleotides 5780 and 5781 in M13/f1 (5781 and 5782 in fd) (71, 208; van Weezenbeek, Ph.D. thesis).

It is interesting to note that hairpin A is rich in G+C. As already mentioned in previous sections, it probably serves as an "anchor" for g7p/g9p and is located at the corresponding end of the filament (135, 282, 327). At the opposite end of a wild-type phage, part of the gene 3 sequence is located, which is also rich in G+C (positions 2284 to 2391). Therefore, G+C-rich DNA sequences are packaged at both ends of a wild-type phage (327).

The sites of secondary structure are specifically distributed in the genome. Besides the hairpins in the IR, Shen and Hearst (281) found several others which could be related to the promoter sites detected in vitro. Phage T4 DNA polymerase uses only single-stranded DNA as template and is delayed at sequences displaying double-stranded character. Huang and Hearts (131, 132), therefore, used this enzyme to detect at least seven stable helical regions in the fd genome. Two of them coincided with the "origin hairpins" B and C.

Other investigators found evidence of "pause sites" in intercistronic regions of the genome. They are kinetic barriers where the host replicative machinery stops during DNA and RNA syntheses. These sites contain hairpin structures and exhibit regulatory properties (214).

This holds especially true for the central terminator immediately distal to gene 8 (positions 1538 to 1564 in M13 DNA; Fig. 5a). It is folded into a tight loop, followed by a unique sequence of eight thymine nucleotides (261, 287). A further characteristic is a GC-rich region upstream from the termination point, followed by an AT-rich region (317; van Wezenbeek, Ph.D. thesis). The hairpin or the polydeoxythymidine sequence or both act as strong rho-independent "stop" signals for RNA polymerase during transcription.

Rho-dependent transcription termination takes place at two main sites in vivo. One signal is hairpin A; the other one (296, 297, 317; van Wezenbeek, Ph.D. thesis) probably is the GC-rich dyad symmetry from positions 3319 to 3341 (Fig. 5b) distal to the gene 3/6 region.

Comparison of the DNA sequences of strains fd and M13/f1 (125, 317; van Wezenbeek, Ph.D. thesis) shows a strong conservation of regulatory elements and gene sizes. The IR is the most variable region in the genome except for its base-paired sections. The nucleotide sequences of the three major hairpins (A, B, and C), of the central terminator, and of all ribosome binding sites are identical in M13 and fd. This is also true for most promoter sequences. The frequency of base substitutions is widely different for the individual phage genes: genes 7, 8, and 9 are fully conserved, whereas gene 4 exhibits the highest frequency of sequence variations. Induced mutations, using restriction endonucleases, give a similar result: genes 1, 3, and 4 and certain

