localization of structural genes and regulatory elements on the genome of bacteriophage M 13

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LOCALIZATION OF STRUCTURAL GENES AND REGULATORY ELEMENTS ON THE GENOME OF BACTERIOPHAGE M13

PROEFSCHRIFT

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CHAPTER I

GENERAL INTRODUCTION

M13 is a filamentous single-stranded DNA bacteriophage, closely related to the phages f1, fd and ZJ/2. These phages infect only male strains of *Escherichia coli* and do not kill the host cell. Their progeny is released continuously through the bacterial cell membrane.

The propagation of the M13 phage requires not only the eight structural genes located on the M13 genome, but also an unknown number of host cell functions. In addition to the structural genes, the M13 genome contains several regulatory elements, *i.e.* regions with a defined nucleotide sequence that contain the signals required for the initiation of DNA replication (origin of replication) or for the initiation and termination of RNA synthesis (promoter and terminator respectively). The localization of such regulatory elements and of the structural genes must be known to further an understanding of the mechanisms involved in the expression and multiplication of the M13 genome.

In this thesis we describe the ordering of specific fragments of M13 double-stranded DNA produced by the action of restriction endonucleases (Chapter II, V and VII). Such restriction fragments we used to determine precisely the localization of the structural genes (Chapter III, IV and V), some promoters (Chapter IV) and the origin of the complementary strand synthesis of the RF replication (Chapter VI) on the M13 genome.

In this Chapter a short introduction to the biology of the F-specific filamentous phages, in particular M13, will be given.

1.1 BACTERIOPHAGE M13

M13 is a filamentous bacterial virus that contains single-stranded DNA in a tubular coat (length 8500 Å, diameter 60 Å) consisting of about 2000 molecules of the major capsid protein (mol. wt. 5,200) which is specified by gene VIII (see Table 1) (1-3). In this tubular coat the capsid proteins overlap each other like shingles, forming a cylindrical shell that surrounds the viral DNA (4). A few molecules of a larger protein (mol. wt. 60,000), encoded by gene III, are present, probably at one end of the filament, and this protein may be involved in the binding of the phage to the bacterial cell or possibly to the F-pili (1, 5-11). It has also been suggested that a third minor protein component (gene VI product?) is present in the phage particle, but solid experimental evidence for this suggestion is not available (2, 12).

Table 1. *The sizes of the M13 gene products and their possible function*

Genes	Function	Size of the protein (daltons)*				
		in vivo		*in vitro*		
I	(phage assembly?) (1,12,14,21)	-		35 - 36,000		(36,41)
II	DNA synthesis (nickase?) (23-27)	(40,000)	(35)	40 - 46,000		(36,41)
III	capsid protein, adsorption (1,8,11,14,28)	56-68,000	(2,36,37)	59 - 68,000		(36,41)
IV	(phage assembly?) (1,12,21)	(45,000)	(38)	48 - 50,000		(36,41)
V	DNA synthesis (1,21,24,29-33)	9,700	(2,39,40)	9 - 10,000		(41,42-44)
VI	(capsid protein?, phage assembly?) (1,2,12,21)	(3,000)	(2,12)	-		
VII	(phage assembly?) (1,12,21)	-		-		
VIII	capsid protein (1,8,21,71)	5,240	(2,3)	5,2 - 5,800		(36,41)

* Most of the molecular weights have been deduced from the mobility of the polypeptides in SDS gels relative to markers and the reported molecular weights may vary with gel system and/or the laboratory.

1.2 GENE PRODUCTS AND GENE FUNCTIONS

The genome of bacteriophage M13, which consists of a covalently closed circular DNA molecule of approximately $2x10^6$ daltons, is one of the smallest viral genomes known (1, 13). By genetic complementation tests using conditional lethal mutants, the presence of eight genes in bacteriophage M13 has been determined (8, 14). The arrangement of these eight genes on the M13 genetic map has been ascertained recently (15-20, Chapter III and V).

At the present time the functions of only four genes are known, namely of the genes II, III, V and VIII (see Table 1). The functions of the remaining genes are unknown, but have been suggested to be involved in phage assembly (1, 12, 21).

The gene products of M13 can be identified in M13-infected cells as well as in an *in vitro* protein synthesis system. The continuation of host protein synthesis after M13 infection, however, has hampered the identification of the products of most phage genes *in vivo* (2). For this reason only three of the eight gene products synthesized *in vivo* have been characterized unequivocally. Two of these proteins are localized in the bacterial membrane (the gene III and gene VIII protein) and one in the cytoplasm (the gene V protein) (2, 34, 71).

By using a DNA-dependent *in vitro* protein synthesis system, it has been possible to identify the products of six of the eight M13 genes, namely the products encoded by the genes I, II, III, IV, V and VIII (for molecular weights see Table 1) (36, 41-44). In this *in vitro* system the products of gene III and gene VIII are larger than those found *in vivo* and may be precursor molecules of these (21, 36).

The products of gene VI and gene VII have not yet been identified (45). A possible explanation for this failure is that they are synthesized, both *in vivo* and *in vitro*, in very small amounts. Another possibility, although less probable, is that these proteins are degraded immediately after their synthesis.

1.3 REPLICATION

After adsorption of the phage to the host cell, the single-stranded M13 DNA penetrates into the cell and is replicated. This replication process occurs in three stages (1, 13):

1. Conversion of single-stranded (SS) DNA into double-stranded parental replicative form (RF) DNA (SS → pRF).
2. Replication of RF DNA (pRF → RF).
3. Synthesis of progeny SS DNA (RF → SS).

The conversion of phage DNA into parental RF DNA takes place during or immediately after its entrance into the cell (11, 28). The pRF thus formed is replicated and a pool of progeny RF is build up. For the pRF replication a functional gene II protein is required (24). At about 15 to 20 minutes after infection, when the RF pool size is about 100 to 200 molecules, the amount of RF replication decreases and the synthesis of progeny SS DNA becomes predominant (21). This change in replication mode requires the presence of the products encoded by gene II and gene V (21, 32). Newly synthesized progeny SS molecules which first accumulate as a pool of single-stranded DNA, are subsequently enclosed in a protein coat in the inner membrane of the host and are then released from the cell (21, 34, 71).

M13 RF DNA exists in two forms in the cell, namely RF-I DNA and RF-II DNA. RF-I DNA is closed, circular, double-stranded replicative form DNA with twists superimposed on the turns of the double helix, whereas RF-II is a non-twisted circular DNA duplex with at least one single-stranded break (1).

1.3.1 CONVERSION OF SS DNA INTO PARENTAL RF DNA

In the first stage of infection SS DNA is converted into pRF DNA by the host replicative system without requiring any newly synthesized phage protein (1, 24). There are, however, indications that gene III protein, present in the phage particle, may perform an essential function in the synthesis of pRF DNA by linking the phage DNA to a cellular replication system in or on the inner cell membrane (11, 28).

In vitro investigations have revealed that the synthesis of the complementary strand is initiated at a specific site on the SS DNA molecule by action of *E.coli* RNA polymerase (48-53). This enzyme synthesizes a primer fragment which is then elongated by the action of DNA polymerase III holoenzyme (DNA polymerase III star and copolymerase III star) (50, 54). The RNA primer is finally removed by the 5' → 3' exonuclease action of DNA polymerase I (50, 52). The completed complementary strand is circularized and closed by the action of DNA ligase (50, 52).

1.3.2 REPLICATION OF RF DNA

The replication of RF-I DNA requires, in addition to DNA polymerase III holoenzyme and some unknown host factors, the phage gene II protein (55-59). After the start of the RF DNA replication a single-stranded break is introduced into the viral strand of the RF-I molecule (25-27). At present it is not

absolutely clear whether the gene II protein itself is directly responsible for this cleavage or whether it somehow works in co-operation with an host endonuclease.

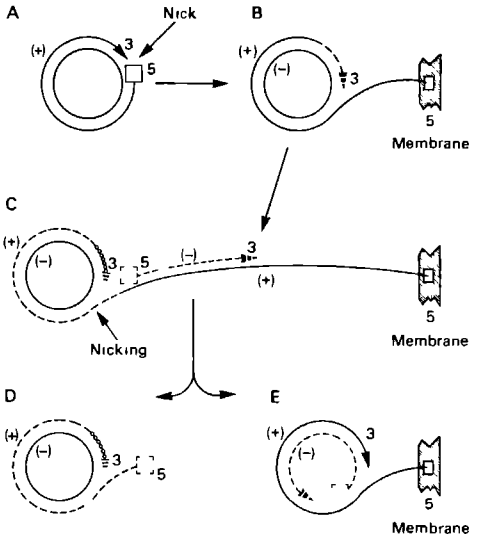

Fig. 1.: *ϕX-174 Replication according to the rolling circle model of Gilbert and Dressler.*

The precise mechanism of the RF replication is unknown. However, most of the experimental data on the M13 RF DNA replication are consistent with the "rolling circle" model proposed by Gilbert and Dressler for the RF replication of bacteriophage ϕX-174 (60). The replication of this icosahedral, single-stranded DNA phage is similar to that of M13 (13). According to this model the RF replication occurs as follows:

1. An endonuclease ("nickase") cleaves the viral (+) strand at a specific and unique sequence (the origin of replication) of the RF-I molecule converting this to RF-II (Fig. 1A).
2. After nicking the 5' end of the viral (+) strand is bound to a specific site on the membrane (Fig. 1B).
3. Viral (+) strand synthesis is started at the 3' end of the "parental" viral (+) strand, followed by elongation of this strand using the "parental" complementary (-) strand as a template (Fig. 1B). This elongation results

in the displacement of the "parental" viral strand (Fig. 1C).

4. The complementary (-) strand synthesis is started, when a unique initiation region is exposed on the displaced viral strand and proceeds in a direction opposite to that of the viral (+) strand synthesis (Fig. 1C).
5. At the end of the first round of replication, the "nickase" splits the replicating molecule into two intermediates: one containing the "parental" viral (+) strand (Fig. 1E) and the other containing the "parental" complementary (-) strand (Fig. 1D).
6. The replicative intermediate, containing the "parental" viral (+) strand (Fig. 1E), is converted into an RF-I molecule, while the other one (Fig. 1D) starts a new round of replication.