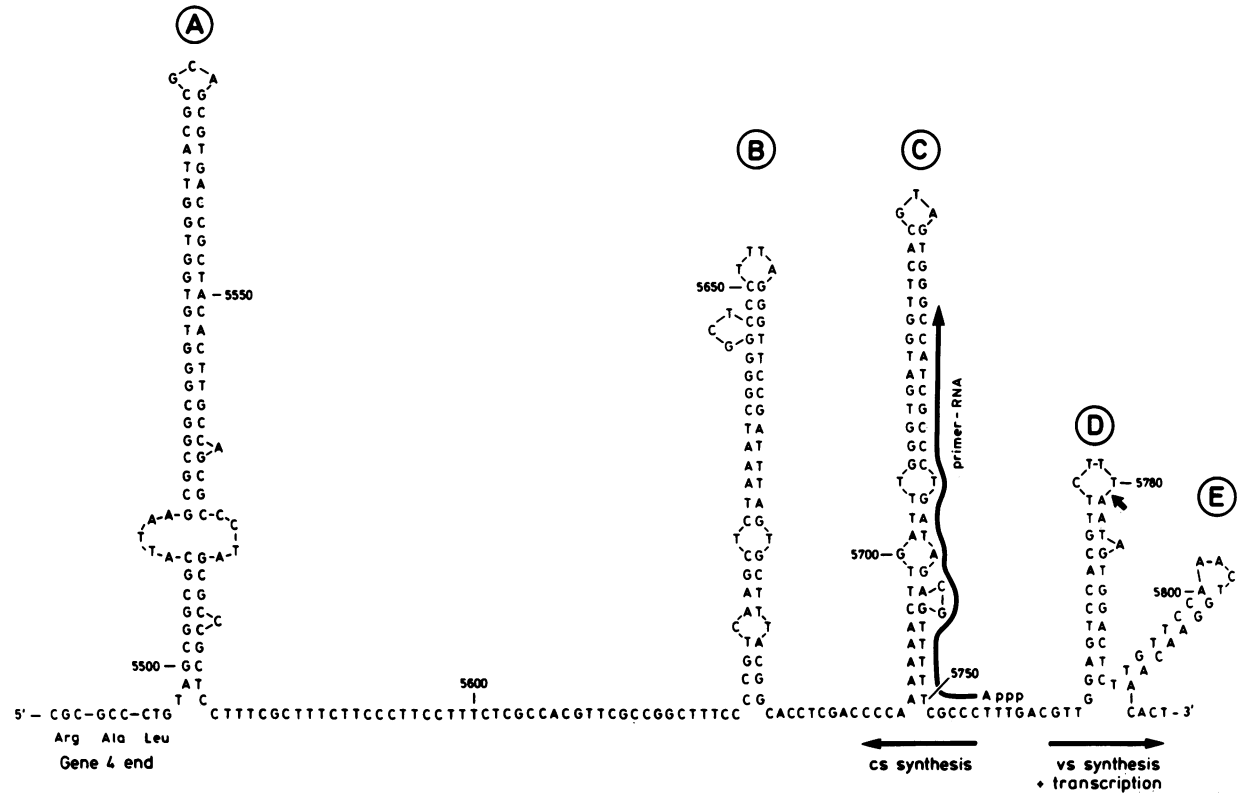

FIG. 4. Nucleotide sequence and secondary structure of the IR surrounding the replication origins of M13 DNA (adapted from reference 317). Only the VS is shown. The end of gene 4, the location of the primer RNA initiating complementary strand synthesis, and the directions of complementary and VS synthesis as well as of transcription are indicated. The heavy arrow points to the gene 2 protein-nicking site (VS origin).

single-stranded sections of the IR are most often altered (19). Ray (257) correlates the conservation of genes with the occurrence of palindromic sequences within them.

Only 12 of the about 190 nucleotide interchanges between fd and M13 cause an amino acid change; thus the bulk of the natural base substitutions is silent. Similar relationships may be established for phage fd and f1 (15, 117). Comparison of all three DNA sequences reveals that M13 and f1 are more closely related than is either one of them to fd.

DNA Replication

In contrast to other viruses which are assembled in the host cell cytoplasm, DNA replication and coat protein synthesis of Ff are not coupled (247). The filamentous phage genome may be regarded as a plasmid or episome; it is essentially dependent on host enzymes for its multiplication. Only three phage-encoded proteins are involved in viral DNA synthesis. Three different stages of this process may be distinguished: parental RF formation, RF multiplication, and progeny VS production.

Stage 1. The infecting viral DNA strand is converted to the parental double-stranded RFI through formation of a minus strand. CS synthesis starts with a priming RNA fragment which is elongated by DNA polymerase. Only host enzymes are involved at this stage.

Since penetration of the phage seems to be tightly linked to replication of its DNA, the question arose as to whether the replication origin segment might be the first to enter the cell. Furthermore, g3p was proposed to be a pilot protein leading the viral DNA to the host replicative machinery (140, 184). These assumptions then were supported by the finding that hairpin A, which is in close vicinity to the origins, is always located at one tip of the phage (135, 282, 327). However, more recent results do not support this "pilot hypothesis." (i) Hairpin A is not involved in initiation of DNA replication, since it can be separated from the origin by inserting 1,000 to 2,000 bases without impairing DNA synthesis (135). (ii) None of the origins for CS and VS synthesis needs to be at one end of the phage (135, 327). (iii) The origin sequence is in itself sufficient for initiation; no pilot protein is needed (144). (iv) g3p is at the end of the particle opposite to the "hairpin end." Therefore, the IR holding the origins is far from the presumed pilot protein (99, 173, 327).

Investigations on the host proteins necessary for the three different stages of viral DNA replication led to the assumption that a novel form of RNA polymerase had been detected (339). The enzyme, denoted RNA polymerase III, was found to be rifampin sensitive like RNA polymerase I, but able to distinguish M13 DNA from other viral single-stranded DNAs. Meanwhile, this "discovery" has been devaluated: it is the σ (sigma) subunit of the well-known RNA polymerase I holoenzyme which confers specificity in priming on M13 DNA; RNA polymerase III does not exist (142). Without the σ subunit (in vitro) the enzyme will also prime ΦX174 DNA replication.

The in vitro conversion of the VS to RFI requires six *E. coli* proteins: RNA polymerase I holoenzyme, single-strand

binding protein, DNA polymerase III*/copolymerase III*, DNA polymerase I, and DNA ligase (92, 319).

RNA polymerase forms an RNA primer (31, 335) of 30 nucleotides in length, starting from nucleotide 5756 near the lower part of hairpin C (90, 104). The enzyme covers hairpins B and C, making them inaccessible for host nuclease digestion (273, 274).

Single-stranded DNA-binding protein (283) is highly specific for single-stranded DNA. Accordingly, it masks the viral DNA except for the origin region, where it does not bind due to the high degree of base-paired secondary structure. Thus, this protein indirectly is responsible for correctly positioning the RNA polymerase at the CS origin and blocking it in transcription of the single-stranded DNA. Without this protein, the polymerase initiates multiple synthesis starts on the VS template (142, 222).

The RNA primer fragment is elongated with deoxyribonucleotides by DNA polymerase III*/copolymerase III* (338), which probably act on the phage DNA as a native holoenzyme. After one round of replication, DNA polymerase I (*polA*) excises the primer RNA due to its 5′ → 3′ exonucleolytic activity and fills the small gap remaining in the CS, thus creating a full-length linear CS consisting exclusively of deoxyribonucleotides (45, 57, 299). The enzyme is indispensable in both of its activities for normal RF synthesis in vitro and in vivo. When the exonuclease function is mutated, a labile RF species still containing the primer RNA accumulates. The unique gap in the CS of RF DNA synthesized in vitro with a soluble enzyme fraction provided the first hint for the specificity of CS initiation (307).

Finally, DNA ligase creates RFIV DNA by joining the ends of the ready-made CS.

Stage 2. RF replication starts approximately 10 to 30 min post-infection. It is initiated after expression of the viral gene 2 which is needed for VS synthesis. This process proceeds according to Gilbert and Dressler's "rolling circle" mechanism (93, 207): the old VS is nicked by g2p at a specific site and "peeled off" as a new one is synthesized, using the free 3′ end of the old VS for joining new nucleotides to it, and using the CS as a repeated template. The CS always remains closed circular; the process thus is called asymmetric replication. After each round of VS replication, new complementary strands are initiated on the displaced viral strands as soon as the CS origin gets exposed (208). They are synthesized as already described.

The result of each replication cycle is one molecule of RFIV, that is, a double-stranded, closed circular, relaxed DNA. It has to be converted to supercoiled RFI by DNA gyrase and, thereupon a new initiation occurs through cleavage of this DNA by g2p in the VS (207, 275).

As is evident from Fig. 4, the origins for both DNA strands are closely spaced (24 nucleotides apart). Because of their relative location, VS and CS syntheses proceed independently of each other, and since both strands are formed in a 5′ → 3′ direction, CS synthesis is clockwise on the circular genetic map while elongation of the VS proceeds counterclockwise (128).

g2p and gXp are the only phage proteins required for RF propagation (88, 206, 245). The function of gXp in DNA synthesis is still obscure; in amber X mutants the rate of VS formation is drastically reduced, pointing to a strong supportive role of gXp during this process (88). Yet the decisive enzyme still is g2p. Not only is it necessary for initiation of VS synthesis, but it also cleaves unit-length genomes from the nascent strands and seals them to closed circles (111, 202, 205). When added to RFI DNA in the presence of Mg^{2+}