The following evidence supports the rolling circle model:

ad 1. Analysis of RF-II DNA, isolated from *E.coli* rep_3^- cells (these cells allow pRF DNA formation, but noc its replication), has demonstrated that under the direction of the M13 gene II protein a strand specific discontinuity is made in the viral strand of pRF-I DNA (25). This observation indicates an endonuclease activity specifically acting on the viral strand.

ad 2. Pulse labeling experiments have shown that replicating M13 RF DNA is associated with the membrane (72, 61, 62).

ad 3. Biochemical analyses of intermediates of the M13 RF replication have shown (61, 64) that some of these intermediates contain viral strands, longer than genome length, as is required for rolling circle intermediates as shown in Fig. 1C. It should be mentioned that intermediates containing viral strands shorter than genome length are also observed (64). Such intermediates are not consistent with a rolling circle model. Transfection experiments with heteroduplex molecules have suggested that the minus strand of a replicating RF molecule is the master template for progeny RF molecules (Fig. 1C) (65).

ad 4. Analysis of RF-I molecules, labeled after the start of the RF replication, has demonstrated that the complementary strand synthesis proceeds in a direction opposite to that of the viral strand synthesis (Chapter VI). This observation suggests that the complementary strand synthesis starts when a "tail" of one genome length of the viral strand is formed. This suggestion is in agreement with the proposed mechanism of complementary strand synthesis (Fig. 1C).

ad 6. The observation that after the start of the RF replication only the complementary strand of the first newly synthesized RF-I molecules is

labeled, indicates that the replication intermediates which contain the "parental" viral strand are converted into RF-I molecules (Chapter VI).

1.3.3 SYNTHESIS OF PROGENY SINGLE-STRANDED DNA

The synthesis of progeny single-stranded DNA, requiring both gene II and gene V protein, occurs by an asymmetric replication of RF-I DNA (21, 24, 26, 30-33, 46, 63, 66-69). This process is initiated by the generation of a 3' end on the viral strand of RF-I DNA, presumably by gene II action (21, 26, 46). The subsequent elongation of the 3' end probably occurs in the same way as elongation of the viral strand during the RF replication (see Fig. 1C) (47, 67, 68). However, the conversion of the single-stranded "tail" into double-stranded DNA is now repressed by the action of the phage specific protein encoded by gene V. This protein, which binds co-operatively to the nascent single strands, inhibits the elongation of the complementary strand (21, 29-33, 38). Segments of SS DNA of genome length are cut from the "tails" of the replicating intermediates, probably by the action of gene II protein. The linear DNA molecules, which are still covered by gene V proteins, are then circularized by host cell enzymes (70). The newly synthesized DNA molecules join an intracellular pool of single-strand molecules before maturing into phages (47). This maturation occurs in the *E.coli* inner membrane and requires probably the products of gene I, III, IV, VII and VIII (21, 34, 71). During this maturation the gene V proteins, bound to the single-stranded DNA, are displaced by the capsid proteins encoded by gene VIII and gene III and can thus be recycled (21, 72).

1.4 TRANSCRIPTION

The transcription process starts immediately after the parental RF molecule is formed (1, 13). The messenger RNA's are all transcribed from the complementary strand of this RF molecule in a direction which is counterclockwise around the genetic map (36, 73, 74, Chapter IV).

Based on studies of *in vitro* transcription of RF-I DNA with *E.coli* RNA polymerase holoenzyme,a model for this transcription has been proposed (75-82). This model postulates that different RNA classes are the result of a transcription process in which initiation of RNA synthesis occurs at different promoter sites and termination at a unique termination site. This implies that all RNA species should contain identical nucleotide sequences at their 3'-terminal end. This model is supported by the following recent observations:

1. *In vitro* transcription of M13 RF-I DNA gives rise to seven RNA species ranging in size from about 8S upto 26S (77). Two of these RNA species (23S and 26S) start with pppA, while the other five (8S, 11S, 14S, 17S and 19S) start with pppG (77). *In vitro* transcription of fd and f1 RF-I DNA also gives rise to transcripts of specific size (75, 76, 80). However, due to the method of separation used, only four classes of RNA could be identified, namely 10S, 13S, 17S and 26S RNA (75, 76, 80).
2. With the aid of DNA restriction fragments, eight promoter sites (RNA initiation sites) have been located on the M13 genome (74, 81, Chapter IV). The positions of these promoters are in agreement with the size of the RNA species observed (77). A generally similar distribution of RNA polymerase binding sites on the genome has been found for the phages fd and f1 (19, 20, 80, 83).
3. Hybridization-competition studies with the four RNA transcripts of phage f1, have indicated that the largest RNA's have sequences in common with the smaller RNA's (80).
4. Studies of *in vitro* protein synthesis, directed by various M13 and f1 RNA species synthesized *in vitro*, have demonstrated that all size classes of RNA are able to synthesize the protein encoded by gene VIII (77, 80). From these results it is infered that the termination site is located immediately distal to this gene (77, 80). This conclusion is furthermore substantiated by transcription studies on M13 restriction fragments which contain the entire gene VIII (77).

Recent studies on *in vitro* protein synthesis, directed by the various M13 specific RNA molecules isolated from infected cells, suggest that a similar transcription process also operates *in vivo* (84).

In cells, infected with bacteriophage M13, the proteins specified by gene V and gene VIII are synthesized in much larger quantities than the other phage encoded proteins (2). Some regulatory mechanism(s) must therefore ensure that the synthesis of these proteins is initiated more frequently than that of the other phage specific proteins. Evidence has been presented for the occurrence of the following regulatory mechanisms on both transcriptional and translational level *in vitro*:

1. RF-I DNA is transcribed into several distinct polycistronic mRNA's of different lengths which have in common the coding information for gene VIII (77, 80). As a consequence, the synthesis of the gene VIII protein is more pronounced than the synthesis of the other phage proteins (transcriptional control).

2. The regions coding for gene V and gene VIII are transcribed more efficiently than the regions coding for all other genes (transcriptional control) (81).
3. The net synthesis of gene V and gene VIII products in *in vitro* protein synthesis systems,directed by the differently sized mRNA's,is always higher than that of the other phage encoded proteins (translational control) (80).

These mechanisms probably also operate *in vivo*, but further studies are needed to determine the contribution of each of these controlling steps to the observed level of synthesis. This information will allow us to determine to what extent the *in vitro* transcription system can be used to study the control of gene expression on the transcriptional level.

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CHAPTER II

STUDIES ON BACTERIOPHAGE M13 DNA

1. A CLEAVAGE MAP OF THE M13 GENOME

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Studies on Bacteriophage M13 DNA

1 A Cleavage Map of the M13 Genome

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A physical map of the bacteriophage M13 genome has been constructed on the basis of specific cleavage of M13 replicative form DNA by bacterial restriction endonucleases The 13 fragments produced by the enzyme from *Haemophilus aphrophilus* (endonuclease R *Hap* II) as well as the 10 fragments produced by the enzyme from *Haemophilus aegyptius* (endonuclease R *Hae* III) have been ordered by analysis of partial digest products and by analysis of overlapping sets of fragments

In addition, the single site in M13 replicative form DNA cleaved by the restriction enzyme from *Haemophilus influenzae* Rd (endonuclease R *Hin* dII) has been located more precisely With this unique site as a reference point, the *H aphrophilus* cleavage sites and the *H aegyptius* cleavage sites have been localized on the map

The genome of the small filamentous bacteriophage M13 consists of a circular, single-stranded DNA ($M_r = 2 \times 10^6$), which, upon infection, is converted into a double-stranded replicative form DNA molecule (for review see [1]) In any attempt to sequence this DNA it seems logical that an initial step would be the production of small unique fragments of the whole molecule The utilization of bacterial restriction endonucleases is very attractive for this purpose since these enzymes make a limited number of duplex cleavages in DNA by recognizing specific nucleotide sequences [2–5], thus providing DNA fragments useful in physical mapping studies [6,7] and in nucleotide sequence analysis Cleavage of DNA into defined fragments can also be used to analyse the structure and function of particular parts of the genome This approach has already been used to study operator [8,9] and promoter regions in DNA of bacteriophage λ, T7, fd and ΦX-174 [9–12], but also in DNA of the Simian virus-40 [10 13] and the human adenovirus-2 [10] In addition, the availability of ordered DNA fragments has allowed the localization of the origins of DNA replication [14–18], as well as the direction of both replication [15–17] and transcription [13] of bacteriophage and viral DNA

We reported earlier that the *Hin* dII restriction endonuclease from *Haemophilus influenzae* has a single cleavage site in the double-stranded, covalently closed replicative form DNA of phage M13 [19] Recently we observed that the restriction endonuclease *Hap* II from *Haemophilus aphrophilus* produces 13 fragments from M13 RF molecules and that a completely identical fragmentation pattern was obtained with the *Hpa* II endonuclease from *Haemophilus parainfluenzae*

In order to use these fragments and enzyme cleavage sites to localize genes and template functions of the M13 genome, it is necessary to order the fragments in the molecule By analysis of partial digest products and overlapping sets of fragments, we have ordered the M13 RF pieces produced by the *Hap* II endonucleases as well as the fragments produced by the restriction endonuclease *Hae* III from *Haemophilus aegyptius* In addition, the unique site cleaved by the *Hin* dII enzyme has also been localized On the basis of these data we have constructed a physical cleavage map of the M13 genome

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Abbreviations *Hap* endonuclease endonuclease R *Hap* II from *Haemophilus aphrophilus* *Hae* endonuclease endonuclease R *Hae* III from *Haemophilus aegyptius* *Hin* endonuclease endonuclease R *Hin* dII from *Haemophilus influenzae* Rd the nomenclature of restriction enzymes follows the convention suggested by Smith and Nathans [24] RF replicative form DNA RF I covalently closed circular double stranded DNA RF II double stranded circular DNA containing at least one single strand nick RF III full length linear double stranded DNA

Enzymes RNAase A or ribonuclease A from pancreas (EC 3 1 4 22) endonucleases (EC 3 1 4 30)

The consequences of this map for the gene order of bacteriophage M13 is discussed in the following paper [20]

MATERIALS AND METHODS

Bacteria and Phages

Escherichia coli C89 (K12, 159F$^+$, *uv*^{s}, *su*$^-$) was used for the cultivation of M13 phage as well as for the preparation of M13 replicative form I DNA

Haemophilus influenzae Rd was obtained from Dr H O Smith and from Dr S W Glover *Haemophilus aphrophilus* strain was a kind gift of Dr M Takanami

Phage M13 (*wt*) was originally obtained from Dr P H Hofschneider

Preparation of ^{32}P-Labeled M13 Replicative Form I DNA

E coli C89 was grown in 200 ml of low-phosphate medium This medium contained per liter 4 0 g bactopeptone (Difco), 12 0 g Tris base, 1 0 g KCl, 1 0 g NH_4Cl The pH was adjusted with HCl to 7 4 After autoclaving, 0 2 g $CaCl_2$, 4 g glucose and 0 3 g $MgSO_4$ were added At a density of 4×10^8 cells/ml the culture was supplemented with 10 mCi of [^{32}P]-orthophosphate (carrier-free, The Radiochemical Centre, Amersham, England) and infected with M13 phage at a multiplicity of 10 After 60 min at 37 C the infected cells were harvested, washed once with 100 ml of 50 mM Tris-HCl, pH 8 0 and resuspended in 8 ml of the same buffer Thereafter 6 ml of 0 1 M EDTA, pH 8 0 and 2 ml of lysozyme (4 mg/ml in 0 25 M Tris-HCl, pH 8 0) were added and lysis was affected by incubation for 30 min at 37 °C To the viscous solution 1 5 ml of 20% sodium dodecyl sulphate was added and the incubation was continued for 15 min at 37 °C Host DNA was removed by careful addition of 5 M NaCl to a final concentration of 1 M and after standing for 3 h at 0 °C, the DNA precipitate was spun down at $78000 \times g$ for 30 min The supernatant, containing the RF, was saved and extracted twice with an equal volume of phenol, saturated with 0 1 M Tris base and 1 mM EDTA The remaining aqueous phase was dialysed exhaustively against Tris-saline-EDTA buffer (0 1 M Tris-HCl, pH 7 6, 0 1 M NaCl, 1 mM EDTA) To the dialysed solution heat-treated pancreatic ribonuclease A (Boehringer Mannheim, Germany) was added to a final concentration of 50 µg/ml and the solution was incubated for 30 min at 37 °C Thereafter the same amount of ribonuclease A was added and the incubation continued for a second 30-min period The solution was then brought to 1 M NaCl and the DNA was denatured by addition of 1 M NaOH to give a pH of 11 8 After 3 min at room temperature, the solution was neutralized to pH 8 and then percolated over a nitrocellulose column (10 × 2 cm), which was equilibrated in 1 0 M NaCl, 0 1 M Tris-HCl, 1 mM EDTA, pH 7 6 All DNA was denatured in this procedure to single strands which were bound to the nitrocellulose column Under the conditions used, covalently closed, supercoiled RF I renatures spontaneously and hence remains in the flow-through fractions of the column The fractions containing ^{32}P-labeled RF I were pooled and the DNA was precipitated with ethanol at −20 °C The precipitate was dissolved in 1 ml Tris-saline-EDTA buffer and layered on a Sephadex G-100 column (25 × 2 cm), which was equilibrated in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7 6) The RF I eluted in the void volume of the column and was completely devoid of low-molecular-weight RNA digest products Finally the ^{32}P-labeled RF was precipitated by the addition of 0 1 vol of 3 M sodium acetate, pH 5 6 and 2 5 vol of ethanol at −20 °C, the precipitate collected by centrifugation at $55000 \times g$ for 15 min and dissolved in Tris-EDTA buffer The quality of the RF I preparation was then verified by sedimentation through a 5−20% alkaline sucrose gradient in 1 M NaCl, 0 1 M NaOH, 3 mM EDTA, pH 12 4 at 56000 rev /min in a SW-56 Spinco rotor for 90 min at 4 °C The specific radioactivity of the M13 RF thus prepared was about $1\,5 \times 10^6$ counts min^{-1} µg DNA^{-1}

Restriction Nuclease Assay

Restriction nuclease activity was assayed by gel electrophoresis of the digestion products In both the assay for *Hap* II endonuclease and *Hae* III endonuclease, 2 0 µg of M13 RF was incubated with appropriate amounts of enzyme in 50 µl of 10 mM Tris-HCl, pH 7 6, 7 mM $MgCl_2$ and 7 mM 2-mercaptoethanol After incubation for 120 min at 37 °C, the reaction mixture was mixed with 5 µl of 70% sucrose, 0 2 M EDTA, 1% bromophenolblue and then applied to a cylindrical polyacrylamide gel (12 cm long, 6 mm internal diameter) Gels were 3% in acrylamide and 0 15% in *N,N* -methylenebisacrylamide and were prepared in 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7 8 After electrophoresis the gels were stained for 30 min in ethidium bromide solution (1 µg/ml) as described by Sharp *et al* [31] and the enzyme activity was evaluated by comparison of the fragmentation patterns obtained One activity unit of restriction endonuclease was defined as the amount of enzyme catalyzing the complete conversion of 1 0 µg of RF into DNA fragments

in 60 min at 37 °C under the standard conditions of incubation

Restriction endonuclease *Hin* dII was assayed under exactly the same conditions using 2 0 µg of T7 DNA as a substrate in an incubation mixture containing 10 mM Tris-HCl, pH 7 6, 50 mM NaCl, 7 mM $MgCl_2$ and 7 mM 2-mercaptoethanol

Cleavage of ^{32}P-Labeled M13 Replicative Form I DNA with H aphirophilus *Restriction Endonuclease*

The *Hap* II endonuclease, used for most of the experiments to be described, was isolated according to the method described by Takanami [20,21] The enzyme was stored in 50% glycerol, 10 mM potassium phosphate, pH 7 6, 2 mM 2-mercaptoethanol at −20 °C

For preparation of complete digests, ^{32}P-labeled M13 RF I supplemented with 1 0 µg of unlabeled RF was incubated with *Hap* II endonuclease at 37 °C in a reaction mixture containing 10 mM Tris-HCl, pH 7 6, 7 mM $MgCl_2$ and 7 mM 2-mercaptoethanol With each new preparation of enzyme, a series of enzyme concentrations was used to determine the optimum conditions for complete digestion of M13 RF Some variation was found for different preparations, but generally incubation of 1 0 µg of RF with 0 6 units of enzyme in a volume of 0 1 ml for 3 h at 37 °C resulted in complete digestion, as determined by electrophoresis of the products All reactions were terminated by the addition of EDTA to a final concentration of 20 mM and chilling the incubation mixture to 0 °C

To obtain partial digests of ^{32}P-labeled M13 RF I, both the ratio of enzyme to DNA and the time of incubation were varied, but in all cases the incubation was performed in a final volume of 0 1 ml at 37 °C in the presence of 1 0 µg of carrier RF in the standard incubation buffer described above For instance, digestion with 0 2 unit of enzyme for 60 min yielded at least 20 intermediate products in addition to some limit products of the reaction Individual, partially digested fragments isolated from the preparative polyacrylamide gel (see below) were completely digested by incubation with *Hap* II endonuclease in excess This was routinely carried out by adding 0 3 unit of enzyme to an incubation mixture of 25 µl of 10 mM Tris-HCl, pH 7 6, 7 mM $MgCl_2$ and 7 mM 2-mercaptoethanol, containing the DNA fragment, but in which case the addition of carrier RF was omitted Incubation for 3 h at 37 °C was generally sufficient to achieve complete digestion

Digestion of individual *H aegyptius* fragments with *Hap* II enzyme was carried out under exactly the same conditions as used for complete digestion of partially digested *H aphirophilus* fragments

Cleavage of ^{32}P-Labeled M13 Replicative Form I DNA with H aegyptius *Restriction Endonuclease*

The enzyme used in these studies was *Hae* III endonuclease and was generously provided by Dr W Fiers The enzyme was stored in 50% glycerol, containing 10 mM potassium phosphate, pH 7 6 and 2 mM 2-mercaptoethanol at −20 °C For preparation of complete digests, ^{32}P-labeled M13 RF I was supplemented with 0 1 µg of carrier RF and incubated with *Hae* III endonuclease at 37 °C in a reaction mixture of 0 05 ml containing 10 mM Tris-HCl, pH 7 6, 7 mM $MgCl_2$ and 7 mM 2-mercaptoethanol The incubation reaction was terminated by the addition of sodium dodecyl sulphate to a final concentration of 1%, followed by incubation for 15 min at 37 °C Optimum conditions for complete digestion of M13 RF were worked out essentially as described for the *Hap* II endonuclease

Individually isolated *H aphirophilus* fragments were completely digested with 0 5 to 0 8 unit of *Hae* III endonuclease for 90 min at 37 °C in 50 µl of standard incubation buffer The addition of carrier RF, however, was omitted

Cleavage of ^{32}P-Labeled M13 Replicative Form I DNA with H influenzae *Restriction Endonuclease*

The enzyme used was isolated according the procedure described by Takanami and Kojo [22] and was followed up to the phosphocellulose chromatography step The DEAE-cellulose step was omitted Instead, Sephadex G-200 column chromatography in the presence of 0 1% bovine serum albumin was used to remove residual contaminating nucleases

Treatment of Simian virus-40 DNA with this enzyme preparation and subsequent polyacrylamide gel electrophoresis revealed 11 sharp bands in the gel as observed earlier by Danna and Nathans [23] This is due to the action of two restriction enzymes, namely *Hin* dII and *Hin* dIII endonuclease [6], and we therefore consider our preparation to contain both restriction enzymes As *Hin* dIII endonuclease has no cleavage site in M13 RF I, (Vereijken and Jansz, personal communication) the enzyme preparation used will be denoted as *Hin* dII for convenience

Incubations were carried out for 1 h at 37 °C in a buffer containing 10 mM Tris-HCl, pH 7 6, 50 mM NaCl, 7 mM $MgCl_2$ and 7 mM 2-mercaptoethanol Generally, incubation of 1 0 µg of M13 RF with 1 0

to 1.5 units of enzyme in a final volume of 0.1 ml resulted in a complete conversion of the supercoiled RF-I into full-length linear RF-III molecules [19].

Vertical-Slab Gel Electrophoresis

Before electrophoresis, samples of digested DNA were made 20 mM in EDTA, 15% in sucrose and 0.1% in bromophenolblue. Electrophoresis was carried out in a discontinuous polyacrylamide gel slab (40 cm × 18 cm × 0.2 cm), composed of a 3% polyacrylamide gel (about 25 cm long) on top of a 10% polyacrylamide gel layer (12 cm height) in electrophoresis buffer containing 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.8. A gel chamber similar to that described by de Wachter and Fiers [25] was used. Electrophoresis was performed at room temperature for 20–24 h with a constant current of 40 mA. Under these conditions bromophenolblue moved to the end of the gel.

Preparative electrophoresis of digestion products was followed by autoradiography of the wet gel to locate the ^{32}P-labeled bands. Gel segments (2 × 0.4 × 0.2 cm) corresponding to these bands were excised, the gel crushed by piercing through a hypodermic syringe and shaken for a few seconds with 0.5 ml of 0.1 × standard saline citrate, pH 7.4. After standing for 2 h at room temperature, the suspension was clarified by centrifugation (5 min at 5000 × *g*), the supernatant saved and the extraction repeated. In preparation for redigestion, the DNA in the combined eluates was precipitated by the addition of 0.1 vol. of 3 M sodium acetate, pH 5.6 and 2.5 vol. of ethanol at −20 °C. The precipitate was recovered by centrifugation (15 min at 80000 × *g*) and then dissolved in 25–50 µl of the appropriate incubation buffer. For autoradiographic analysis of DNA fragments, the wet gels were placed in contact with Kodak RP/R 54 medical X-ray film and exposed for 1–2 days. Satisfactory radioautographs were obtained with approximately 300 counts/min or more of the ^{32}P-labeled DNA fragments.