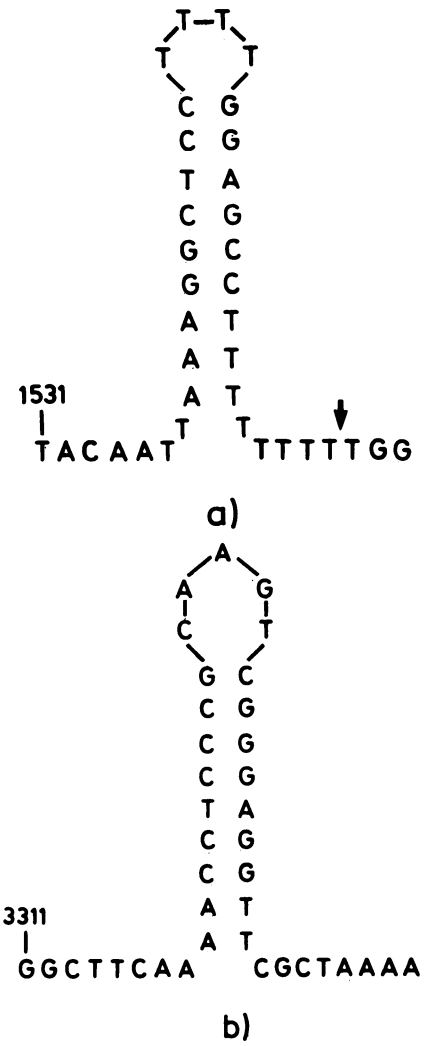

FIG. 5. Termination signals of the phage genome (adapted from reference 317). (a) Secondary structure of the rho-independent terminator of transcription distal to gene 8. (b) Secondary structure of the postulated rho-dependent termination site distal to gene 6. The arrow indicates the position at which mRNA synthesis terminates.

in vitro, g2p converts its substrate to about 60% RFII and up to 40% RFIV (205). Without Mg^{2+}, but in the presence of Mn^{2+}, RFIII (linear RF) results.

It is not known if the enzyme remains associated with its DNA after initial recognition. In the case of ΦX174 DNA replication the homolog enzyme covalently binds to its substrate; similar events have been proposed for g2p (8, 207).

The host functions needed for VS synthesis are not as clear as in the case of CS synthesis. Staudenbauer et al. (299, 302) used *E. coli* mutants in *dnaE* (DNA polymerase III) and *polA* or *polB* (DNA polymerase I or II) to study replication in vivo. They found that DNA polymerase III is not indispensably required for conversion of the VS to RFI in vivo (probably another polymerase can take over), yet it is most important for normal RF replication and for VS synthesis (stage 3). Also, the *E. coli dnaB* function is not needed for parental RF formation or for progeny VS synthesis, but is required for early RF replication (238, 257, 300–302). The

dnaG function (rifampin-resistant RNA polymerase) has also been supposed to be required for RF replication, but its role is obscure (257). Meyer and Geider (207) reconstituted fd RF replication in vitro, using g2p and the following *E. coli* proteins: DNA polymerase III holoenzyme, single-strand binding protein, *rep*-helicase, and, with RFIV as a substrate, DNA gyrase.

Termination is different for VS and CS. For the VS, the origin sequence simultaneously holds a termination signal. Synthesis stops as soon as this typical sequence is reached (68, 124). This implies that two functional VS origins are not allowed in one genome. When the replicative machinery encounters a second origin, inserted artificially in vitro, synthesis is terminated and one origin and the DNA sequence between the two origins are excised (124).

On the other hand, multiple copies of the CS origin are possible in one genome. CS synthesis evidently is terminated when the 3′ end of the new strand "runs" into its own 5′ end after one full round of replication (144).

This was confirmed by findings on the genome of miniphages. Miniphages behave as autonomous replicons, since their genome only consists of the IR and the proximal parts of genes 4 and 2 and accordingly contains all of the genetic information necessary for propagation (81, 83, 106). In the presence of a helper phage which provides the proteins, these defective DNAs are packaged into infectious particles and rapidly overgrow wild-type phages. Only certain mutants in gene 2 are resistant to miniphage interference (85). In studies by Chen and Ray (46), the RF DNA of four cloned M13 miniphage contained multiple copies of the replication origins. Only one origin/terminus for VS synthesis was active; the other copies were not functional. The CS origin, however, may be present in the miniphage genome in several active copies and contribute to the vast proliferation of these particles (125).

The same applies to the genome of maxiphage. The IR does not need to be intact to allow DNA replication. The possibility of inserting genetic material into this part of the genome constitutes the basis for the use of filamentous phage as a versatile cloning vehicle. Gene 8 with its proximal promoter and terminator sequences has been transposed into the IR and is fully expressed (211, 212). The same holds true for other inserts.

The peculiarities of the replication origins are currently under intense investigation. It is clear that hairpins C and D are sufficient as origins for both DNA strands. Neighboring sequences per se are not needed for replication, yet they may have supporting functions (van Wezenbeek, Ph.D. thesis). Cleary and Ray (47) found the sequence between hairpin D and gene 2 to be necessary for the isolated origin fragment to be fully active in a plasmid system: the plasmid DNA efficiently transformed cells only when it contained a stimulatory sequence of about 40 nucleotides downstream to the g2p nicking site in addition to the origin segment (48).