Nomenclature for DNA Fragments

The designation of DNA fragments produced by restriction endonucleases follows the convention suggested by Danna *et al.* [6] and Smith and Nathans [24]. In addition, arabic numerals are used to designate enzyme cleavage sites, with a prefix indicating the enzyme used to cleave this site. The direction of numbering these sites is clockwise, using the single cleavage site of the *Hin* dII enzyme as a zero point.

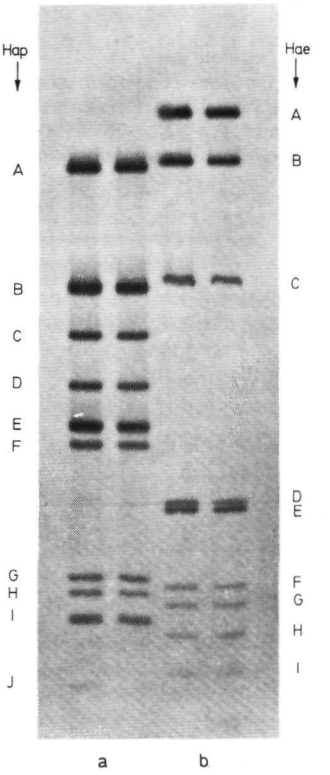

Fig. 1. *Autoradiograph of a polyacrylamide gel fractionation of* ^{32}*P-labeled M13 RF I fragments, produced by cleavage with* Hap II *endonuclease and* Hae *III endonuclease.* For preparation of digests, 0.02 µg of ^{32}P-labeled M13 RF I (10^6 counts min^{-1} μg^{-1}), supplemented with 1.0 µg of unlabeled M13 RF, was incubated with varying amounts of restriction endonuclease in a volume of 0.1 ml of 10 mM Tris-HCl, pH 7.6, 7 mM $MgCl_2$, 7 mM 2-mercaptoethanol for 3 h at 37 °C. Thereafter the digests were subjected to electrophoresis on 3% discontinuous polyacrylamide slab gels for 20 h at 40 mA. Conditions for electrophoresis and autoradiography are described under Methods. (a) RF fragments produced by incubation with 0.5 unit (left) and 1.5 units (right) of *Hap* II endonuclease. (b) RF fragments produced by 0.4 unit (left) and 1.0 unit (right) of *Hae* III endonuclease

RESULTS

^{32}P-labeled M13 RF was incubated with varying amounts of *Hap* II restriction endonuclease and the ensuing digests were subjected to polyacrylamide gel electrophoresis. Autoradiographs of these gels show 10 distinct bands, designated *Hap*-A to *Hap*-J (Fig. 1). Incubation with higher enzyme concentrations did not change this pattern, which argues, therefore, that the fragments represent terminal digestion products.

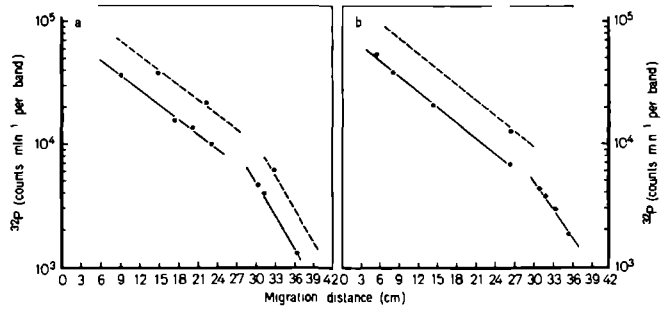

Fig 2 *Relationship between mass and electrophoretic mobility for M13 RF I fragments produced by cleavage with* Hap *II endonuclease and* Hae *III endonuclease* ^{32}P radioactivity present in each band has been plotted on a logarithmic scale against their distance of migration during electrophoresis on a 3% discontinuous polyacrylamide gel The dashed lines in parallel are calculated mass *versus* mobility lines for radioactive bands with twice the number of fragments as those bands falling on the solid lines (a) *Hap* II fragmented RF I, (b) *Hae*-III-fragmented RF I

Table 1 *Chain lengths of M13 RF I fragments produced by cleavage with restriction endonuclease from* H aphrophilus *and* H aegyptius
^{32}P labeled M13 RF I was digested with either *Hap* II endonuclease or *Hae* III endonuclease as described under Methods and the digest products were separated on 3% discontinuous polyacrylamide gel slabs Individual bands were excised the gel solubilized with periodate [20] and the radioactivity determined Relative molecular weights were evaluated by expressing the radioactivity values of each band as a fraction of the total summed radioactivity Chain lengths are given in base pairs and are calculated from the molecular-weight fraction of the fragments by assuming a total of 6400 base pairs for the M13 RF molecule Figures represent average values obtained from analysis of six *Hap* II and four *Hae* III fragmented RF I preparations The designation of bands corresponds to Fig 1

Hap fragments	Relative molecular weight	Chain length in base pairs	*Hae* fragments	Relative molecular weight	Chain length in base pairs
	% total			% total	
A	23 8 ± 0 7	1530	A	39 0 ± 0 9	2500
B_1	12 8 ± 0 4	820	B	25 5 ± 0 8	1630
B_2	12 5 ± 0 4	800	C	12 7 ± 0 8	820
C	10 1 ± 0 3	650	D	4 9 ± 0 6	310
D	8 7 ± 0 4	560	E_1	9 0 ± 0 3	290
E_1	7 1 ± 0 4	460	E_2		290
E_2	7 0 ± 0 3	450	F	2 9 ± 0 1	190
F	6 4 ± 0 4	410	G	2 5 ± 0 1	160
G	3 1 ± 0 2	200	H	1 8 ± 0 1	120
H	2 7 ± 0 2	170	I	1 2 ± 0 1	70
I_1	2 2 ± 0 2	140			
I_2	2 2 ± 0 2	140			
J	0 8 ± 0 1	50			

A logarithmic plot of ^{32}P radioactivity present in each band *versus* the distances migrated during electrophoresis shows that most of the points fall on two straight lines (Fig 2) The biphasic nature of the curve is a consequence of the discontinuous gel system used, of which the transition between the 3% and 10% gel layers is located between the position of band *Hap*-F and *Hap*-G The ^{32}P radioactivity in *Hap*-B, *Hap*-E and *Hap*-I fall on a line parallel to the single fragment *versus* mobility line, corresponding to the radioactivity expected for bands containing two fragments of similar size We conclude from this that bands B, E and I contain two fragments, which further will be designated as *Hap*-B_1 and *Hap*-B_2, *Hap*-E_1, *Hap*-E_2, *Hap*-I_1 and *Hap*-I_2 In fact a better resolution of these partially resolved doublets could be obtained by performing longer electrophoretic runs on 3% and 10% polyacrylamide gels [20] Therefore, cleavage of M13 RF by *Hap* II restriction endonuclease yields 13 specific limit fragments On the basis of the relative yield of each fragment from uniformly ^{32}P-labeled RF, the size of each fragment has been estimated (Table 1) Assuming a total of 6400 nucleotide pairs per M13 RF molecule [1], this resulted in a range of 1530 nucleo-

Fig 3. *Electrophoretic analysis of partial digestion fragments of* ^{32}*P-labeled M13 RF I after complete digestion with* Hap *II restriction endonuclease* (a) Partial *Hap* II digest of M13 RF I 0 2 μg of ^{32}P-labeled M13 RF I (10^6 counts min^{-1} μg^{-1}), supplemented with 1 0 μg of unlabeled RF were incubated for 60 min at 37 °C with 0 2 unit of *Hap* II endonuclease in a final volume of 0.1 ml of 10 mM Tris-HCl, pH 7 6, 7 mM $MgCl_2$ and 7 mM 2-mercaptoethanol After addition of EDTA to 0 02 M, the partial digests were electrophoresed for 23 h at 40 mA (b to e) Complete digestion of partial digest products with *Hap* II endonuclease Each partial digest product (designated 1,2,3 *etc* corresponding to their position in a), was eluted from the preparative gel, the DNA fragment was concentrated by ethanol precipitation and then completely digested with *Hap* II enzyme in excess in a volume of 0 05 ml of standard incubation buffer Conditions for elution, digestion and electrophoresis are described under Methods The redigested partial products were electrophoresed in a 3% discontinuous polyacrylamide slab gel, together with a complete *Hap* II digest of M13 RF I as a marker (M) Each plate is a autoradiograph of a single gel slab following electrophoresis The origin is at the top

tide pairs for *Hap*-A to about 50 nucleotide pairs for the smallest fragment *Hap*-J.

The general approach for ordering DNA fragments in a physical map was the separation by polyacrylamide gel electrophoresis and location by autoradiography of individual ^{32}P-labeled fragments incompletely digested with the *Hap* II endonuclease. Each partial product was eluted from the gel, subsequently digested with an excess of enzyme and the final products identified by their electrophoretic mobilities. In this way several sets of overlapping fragments were obtained and the order of all 13 *Hap* II fragments deduced.

Partial digests of ^{32}P-labeled M13 RF were prepared under standard conditions with either small quantities of enzyme or short incubation periods. Either procedure led to the accumulation of numerous intermediate products, the average size of which depended on the time of incubation and enzyme concentration. A typical result of partial digestion with *Hap* II endonuclease is shown in Fig. 3a. Each fragment was eluted from the gel, then further purified and concentrated and subsequently redigested with an excess of enzyme. For autoradiographic analysis, each redigested partial product was electrophoresed in a slab gel in parallel with a complete digest as a marker, in order to identify the limit products derived after redigestion. Several examples of these analyses are visualized in Fig. 3. For instance, sample 8 (Fig 3c) yielded fragments *Hap*-D and *Hap*-J upon redigestion; sample 11 (Fig. 3d) yielded fragments *Hap*-D, *Hap*-I_2 and *Hap*-J while sample 16 (Fig. 3d) produced fragments *Hap*-D, *Hap*-F, *Hap*-I_2 and *Hap*-J upon redigestion.