Kim et al. (146) constructed a series of mutants with viable deletions in the region of the CS origin. Most interestingly, they found that the sequence coding for the primer RNA as well as both of the RNA polymerase-protected hairpins B and C are not essential for replication. The mutants which had lost this whole section of their IR were drastically impaired in growth compared to the wild type, yet were still viable. Evidently, alternative origin sequences in their genomes took over the role of initiating CS synthesis. VS synthesis, on the other hand, was found to be strictly dependent on the presence of the g2p nicking site. Moreover, in all mutants tested a sequence of 13 nucleotides immediately preceding this site was preserved. Therefore, g2p requires a well-defined nucleotide sequence to exert its specific functions. Dotto and co-workers confirmed and refined these results (66, 69–71). They discerned a VS origin "core region" (domain A) of 40 nucleotides in length and a "secondary region" (domain B) also called "enhancer sequence," which is about 100 nucleotides long. Domain A contains the g2p recognition sequence and an initiation and a termination signal, which are distinctly different but partially overlapping. Domain B is only needed for initiation of VS synthesis and is dispensable to some extent.

The g2p recognition sequence extends from four nucleotides on the 5′ side of the nicking site to 11 to 29 nucleotides on the 3′ side. Hairpin D is not necessary for recognition. It is, however, required for biological activity of the VS origin. The stretch of 12 to 13 bases preceding the g2p cleavage site on its 5′ side is part of the signal for termination, which coincides on its 3′ side with the recognition sequence. The signal for initiation finally includes the g2p recognition sequence and extends for about 100 nucleotides downstream into domain B. If the topography of one of these three signals is disturbed by inserts, deletions, or base substitutions, the biological activity of the VS origin drops to very low levels (1% or less). The minimal sequence required for VS initiation, however, may be reduced to domain A by quantitative or qualitative changes of g2p (73, 74, 147). A regulatory mutation in g5p (73) as well as a mutation in the g2p mRNA leader, the target of g5p repression of g2p synthesis (74), both result in a vast overproduction of g2p. Also, two mutants in the coding part of the g2p mRNA leading to an altered protein species have been described (74, 147). The mechanism of how the enhancer sequence is rendered absolutely unnecessary by these different mutations is not known.

Stage 3. Late in infection (about 60 min after penetration of the phage), there is still a certain amount of RF replication (164), but the bulk of DNA synthesis concerns VS. The double-stranded RF molecules serve as biosynthetic precursors of the progeny VS which are packaged into new particles (255, 256, 260). Meanwhile accumulated g5p switches replication from RF to single-strand synthesis by binding to the displaced VS and preventing formation of the CS (245, 271).

VS synthesis requires the viral gene 2, gene X, and gene 5 products and expression of the host functions *dnaC*, *dnaE*, and *polA* (88, 257). Hairpin A is not needed at this stage; single-strand synthesis and the formation of the g5p/DNA complex also proceed in its absence (101).

Regulation of DNA synthesis is brought about in the ways already described above. g5p is the main control element for expression of g2p and gXp and, hence, for replication of any type of DNA (209, 349).

The phage DNA undergoes recombination and segregation like any other genome. When *E. coli* is transfected with an in vitro constructed heteroduplex f1 DNA, segregation is strongly asymmetrical (82); it is mainly the CS which determines the progeny genotype. Mixed infections with two amber mutants lead to viable progeny, since the parental genomes recombine and segregate via a heteroduplex DNA intermediate (84). A part (about 25%) of the double-length (diploid) progeny of mixed infections with two amber mutant phages is heterozygous. These particles contain two different, circular, and single-stranded viral DNA molecules (13) and thus are viable. Recently, a transitory recombination between phage M13 DNA and a plasmid (pHV33) has been reported (55).

Transcription and Translation

When viral RF DNA is multiplied, probably all or most of these molecules are actively transcribed (191 and citations therein). However, the parental RFI is also active in and sufficient for protein synthesis. For example, when g2p is defective and therefore DNA replication is blocked beyond the stage of parental RF formation, phage proteins accumulate in almost normal amounts (114, 191).

Only the CS of the double helix is transcribed; transcription proceeds counterclockwise on the circular map (210, 312). This means that the polarity of gene expression is the same as the gene order on the VS (Fig. 2).

Transcription in vivo is much more complex than in vitro. The promoter sites identified in vitro do not all coincide with those found for in vivo gene expression. An activity in the infected cell has only been demonstrated for promoters $G_{0.92}$, $G_{0.06}$, $G_{0.12}$, $G_{0.19}$, $X_{0.25}$, and $A_{0.64}$, preceding genes 2, X, 5, 8, 3, and 4, respectively, and for the terminators $T_{0.25}$ and hairpin A (261, 296, 297; van Wezenbeek, Ph.D. thesis). Cashman and Webster found eight phage specific mRNAs in f1-infected cells (36, 37). Smits et al. (297) detected at least 11 species in vivo. Smits et al. (298) used a minicell-producing *E. coli* strain to demonstrate in vivo activity of most of the G promoters identified in vitro. The mRNAs produced in normally infected cells are the same as those found in the minicell system (i.e., in vivo), but there are only three coincidences with mRNAs synthesized in vitro with RF DNA as template (297, 298). Only the most abundant mRNA, which codes for g8p and makes up more than 2% of the total cellular RNA (!), has been shown to be identical to its in vitro counterpart (261).

The half-life for the mRNAs decreases with increasing molecule lengths, ranging from ca. 0.5 to 11 min (37). The mRNA for g5p has a half-life of approximately 2.5 min. Two half-lives have been reported for the gene 8 messenger, 2.5 and 10 to 11 min, suggesting the presence of two mRNA species for g8p (160, 261). Early in infection, all of the mRNAs synthesized for this protein are probably of the long-lived type.

Nevertheless, one should keep in mind the difference between physical and biological half-lives (160, 257): an mRNA molecule may be physically intact for a while after having lost its functionality.

Since the phage proteins are needed in different quantities at different stages of the viral life cycle, gene expression has to be regulated. Based mainly on in vitro investigations, a "cascade mechanism" for regulation of transcription has been developed (78, 297, 312). According to this model, mRNA synthesis starts at several promoter sites and is generally terminated at one site, the central terminator $T_{0.25}$. This leads to a "cascade" of mRNA molecules, increasing in number and decreasing in length as $T_{0.25}$ is approached. Consequently, genes located in front of the central terminator are transcribed more frequently than those at greater distances. This hypothesis is derived from and consistent with the following experimental findings: the viral genome contains sections of frequent as well as rare transcriptional activity (297), the former being congruent with the genes for proteins needed in high amounts (genes 5 and 8); in vitro transcription is initiated at several active (G) promoters, but terminated at only one site, the rho-independent $T_{0.25}$ (which is also active in vivo) (36, 37, 297); and expression of genes 5 and 8 seems to be regulated mainly via the amount of corresponding mRNA synthesized.

Other results, however, do not rigorously fit the cascade model in the form as it was proposed. Cashman and Webster (36, 37) and Smits et al. (297) reported that most small mRNA species are processing products of larger precursor molecules all having the same 3′ end, the sequence coding for g8p. Thus, not all mRNA molecules arise from an extra initiation at a specific promoter site.

The poor expression of genes 7 and 9, which are located right in the middle of the high transcription region, cannot be explained by the cascade model. Furthermore, gene 8, together with its most proximal promoter and the central terminator sequence, has been successfully transposed to the IR and is expressed there at high levels, without the aid of any mRNA cascade (211, 212).

LaFarina and Model (161) studied the messenger populations in f1-infected cells and found a rather complex pattern of gene regulation in vivo. They concluded that transcription in vivo proceeds differently with regard to frequency and organization in each of two functional parts of the genome: the F-region (= frequently transcribed), ranging from the IR to the central terminator, consisting of all of the (strong) G-promoters and genes 2, X, 5, 7, 9, and 8; and the I-region (= infrequently transcribed), holding the (weaker) A-promoters and genes 3, 6, 1, and 4. That means that the A-promoters initiate synthesis of mRNAs for proteins required in only low quantities. In the F-region, much mRNA processing takes place, resulting in a population of overlapping, small molecules; a gradient of increasing transcriptional activity runs parallel to a gradient of increasing messenger stability from genes 2 to 8. No such gradient is observed in the I-region, where proximal and distal sequences are almost equally expressed and at low rates. One large mRNA (4 kilobases), spanning the entire I-region, as well as smaller RNAs overlapping at their 5′ termini were detected.