In some instances, the putative partial products isolated from preparative gels were actually limit products. For example, sample 10 (Fig. 3e) from the partial digest had the same electrophoretic mobility as the marker fragment *Hap*-C, and after redigestion, yielded no other fragments. The same results were obtained with putative partial products that had mobilities identical to those of fragment *Hap*-B_1,

Table 2 *Redigestion of partial digestion products of M13 RF I with* H aphirophilus *endonuclease*
Partial digestion fragments, numbered according to Fig 3, were eluted from the preparative gel and further purified as described under Methods After redigestion with an excess of *Hap* II endonuclease, each digest was analysed by 3% polyacrylamide gel electrophoresis Molecular weights of the partial fragments were estimated graphically from a plot log (molecular weight) *versus* mobility, in which the complete digest products either present in the partial digest or run in parallel, were used as reference molecular weights Calculations were made using the molecular weight values of the limit products, as listed in Table 1 When two readily distinguishable groups of final products were derived from a partial product, the groups are shown on two separate lines in the table

Undigested partial product		Redigested partial product		
Partial fragment number	estimated molecular weight	products	sum of product molecular weight	overlapping orders
	% total		% total	
7	9.0	F, I_2	8 6	F I_2
8	9 6	D, J	9 5	J D
9	10 3	G, E_1	10 1	E_1 G
	10 3	F, I_2, J	9 5	F I_2 J
11	11 8	D, I_2, J	11 7	I_2 J D
12	12 5	B_2	12 5	B_2
	12 5	C, I_1	12 3	C I_1
14	15 0	B_2, I_1	14.7	I_1 B_2
15	15 9	B_1, H	15 6	H B_1
16	18.2	D, F, I_2, J	18 5	F I_2 J D
17	19.1	B_2, E_2	19 5	B_2 E_2
	19.1	C, D	18 8	D C
18	19 9	B_1, E_1	19 9	B_1 E_1
21	22 3	B_1, E_1, H	22.6	H B_1 E_1
22	23 8	A	23.8	A
	23.8	B_2, C, I_1	24 8	C I_1 B_2
23	25 5	A, G	26.9	G A
	25 5	C, D, F, I_2, F	27.2	F I_2 J D C
24	27.2	A, F	30 2	A F
	27.2	B_2, C, E_2, I_1	31.8	C I_1 B_2 E_2
25	28.2	A, G, E_1	34 0	G A E_1
	28 2	A, F, I_2	32.4	A F I_2

Hap-D, *Hap*-E and *Hap*-F (Fig. 3) and *Hap*-G, *Hap*-H, *Hap*-I and *Hap*-J (data not shown), confirming that these fragments do not contain additional cleavage sites for the *Hap* II enzyme.

Some partial products were clearly a mixture of two partial fragments of approximately equal length. Upon redigestion, two groups of limit products were formed, readily distinguished by the intensity of bands in the autoradiograph. For example, sample 17 (Fig. 3c) is actually composed of a fragment consisting of *Hap*-B_2 and *Hap*-E_2 and another fragment, which is composed of *Hap*-C and *Hap*-D. In some cases, the apparent limit products isolated from the partial digest gels were actually composed of a limit product, contaminated with a partial fragment of equal size. For example, sample 22 from the partial digest had the same mobility as the marker fragment *Hap*-A, but upon redigestion, the presence of the limit product *Hap*-A as well as fragments *Hap*-B_2, *Hap*-C and *Hap*-I_1 were clearly detected. In analogy, sample 12 showed fragments *Hap*-C and *Hap*-I_1 as one group and the limit product *Hap*-B_2 as the other constituent.

As a further check on the composition of the partial products, we compared the molecular weight of each partially digested fragment with the sum of the molecular weights of the products derived from it. For these calculations we used the molecular weight values for the limit products listed in Table 1. The molecular weight of each partial digest product was estimated graphically from a plot relating molecular weight *versus* mobility, using the complete digest marker fragments, run in parallel, as a reference. Table 2 summarizes the data from the partial products analysed. The results were straightforward, the size of each partial digest product being identical to or close to the sum of the molecular weights of the products derived from it. Slight discrepancies were apparent, particularly for larger fragments, which might be due to an increasing non-linearity of the relationship between log (molecular weight) *versus* mobility and the consequent difficulty in estimating molecular weights for such fragments. To deduce the physical order of the *Hap* fragments from the data in Table 2, we have arranged the limit products derived

from each partial fragment in overlapping positions. The order of the fragments, deduced from these analyses, is therefore:

A F I_2 J D C I_1 B_2 E_2 H B_1 E_1 G

with A and G being contiguous in the circular map.

The restriction endonuclease *Hin* dII from *H. influenzae* has been shown to produce one specific double-strand break in RF I of bacteriophage M13 [14, 19] and fd [22]. Such a unique cleavage site might be valuable as a reference point in the circular genome. For this reason we felt it worthwile to localize this site more precisely on the cleavage map. First we determined which *Hap* fragment contains the *Hin* site by comparing a *Hap* II enzyme digest of covalently closed, circular M13 RF I with a *Hap* II enzyme digest of RF I, which was predigested with the *Hin* dII endonuclease. Electropherograms of the two digests are shown in Fig. 4. The only difference observed was the absence of *Hap*-D in the double digest of M13 RF and the appearance of two smaller fragments, *i.e.* *Hap*-D · *Hin*-1 and *Hap*-D · *Hin*-2, located between band *Hap*-F and *Hap*-G. From their electrophoretic mobilities their size was estimated and was found to be 300 and 240 nucleotide pairs, respectively. It is evident, therefore, that the unique *Hin* site is within *Hap*-D. This has been confirmed by direct cleavage of the isolated *Hap*-D fragment with *Hin* dII endonuclease. Identical results were recently obtained by Tabak *et al.* [14], using restriction endonuclease *Hpa* II for which it is known that the recognition sequence is identical to the *Hap* II enzyme, and by Takanami [21] using fd RF. In the latter case the *Hin*-cleavage site was found to be located in a fd fragment, of which the size was approximately 60 base pairs smaller than the corresponding M13 fragment (see Discussion).

To determine which end of fragment *Hap*-D is closest to the *Hin* site, we digested larger pieces of DNA containing the *Hap*-D fragment and determined whether fragment *Hap*-D · *Hin*-1 or *Hap*-D · *Hin*-2 is contiguous to *Hap*-C, the fragment adjacent to *Hap*-D. This was done by cleaving the partial fragments 11, 16 and 17 (Fig. 3 and Table 2) with *Hin* dII endonuclease and analysing the products. As shown in Table 3, fragment 17, which is composed of *Hap*-C and *Hap*-D, was split into two fragments. The smallest fragment appeared to be identical with the *Hap*-D · *Hin*-1 fragment of about 300-nucleotide-pairs long. In contrast, digestion of partial fragments in which *Hap*-D was at the proximal end gave rise to *Hap*-D · *Hin*-2 of about 240-nucleotide-pairs long. Therefore, the *Hin* site is in *Hap*-D and nearest to the *Hap*-cleavage site-1, located between D and J.

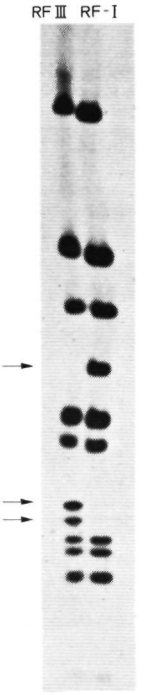

Fig. 4. *Autoradiograph of ^{32}P-labeled DNA fragments, produced by cleavage of covalently closed M13 RF I and of full-length linear M13 RF III with* Hap *II endonuclease.* For preparation of RF-III digest, 0.04 µg of ^{32}P-labeled M13 RF I (2×10^5 counts min^{-1} μg^{-1}), supplemented with 1,0 µg of carrier RF was incubated for 60 min at 37 °C with 1.5 units of *Hin* dII endonuclease in a volume of 0.05 ml of 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 7 mM $MgCl_2$ and 7 mM 2-mercaptoethanol. Thereafter *Hap* II endonuclease was added in excess and the incubation continued for 3 h at 37 °C. For preparation of RF I digest, an identical amount of ^{32}P-labeled M13 RF I, enriched with carrier RF, as given above, was incubated for 3 h at 37 °C with 0.8 unit of *Hap* II endonuclease in a final volume of 0.05 ml of standard incubation buffer. Electrophoresis was carried out for 19 h at 40 mA. Further conditions for electrophoresis and autoradiography are described under Methods

The foregoing data are summarized in a cleavage map of the M13 genome, incorporating the various cleavage sites and approximate size estimates of the fragments in relation to the *Hin* site as the zero point of the map (Fig. 5).

Cleavage of ^{32}P-labeled M13 RF I by the restriction endonuclease from *H. aegyptius* yields 9 distinct bands (designated *Hae*-A to *Hae*-I), readily separable by polyacrylamide gel electrophoresis (Fig. 1). A comparison of label content to electrophoretic mobility

Table 3 *Analysis of partial digest* Hap *fragments, containing the* Hin *site, after cleavage with* H influenzae *restriction endonuclease*
Partial *Hap* fragments, of which the numbers refer to Fig 3 and Table 2, were digested with *Hin* dII endonuclease in excess and then analysed by 3% polyacrylamide gel electrophoresis The presence of *Hin* products were detected by autoradiographic comparison of the *Hin* dII digest products of the partial fragments with the products of a double *Hap*-II – *Hin*-dII digest of M13 RF I as a marker Molecular weights of the *Hin* products were estimated graphically from a plot relating log (molecular weight) *versus* mobility, in which the molecular weights of the double-digest products were used as a reference

Partial fragment number	Order of fragments	Molecular weight of *Hin* products detected	Order of *Hin* fragments
		% total	
11	D I_2 J	6 8 + 4 6	(*Hap*-D *Hin* 1) (*Hap*-D *Hin* 2 I_2 J)
16	D I_2 J F	4.5 + 13 2	(*Hap*-D *Hin* 1) (*Hap*-D *Hin* 2 I_2 J F)
17	C D	15 0 + 3 7	(C *Hap*-D *Hin* 1) (*Hap*-D *Hin* 2)

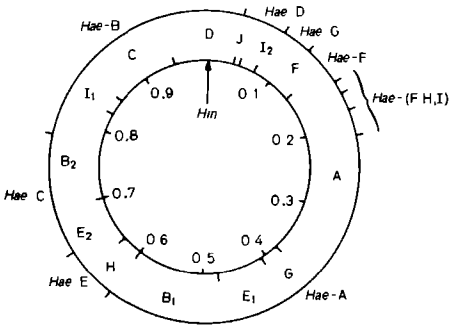

Fig. 5. *A cleavage map of the M13 genome, incorporating the various cleavage sites for* Hap *II endonuclease (inner circle) and* Hae *III endonuclease (outer circle)* Chain lengths of fragments are given in map units or fractional length of M13 RF, with the *Hin* site as zero point of the map

indicated the presence of a single fragment in each band except for band *Hae*-E, which appeared to be a doublet (Fig. 2). Thus, *Hae* III endonuclease produces 10 specific limit fragments of M13 RF I.

To orient these fragments in the RF molecule, we have determined which *Hap* fragments are present in each of the *Hae* fragments In addition, each *Hap* fragment was digested with the *Hae* III endonuclease and subsequently analysed, in order to determine which *Hap* fragments contain a cleavage site for this enzyme. At the same time, constructing a cleavage map of the *Hae* fragments by means of analysis of overlapping sets of fragments will give additional support to the physical order of fragments deduced by partial digestion product analysis with the *Hap* II enzyme.

In order to determine the overlaps between both types of restriction fragments, we isolated each single digest fragment following electrophoresis in a 3% polyacrylamide gel and sequentially digested them with the second enzyme. The results are shown in Fig. 6. Upon digestion with *Hap* II enzyme, the largest fragment *Hae*-A yielded fragment *Hap*-B_1, *Hap*-E_1, *Hap*-G and a new rather large fragment, which was located on the gel between band *Hap*-A and *Hap*-B (Fig. 6c). On the basis of its size, which was estimated to be 15.6% of M13 RF *i.e.* 1000 base pairs (Table 4), we have to conclude that this new fragment must be derived from *Hap*-A and therefore must form the overlap *Hae*-A · *Hap*-A. Direct evidence for this conclusion was obtained by digesting the purified fragment *Hap*-A with the *Hae* III enzyme, which resulted in the appearance of this large overlap fragment just described (Fig. 6a). Therefore we conclude that the position of fragment *Hae*-A within the physical map is such that one of the *Hae*-cleavage sites is in *Hap*-A (map position 0.22), the other site being very close to *Hap* site-8 (map position 0.60), between *Hap*-B_1 and H. (Although we expected to detect a small fragment, derived from *Hap*-B_1 or H, we did not observe this product.) At the same time, four smaller products could be detected in the *Hap*-A digest, of which three of them showed electrophoretic mobilities identical to those of the limit fragments *Hae*-F, *Hae*-H and *Hae*-I (Fig. 6a) The fourth fragment, with an estimated size of 3.1% genome length (Table 4), could be identified as the overlap fragment *Hap*-A · *Hae*-E. It appeared, therefore, that the fragments *Hae*-F, H and I as one group have to be positioned in *Hap*-A, while fragment *Hae*-E must be contiguous to this group (Fig. 5) By analogy, fragment *Hae*-B yielded *Hap*-C, *Hap*-D, *Hap*-I_1 and a new fragment of about 4.7% genome length (Fig. 6c and Table 4). The latter fragment was tentatively identified as a split product of *Hap*-B (*i.e.* B_1 and B_2 together). As, however, no *Hae*-cleavage site was apparent in *Hap*-B_1, the fragment most probably must be derived from *Hap*-B_2 Therefore, we conclude that the position

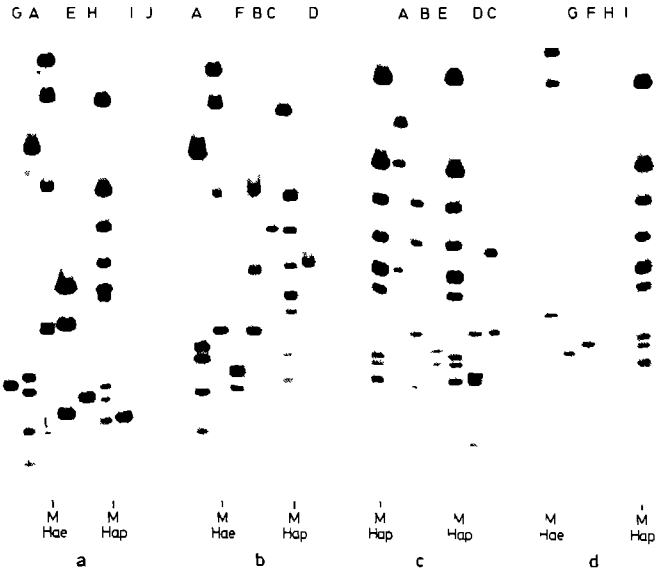

Fig 6 *Electrophoretic analysis of the individual* Hap *II fragments of* ^{32}*P-labeled M13 RF I after cleavage with* Hae *III endonuclease and of the* Hae *III fragments of RF I after cleavage with* Hap II *endonuclease* (a, b) Cleavage of *Hap* fragments with *Hae* III enzyme Each *Hap* fragment, designated on top of this figure according to Fig 1, was digested in 0 05 ml of standard buffer with 0 8 unit of *Hae* III endonuclease for 90 min at 37 °C (c d) Cleavage of *Hae* fragments with *Hap* II enzyme Each *Hae* fragment, designated on top, was digested in 25 µl of standard incubation buffer with 0 3 unit of *Hap* II endonuclease for 3 h at 37 C In both types of analysis, the double digest products were electrophoresed in 3 % discontinuous polyacrylamide slab gels with both a complete *Hap* II digest (M_{Hap}) and a complete *Hae* III digest (M_{Hae}) of M13 RF as markers

of *Hae*-B within the physical map is such that one of the *Hae*-cleavage sites is in fragment *Hap*-B_2 (map position 0 78) and the other site is located very close to the *Hap* site-1 (map position 0 04), between *Hap*-D and *Hap*-J

Further, two *Hae*-cleavage sites were detected within fragment *Hap*-F, one site was apparent in *Hap*-E_2, while no cleavage sites could be demonstrated in the remaining *Hap* fragments H, J and I_2 By combining the data obtained by individually digesting purified *Hap* fragments, together with the results obtained by digesting the individual *Hae* fragments, the orientation of the *Hae* fragments in the M13 RF molecule could be deduced The data are summarized in Table 4 Except for the small *Hae* fragments H, F and I, the mutual position of which has not yet been solved unambiguously, the order of *Hae* fragments is

D G E (F, H, I) A E C B

with D and G being contiguous in the circular map (Fig 5) (see Note Added in Proof)

DISCUSSION

A cleavage map of the circular genome of bacteriophage M13, such as shown in Fig 5, is a physical map based on specific sites susceptible to three restriction endonucleases, *i e* , the *Hap* II, *Hae* III and *Hin* dII endonuclease

Restriction enzymes are known to cleave DNA by recognizing unique nucleotide sequences with rotational symmetry Such sequences have been determined now for an increasing number of restriction endonucleases [26], including the *Hin* dII [2], the *Hap* II [4] and the *Hae* III endonuclease (K Murray, personal communication), used in this study All recognition sites mentioned differ in length or nucleotide sequence In agreement with this is our finding that *Hap* II endonuclease yields 13 fragments of M13 RF I, the *Hae* III enzyme yields 10 fragments and *Hin* dII enzyme has only a single cleavage site on the circular molecule All fragments differ in length (Table 1) and have specific positions in the cleavage map

Table 4 *Redigestion of* Hap *fragments with* H aegyptius *endonuclease and of* Hae *fragments with* H aphirophilus *endonuclease*
Single-enzyme digest fragments of ^{32}P labeled M13 RF I were isolated from polyacrylamide gels and subsequently double-digested with the second endonuclease in excess Conditions for isolation of fragments, digestion and electrophoresis are given under Methods The presence of overlap fragments was detected by autoradiographic comparison of the double-digest products of the fragments with the products of RF fragmented with both *Hap* II and *Hae* III as markers Molecular weights of the overlap fragments were estimated graphically from a plot relating log (molecular weights) *versus* mobility, in which the molecular weights of the single digest products were used as a reference The molecular weights of the overlap fragments are given as fractions of M13 RF, the overlap fragments are designated by letters between brackets

Hap fragment	Terminal *Hae* fragment detected	Molecular weight of *Hap Hae* fragments	Order of fragments
		% total	
A	F H, I	15 6 (a) 3 1 (b)	(b) (F, H I) (a)
B_1	none	none	
B_2	none	7 8 (c), 4 6 (d)	(c) (d)
C	none	none	
D	none	none	
E_1	none	none	
E_2	none	4 8 (e), 2 2 (f)	(f) (e)
F	G	2 4 (g), 1 8 (h)	(h) G (g)
H, I, J	none	none	

Hae fragment	Terminal *Hap* fragment detected	Molecular weight of *Hap Hae* fragments	Order of fragments
		% total	
A	B_1, E_1, G	15 6 (a)	(a) G E_1 B_1
B	C, D, I_1	4 7 (d)	(d) I_1 C D
C	none	7 8 (c), 4 7 (e)	(e) (c)
D	I_2, J	1 8 (h)	J I_2 (h)
E	H	2 4 (g), 2 2 (f), 3 1 (b)	H (f) (g) (b)
F	none	none	
G, H, I	none	none	

Similar fragmentation patterns have been obtained for RF of bacteriophage fd [21] and of ZJ2 (unpublished results), emphasizing the high degree of homology between the DNA molecules of the filamentous F-specific coliphages Moreover, a cleavage map very similar to the one shown in Fig 5 has been obtained for f1 and fd (Seeburg and Schaller, Takanami, personal communications), by using different methods for ordering the DNA fragments It is worth mentioning, however, that fd differs slightly from M13 (and f1), in such a way that the *Hap*-cleavage site-6 is missing in fd and consequently, cleavage between *Hap*-E_1 and *Hap*-G does not occur On the other hand, an additional *Hap*-cleavage site, not present in M13 and f1, is located in *Hap*-D (map position 0 03), giving rise to a smaller *Hap*-D fragment and an extra small fragment contiguous to *Hap*-J

Several features of the M13 cleavage map deserve comment First, the unique *Hin* site has been selected as a zero point of the map, and map distances are given as fractions of the length of M13 RF Fractional length rather than molecular weight or size has been chosen as the former has to be considered more reliable, since the exact number of base pairs of the circular RF molecule is not known Secondly, cleavage at this *Hin* site results in full-length linear molecules, which then are an excellent tool for ordering of fragments by electron microscopic examination of heteroduplexes formed between a fragment and the linear molecule Alternatively, M13 DNA fragments produced by other restriction endonucleases can now be ordered using the *Hin* locus as a reference point

Heteroduplex electron microscopic analysis have already been carried out with mini-M13 DNAs [15], circular, single-stranded DNA molecules of 0 2 to 0 5 the normal length, derived from miniparticles present in M13 phage stocks Heteroduplexes were formed by annealing mini-DNA with linear, complementary (−) strands derived from either M13 RF II synthesized *in vitro* [14] or RF II synthesized *in vitro* which was predigested with *Hin* dII endonuclease Subsequent electron microscopic analysis revealed that all mini-DNA molecules start their sequence very close to, and include, the *Hin* site and are extended for a variable distance in only one direction of this site These results, therefore, direct the zero point of the map very near to the origin of replication of these mini M13-DNA molecules

In addition, Tabak *et al* [14] and Griffith and Kornberg [15] have produced strong evidence that

the origin of conversion of M13 viral DNA into the replicative form *in vitro* also is located very near to the zero point of the map and is confined to a region from 0 05 to 0 1 map units in distance from this locus. This would argue that the origin of complementary single-stranded DNA synthesis is located in or very near to fragment *Hap*-J and the location of the RNA fragment that initiated this strand is encompassed by the fragments *Hap*-J, I_2 and a small part of F It is worth mentioning here than our studies on the gene order of M13 [20] have produced evidence that fragment *Hap*-D, containing the *Hin* site, is located in gene II, the gene product of which is involved in phage DNA replication [27] In analogy to gene A of phage ΦX-174 [16,28] and in analogy to other systems where genes involved in DNA replication are located in (or very close to) the origin site of replication, one might argue that the extended region, encompassed by the fragments *Hap*-D (or part of it), I_2, J and F, as a whole is essential for RF replication and single-strand DNA synthesis The hypothesis that this region is of high functional importance in all stages of M13 replication receives additional support if one accepts that this particular part of the genome is the one, as already suggested [15], which readily replicates once it is formed, giving rise to mini-DNA molecules of 0 2 the normal length and hence to mini-M13 particles.