Since no mRNA has been found which spans the genome altogether, a termination site distal to gene 4 was postulated and its existence was proven recently (162, 163, 213). In vivo under normal conditions hairpin A (Fig. 4) strictly separates the I- and F-regions from each other. In the presence of rho factor, the mRNAs mainly terminate at nucleotide 5565, while in the absence of rho transcription is stopped later in the IR. The origin sequence alone is sufficient as a stop signal in vivo, prohibiting a readthrough of the IR by RNA polymerase. This, however, is not the case in vitro; without rho factor, transcription proceeds even beyond hairpin E.

From genetic complementation studies an operon composed of genes 3→6→1 was deduced (187, 248). No definitive proof for this operon in vivo has been reported, although LaFarina (159) found small amounts of a very large mRNA species (more than 23S in sucrose gradients) which contained the sequences of genes 3, 6, and 1. The gene 3 promoter $X_{0.25}$ is completely included in the central terminator sequence (79) and might well be influenced by the level of gene 8 expression. Termination at $T_{0.25}$ appears not to be stringent: ca. 10% of the RNA polymerase molecules were found to "leak" through it (78), but this could be a consequence of the overlapping promoter sequence as well.

Smits et al. (296) investigated transcription of the I-region in an *E. coli* minicell system. They report expression of genes 3 and 6 on a common messenger starting at promoter $X_{0.25}$ and extending up to the proximal part of gene 1. The termination point probably is at nucleotide 3343 (Fig. 5b). Transcription of gene 1 seems to be mainly accomplished by readthrough of this rho-dependent stop site (123). Gene 4, on the other hand, has a promoter of its own immediately preceding the structural gene.

In vitro, an activity of promoters directly in front of genes 6 ($A_{0.44}$) and 1 ($A_{0.49}$) was demonstrated (79) which, until now, could not be confirmed in vivo. In the infected cell, no small mRNA molecules corresponding to the size of gene 6 and derived from the I-region have been detected (159, 161).

The cascade model looks a bit out-of-date in the light of these findings, but it might nevertheless hold true for expression of the genes in the F-region. Obviously, regulation of expression of the viral genome cannot be explained by one general model but rather by a mixture of regulatory events on the levels of both transcription and translation. A set of effects may act in concert, for example, the location and strength of a given promoter as well as mRNA stability and processing. Significant regulation may occur on the translational level in the form of different translational rates of certain mRNAs as well as repression of translation (for example, g2p/gXp).

Expression of genes 7 and 9 probably is regulated via translation (133, 290). Until now, no specific promoters for these two small genes have been found (211). They share the same operon with gene 5 and are transcribed as a polycistronic mRNA of genes 5→7→9. In consequence, their expression depends strictly on gene 5 expression (133, 289, 290).

A similar situation is found with gene 1 (123). The production of g1p seems to be rigidly down-regulated because of the fatal effects this protein exerts on the host cell. The possible mechanisms for this regulation could include transcription (presence of a terminator in the proximal part of the gene, absence of any detectable promoter activity, overlap of gene 1 with the promoter, and ribosome-binding site of gene 4) as well as translation, since gene 1 contains 32% rare codons (123, 150). Use of infrequently utilized codons has been suggested as a means to limit gene expression ("translational modulation") (150).

As mentioned in a previous section, most of the genes are closely spaced. Therefore, the short silent regions as well as the overlaps between the translational start and stop signals of the genes probably have regulatory functions. They might serve as pause sites to allow a reading frame change, especially within gene clusters (operons 5→7→9, 3→6→1). This would guarantee the translation of the successive gene or, in other cases, might assure termination after the gene just expressed (van Wezenbeek, Ph.D. thesis).

The synthesis of most phage proteins has been demonstrated by in vitro coupled transcription-translation systems (79, 151–153, 160, 210, 312). The only proteins never found in vitro are the products of genes 6 and 7. The product of gene 9, on the other hand, was successfully produced in a cell-free system (290). The products of genes 3, 4, 5, and 8 are also synthesized in minicells containing only viral DNA (298).

Cloning Vectors

Filamentous phages have found widespread use as cloning vectors. Their length is determined by the length of their DNA. Genetic material which is significantly larger than a wild-type genome is packaged into infectious particles (116). Accordingly, foreign DNA fragments of considerable length may be inserted into their genome. The IR especially fits this purpose: it does not code for any protein, its integrity is not a prerequisite for replication of viral and complementary DNA strands, and, as expression of the viral genes is also unaltered, insertion does not impair phage viability and hence propagation of the fragment. Most Ff vectors are helper independent.