The availability of ordered sets of phage DNA fragments is proving useful in analysing functions of the phage genome For example, the physical order of fragments as given in Fig 5 has been used to map genes in the M13 chromosome [20]. Furthermore, such physical maps alligned to the genetic map have been of great value in our protein synthesis studies *in vitro*, in which restriction fragments were used as a template for coupled transcription and translation In analysing the protein products encoded by these fragments, promoters could be localized in front of gene IV (*Hap*-A), in front of gene VIII (*Hap*-B_2) and in fragment *Hap*-C [29,30], producing evidence for a direction of transcription counter-clockwise on the physical map

Finally, the cleavage maps will provide a framework for relating the results of nucleotide sequence analysis of individual fragments to the overall structure of the genome

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Note Added in Proof (February 14 1975) The *Hae* III fragments F H and I the mutual position of which was ambiguous, have now been ordered exactly in the enzyme cleavage map by means of primed DNA-synthesis studies in which the individual *Hae* fragments were used as a primer From the results obtained an arrangement E–I–H–F–A could be established

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CHAPTER III

STUDIES ON BACTERIOPHAGE M13 DNA

2. THE GENE ORDER OF THE M13 GENOME

Eur J Biochem 53 559–567 (1975)

Studies on Bacteriophage M13 DNA

2 The Gene Order of the M13 Genome

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The double-stranded replicative form DNA of bacteriophage M13 was cleaved into 13 specific fragments by the restriction endonuclease from *Haemophilus aphrophilus* The individual DNA fragments from wild-type replicative form molecules were then annealed to circular, single-stranded DNAs of phage M13, bearing amber mutations as genetic markers When such DNA hybrids infected competent *Escherichia coli* cells, only those duplexes which were genetically heterozygous gave rise to wild-type phages in the progeny In this way, the genetic markers carried on the individual DNA fragments could be determined In addition, marker rescue in each gene was obtained with the 10 specific fragments of M13 replicative form DNA, produced by cleavage with the restriction endonuclease from *Haemophilus aegyptius*

From these results and the enzyme cleavage maps of both types of restriction fragments a distribution of genetic markers along the physical map could be obtained, which allowed an arrangement of M13 genes into a genetic map Evidence is presented that the gene order of M13 is IV–(I VI)–III VIII–VII–V–II with II and IV being contiguous on the circular map

The genome of the F-specific filamentous coliphage M13 is a covalently closed, circular molecule of single-stranded DNA with a molecular weight of about 2×10^6 (for review, see [1]) The M13 chromosome is known to code for at least eight gene products, some of which have been well characterized regarding their biological function In particular, the proteins encoded by gene II and gene V are functional elements in the process of viral DNA replication [2–10], the proteins encoded by gene III and gene VIII are the sole protein constituents of the mature phage particle [1,11,12] With the exception of gene VI and VII, all phage gene products have been characterized regarding their molecular weight [12–17]

We have been analysing this viral chromosome in more detail by means of bacterial restriction endonucleases These enzymes cleave the double-stranded M13 replicative form I DNA (RF I) at specific sites and yield a distinct number of electrophoretically separable fragments of this DNA The physical ordering of M13 RF I fragments, produced by the *Hap* II restriction endonuclease from *Haemophilus aphrophilus* and the *Hae* III endonuclease from *Haemophilus aegyptius* has been described [18] This report deals with the genetic characterization of the individual fragments and the ordering of the M13 genes with respect to the enzyme cleavage map

In order to determine which part of the M13 genome is represented in each restriction fragment, we used an assay [19,20] in which wild-type minus strand fragment was annealed to intact plus strand circles, derived from genetically marked amber mutant phages, the hybrid product was then used to infect calcium-chloride-treated *Escherichia coli* cells [21] The production of wild-type phage in the progeny then indicates the presence of the wild-type allele in the restriction fragment From these genetic data a distribution of genetic markers along the physical map could be obtained, which allowed the arrangement of all known M13 genes into a genetic map With respect to the genetic map of bacteriophage f1,

Abbreviations *Hap* endonuclease endonuclease R *Hap* II from *Haemophilus aphrophilus* *Hae* endonuclease endonuclease R *Hae* III from *Haemophilus aegyptius* *Hin* endonuclease endonuclease R *Hin* dII from *Haemophilus influenzae* Rd RF replicative form DNA RF I double stranded circular replicative form I with both strands covalently closed

Definition A_{260} (A_{660}) unit the quantity of material in 1 ml of a solution which has an absorbance of 1 at 260 nm (660 nm) when measured in a 1-cm pathlength cell

Enzyme Endonucleases (EC 3 1 4 30)

as reported by Lyons and Zinder [22], a deviation was apparent in that the order of gene V and gene VII have to be reversed.

MATERIALS AND METHODS

Bacteria and Phages

E. coli C89 (K12, 159F$^+$, *uv*^{s}, *su*$^-$) was used for the preparation of calcium-chloride-treated cells as well as for the preparation of M13 replicative form I DNA.

E. coli K37 (*su*1$^+$) and *E. coli* S26R1D (*su*2$^+$), permissive hosts for M13 amber mutants, and *E. coli* K38, the non-permissive host, were obtained from Dr D. Pratt

Phage M13 (*wt*) was obtained from Dr P. H. Hofschneider; the M13 nonsense mutants *am*1-H7 (gene I), *am*2-H2 (gene II), *am*3-H5 (gene III), *am*4-H38 (gene IV), *am*5-H3 (gene V), *am*6-H1 (gene VI), *am*7-H2 (gene VII) and *am*8-H1 (gene VIII), the characteristics of which have been described [2, 11, 12, 23], were kindly provided by Dr D. Pratt.

The f1 nonsense mutant R68 (amber in gene VII) was obtained from Dr J. Woolford.

Media

E coli C89 was cultivated in M3-antibiotic medium (Difco) for preparing calcium-chloride-treated cells. For the preparation of amber mutant M13 phages, *E. coli* K37 (or S26R1D) was grown in Hershey broth containing 8 g of Bactonutrient broth (Difco), 5 g of NaCl and 5 g of Bactopeptone (Difco) per liter. The agar used contained per liter: 30 g of casamino acids (Difco), 20 g of glycerol, 1 g of yeast extract (Oxoid), 1 g of $MgSO_4 \cdot 7\,H_2O$ and 10 g of Oxoid agar. After autoclaving 5 ml of 1 N NaOH was added.

Growth and Purification of M13 Amber-Mutant Phages

E. coli K37 was grown with aeration in 100 ml of Hershey broth. At a density of 2×10^8 cells/ml the culture was infected with a single plaque of the amber phage. Incubation was continued for 16 h at 30 °C, after which time the cells were removed by centrifugation (10 min at $10000 \times g$). The titers obtained were about $1-4 \times 10^{12}$ p.f.u./ml for the amber phages used The reversion rate was less than 10^{-6}. Phage M13 *am*-H38 was propagated in *E. coli* S26R1D under further identical conditions. The phages were recovered from the supernatant according to the method described by Yamamoto *et al.* [19] with the modification than an additional centrifugation step was introduced (30 min at $55000 \times g$), followed by a second precipitation with 5% poly(ethyleneglycol) in 1 M NaCl. The phages were finally purified by CsCl–buoyant density centrifugation.

Preparation of M13 DNA and M13 Amber-Mutant DNA

Viral DNA was extracted from purified virions by the hot phenol–sodium dodecyl sulphate method described by Marvin and Schaller [25] The DNA was recovered from the aqueous phase by ethanol precipitation at −20 °C and finally dissolved in 10 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 1 mM EDTA.

Alkaline sucrose gradient analysis [26] revealed that at least 95% of the viral DNA was in the circular form.

Isolation of M13 Replicative Form I Fragments Produced by the Restriction Endonucleases from H. aphrophilus *and* H aegyptius

M13 RF I (50 µg), supplemented with ^{32}P-labeled M13 RF I (0 8 µg, 100000 counts/min) as a marker, was digested with either *Hap* II restriction endonuclease or *Hae* III endonuclease in a final volume of 5 0 ml of 10 mM Tris-HCl, pH 7 6, 7 mM $MgCl_2$ and 7 mM 2-mercaptoethanol. Incubation with 40 units [18] of *Hap* II enzyme or 60 units of *Hae* III enzyme for 3 h and 2 h at 37 °C, respectively, was sufficient to achieve complete hydrolysis of M13 RF as determined by electrophoresis of the products. The reaction was terminated by the addition of EDTA to a final concentration of 20 mM Thereafter the digest mixture was extracted twice with phenol, the aqueous phase was collected and then dialysed against 10 mM Tris-HCl, pH 7 6, 0 1 M NaCl, 1 mM EDTA. Two volumes of ethanol were added and the DNA fragments were precipitated at −20 °C for a minimum of 5 h. The precipitate was collected by centrifugation (15 min at $110000 \times g$) and then dissolved in 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.8. After the addition of one-fifth volume of 70% sucrose, 0 1 M EDTA, 1% bromophenolblue, the DNA fragments were separated by electrophoresis in a discontinuous polyacrylamide gel slab ($40 \times 20 \times 0.2$ cm) filled to a height of 12 cm with a 10% aqueous acrylamide solution and topped by 25 cm of a 3% acrylamide solution in electrophoresis buffer, consisting of 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.8. Electrophoresis was carried out under exactly the same conditions as described in the preceding paper [18]. Preparative electrophoresis was followed by autoradiography of the wet gel to locate the [^{32}P]DNA bands. Gel segments corresponding to these bands were excised and the

individual DNA fragments were recovered from the gel according to one of the following methods