Only VS are used for virus assembly. This allows an easy strand separation of a double-stranded cloning probe (which may be integrated in both orientations) (115, 118); single-stranded vector DNA can thus be obtained in high yield. As the phage genome is small and well characterized, especially with respect to endonuclease cleavage sites, the location of inserted fragments can easily be determined.

The original genome has been diversely modified to meet the different requirements of genetic engineering: new restriction sites have been introduced or existing ones have been eliminated (10, 19, 24, 115, 118); genes determining resistance to several antibiotics or marker genes to allow selection of transformed subclones have been inserted (10, 115, 116, 118, 219, 226, 254, 259, 350), and deletion mutants in the CS origin were constructed to select foreign DNA sequences capable of initiating DNA replication on a single-stranded template (143, 144, 223–225, 258). Messing and co-workers even developed a whole vector system, the M13mp series (89, 108, 112, 129, 197–199, 227, 265).

Insertion of a foreign DNA fragment into gene 3 has also been described (294). The fusion protein becomes part of the phage coat and is immunologically accessible on the surface.

The size limit for fragments cloned in the M13/fd vector system is in the order of 5 kilobases (115). Large inserts are subject to rapid deletion during propagation; especially transposons are quickly lost (116, 118). Stabilization by concomitant insertion of resistance genes and submission to selective pressure is used to counteract these spontaneous reversion processes.

Segments of the phage genome have also been cloned into other vectors: M13 DNA fragments ranging from within gene 3 to gene 6 or 1, respectively, have been inserted into pBR317 (145). Restriction fragments of the M13 IR were cloned into pBR322 and allowed functional analysis of different segments in the vicinity of the replication origins (47, 48). fd gene 2 was cloned into pBR325 or phage λ DNA and further used to construct safe cloning vectors (91, 206). The gene was also inserted into plasmid pING1 to achieve a high-level expression of g2p from the resulting polycistronic mRNA (141).

Novel plasmids containing the viral replication origins and packaging signal may be used in conjunction with the infection of a helper phage to obtain phagelike particles which harbor single-stranded plasmid genomes (91, 244).

The reader interested in the methodology of Ff vectors is referred to the excellent review by Zinder and Boeke (354). It presents a wealth of practical information and detailed instructions for choice of the appropriate vector and host, preparation of phage and DNA for cloning, construction of recombinants, etc.

A methodical approach for fast and simple sequencing of DNA cloned in M13 has also been published (277).

CONCLUDING REMARKS

The Ff coliphages have stimulated enormous research interest. Their morphology and structure, as well as the organization, replication, and expression of their genomes, are understood in detail. However, other facets of this viral system are still largely unknown or in dispute. The majority of unanswered questions concern the two vital processes of host infection and assembly of progeny phage.

With regard to penetration, the exact function the pilus serves is still unclear. Why do Ff phages bind only to the tips

of the pili? What role does pilus retraction play in successful infection? How are the *E. coli* membranes transversed? Is the formation of membrane pores a necessity for penetration of the viral DNA? When yes, do the coat proteins play a role?

The assembly process, on the other hand, poses its own questions. Evidently *E. coli* thioredoxin is involved in assembly, but what is its role? Does it alter or activate some protein(s) and, if yes, which one(s)? Is it in itself essential and sufficient for assembly or needed only indirectly as a kind of "catalyst"? What is the nature of its possible interaction with g1p/g4p? The exact role of g1p and g4p in assembly is completely unclear. Also, the precise mode of progeny "construction" is not known. How does the primary complex of g5p and single-stranded DNA formed in the cytoplasm get to the assembly site? This site has to be of membranous nature since all coat proteins as well as g1p and g4p are believed to be membrane bound, and ready-made particles have never been found in the cell interior. Do the adhesion zones between the inner and outer membranes play a morphogenetic role in phage assembly, or do they merely constitute some kind of "transport channel" out of the cell? In which way is g5p replaced by the coat proteins during DNA extrusion?

Apart from being an attractive system themselves, the filamentous bacteriophages can be used to solve other problems. In this context, an important advantage of this system is that the host cell is not lysed. Hence, for example, the inner compartmentation of *E. coli* and the metabolism of its membrane components may be studied with the help of Ff. Research into the replication of the viral DNA could also provide information about certain steps in the replication process of the host genome. Last, but not least, the use of Ff as cloning vectors facilitates the genetic study of bacteria as well as viruses or plasmids. In conclusion, these phages are a versatile research tool for a wide variety of experimentation. It seems that their time has just begun.

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