Gel Electrophoresis Method The DNA fragment was eluted from the gel electrophoretically, essentially as described by Sharp *et al* [27]

Gel Solubilization Method Preparative electrophoresis was carried out on slab gels, in which the regular crosslinker *N,N'*-methylenebisacrylamide was replaced by 0 1 % (w/w) of *N,N*-diallyltartarediamide [28] In the latter case the gels can be dissolved by oxidation with periodate, The gel segments (0 2 × 0 5 × 2 cm) containing the DNA fragments were solubilized by 15-min incubation with 0 3 ml of 1 M sodium periodate Ethyleneglycol (0 2 ml) was added to remove the excess of periodate and the incubation was continued for 10 min The reaction mixture was diluted with 2 5 ml of 10 mM sodium phosphate buffer, pH 6 8 and then percolated over a small hydroxyapatite column (2 × 0 4 cm) After washing the column with 1 ml of 10 mM sodium phosphate, pH 6 8, the DNA was eluted with two 0 5-ml portions of 0 3 M sodium phosphate, pH 6 8, then dialysed against 10 mM Tris-HCl, pH 7 6, 0 1 M NaCl, 1 mM EDTA and finally concentrated by ethanol precipitation

Gel Extraction Method In this procedure the DNA fragments were extracted from the gel as already described [18] For our enzymic as well as our protein-synthesis studies on the DNA fragments [17,29], it appeared to be crucial to purify the fragments even further This was achieved succesfully by centrifugation of the DNA fragment on a linear sucrose gradient (5–20%) in 1 M NaCl, 50 mM Tris-HCl and 3 mM EDTA at pH 7 6 for 3 h at 420000 × *g* The radioactive fractions, containing the purified DNA fragment, were collected and the DNA was further concentrated by ethanol precipitation

Preparation of E coli *Competent Cells*

Competent cells were prepared according to the method described by Taketo [21] *E coli* C89 or K37 was grown with aeration in M3-antibiotic medium At an absorbance of 0 7–0 9 at 660 nm, the cells were collected by centrifugation and resuspended in half the original volume of chilled 0 05 M calcium chloride After standing for 15 min in ice, the bacteria were sedimented by centrifugation and resuspended in 0 1 volume of 0 05 M calcium chloride The $CaCl_2$-treated, competent cells could be stored at 0 °C for at least one week without loss of activity

Fragment Bioassay

Aliquots of each DNA fragment (0 05 pmol), dissolved in 50 µl of 50 mM Tris-HCl, pH 7 6 and 1 mM EDTA were heated for 8 min in a boiling-water bath and then rapidly quenched in ice Immediately thereafter, 50 µl of amber mutant phage DNA (20 µg/ml in 10 mM Tris-HCl, pH 7 6, 0 1 M NaCl, 1 mM EDTA) were added to each tube The tubes were then placed for 15 min in a 57 °C water bath and subsequently incubated for 20 h at 25 °C For assay, 50 µl of the annealing mixture was placed in ice and mixed with 100 µl of an ice-cold suspension of competent *E coli* cells After 10 min at 0 °C, the mixture was incubated for 2 5 min at 37 °C, chilled and then mixed with 5 ml of the agar described above and selectively assayed for *wt* infective centers by plaque assay at 37 °C on *E coli* C89 (*su*$^-$) indicator

Miscellaneous Methods

The preparation of *Hae* III endonuclease and of *Hap* II endonuclease used in this work were those described in the preceding article [18] ^{32}P-labeled M13 RF I was prepared as described there [18], unlabeled M13 RF I, derived from wild-type phage and from *am*4-H38 mutant M13 phage, were prepared by the method described previously [16]

RESULTS

The restriction endonuclease *Hap* II from *Haemophilus aphrophilus* cleaves the double-stranded replicative form I DNA (RF I) of phage M13 into 13 unique fragments (designated *Hap*-A to *Hap*-J in order of fragment size), which can readily be separated by polyacrylamide gel electrophoresis [18] The chain length of each DNA fragment has been estimated and resulted in a range of about 1530 base pairs for the largest fragment *Hap*-A to about 50 base pairs for the smallest fragment *Hap*-J

In order to determine which genetic markers are located on the individual DNA fragments we used a bioassay which was similar to the methods described by Weisbeek *et al* [19] and Hutchison and Edgell [20] The procedure consists of mixing a denaturated wild-type *Hap* fragment under annealing conditions with intact viral single-stranded DNA, bearing an amber mutation as a genetic marker Most of the *Hap* fragments used will give rise to annealed structures, composed of the mutant puls-strand base-paired with minus-strand fragment which does not cover the site of mutation One of the DNA fragments, however, will form a hybrid which should be genetically heterozygous, being composed of a wild-type minus-strand fragment which covers the site of the amber mutation in the plus-strand circle Such annealed complexes are infective to *E coli* spheroplasts or

Table 1 *Efficiency of transfection of $CaCl_2$-treated* E coli *cells*
Bacteria were grown at 37 °C in M3-antibiotic medium to an absorbance of 0.85 at 660 nm, then harvested and treated with 50 mM $CaCl_2$ at 0 °C. The competence for transfection by viral nucleic acids was determined by the addition of increasing amounts of DNA to a suspension of $CaCl_2$-treated cells, derived from either *E coli* K37(su^+) or *E coli* C89(su^-). After a heat shock for 2.5 min at 37 °C, all infection mixtures were plated together with *E coli* C89(su^-) as indicator strain. The figures listed are the scores for infective centers obtained with 1.0 A_{260} unit of DNA

Nucleic acid	Infective centers	
	E coli C89 (su^-)	*E coli* K37 (su^+)
M13 DNA	4×10^5	4×10^6
M13 RF I	5×10^6	6×10^7
M13 DNA (*am*4-H38)	$<10^2$	3×10^6
M13 RV I (*am*4-H38)	$<10^2$	4×10^7

competent cells, giving rise to progeny virus which bear the wild-type marker from the RF fragment. The production of phages bearing such salvaged markers can be selectively measured as infective centers by performing a plaque assay under conditions which are restrictive for the amber mutation in the viral strand.

For the genetic assay we used *E. coli* C89, the non-permissive host for M13 amber mutants. Cells were made competent by pretreatment with 0.05 M $CaCl_2$ at 0 °C as described by Taketo [21]. The competence of such cells for transfection by viral DNA is given in Table 1. Wild-type single-stranded DNA was approximately 10-fold less infective than double-stranded RF I, while M13 amber-mutant DNA was about 10^4 less active as compared to its wild-type counterpart. From these data it might be emphasized, that the bioassay using $CaCl_2$-treated *E. coli* cells is an attractive alternative to spheroplast assays, which because of its simplicity is potentially useful as a specific assay to aid in the purification and characterization of genetically defined DNA and DNA fragments.

To analyse the genetic content of the fragments, produced by cleavage of M13 RF I with the *Hap* II restriction endonuclease, each individual fragment was annealed to single-stranded DNA, derived from the M13 amber mutants *am*1-H7 (gene I), *am*2-H2 (gene II), *am*3-H5 (gene III), *am*4-H38 (gene IV), *am*5-H3 (gene V), *am*6-H1 (gene VI), *am*7-H2 (gene VII) and *am*8-H1 (gene VIII). Each heteroduplex thus formed was then added to $CaCl_2$-treated cells and tested for plaque formation. The results obtained with the individual *Hap* fragments are presented in Table 2.

The data clearly demonstrate that the annealing procedure does result in salvaging the wild-type allele from *Hap* fragments in the case of all eight amber-mutant markers. The residual infectivity of the fragments (data not shown) and the level of wild-type revertants in the control assays of plus-strand amber-mutant DNA only were very small compared to the level of infective centers obtained from the complete annealing mixture. Furthermore, the conclusion may be drawn that the recovery of the wild-type alleles exhibits the expected genetic specificity. This is demonstrated with fragment *Hap*-A (*am*4-H38), the restriction fragment, derived from M13 RF I bearing an amber mutation in gene IV. In contrast to its wild-type analogue, this amber-mutant fragment did not produce wild-type progeny phage when annealed to plus-strand circles carrying the same mutation. A negligible infectivity was apparent which was of the same order of magnitude as was found with the annealed complexes of which no salvaging activity could be recovered (Table 2). The data also demonstrate that the wild-type allele of each of the eight amber-mutant markers is salvaged from a single, unique fragment only. This is consistent with the conclusion that the fragments tested do not contain uncleaved sites for the restriction endonuclease and, therefore, are terminal digestion products.

The terminal fragments *Hap*-D, *Hap*-H, *Hap*-A and *Hap*-I cover only a single genetic marker *ie*, *am*2-H2, *am*3-H5, *am*4-H38 and *am*5-H3, respectively. In contrast, the specified genetic markers in gene I, gene VI, gene VII and gene VIII are all salvaged from fragment *Hap*-B. Previously we demonstrated, however, that the electrophoretic bands containing the fragments *Hap*-B and *Hap*-I are in fact doublets composed of two limit digestion fragments of nearly equal size [18]. To locate the genetic markers *am*5-H3 on one side and *am*1-H7, *am*6-H1, *am*7-H2 and *am*8-H1 on the other more precisely, longer electrophoretic runs were performed in order to obtain a better resolution of the partially resolved doublets. The doublet fragments *Hap*-B_1 and *Hap*-B_2 could be separated on 3% acrylamide gel slabs after electrophoresis for about 30 h, the doublet *Hap*-I_1 and *Hap*-I_2 was separated on 10% gels under further standard conditions. After extraction from the gels, the separated fragments were annealed to amber-mutant plus strands and tested for salvaging activity. As shown in Table 2, the wild-type allele to the *am*5-H3 marker is salvaged now from fragment *Hap*-I_1 only. The mutant markers *am*1-H7 and *am*6-H1 are rescued specifically by fragment *Hap*-B_1, while fragment *Hap*-B_2 displaying a negligible activity for both markers in gene I and VI, demonstrates high salvaging activity for *am*7-H2 and *am*8-H1.

Although the fragments *Hap*-H and *Hap*-I_1 undoubtedly demonstrate genetic activity after annealing

Table 2 *Biological activity recovered from* H aphrophilus *fragments of M13 replicative form I DNA*
A suspension of $CaCl_2$-treated *E coli* C89(*su*$^-$) cells (0 8 A_{660} unit) was infected with DNA hybrid constructed from 0 025 pmol of wild-type M13 *Hap* fragment and 0 25 pmol of circular amber-mutant phage DNA Similar conditions were used to construct the double amber-mutant hybrid All infection mixtures were plated together with *F coli* C89(*su*) as indicator strain and the plates were scored for infective centers The figures listed are the mean value of results compiled from at least two assays The deviation of scored figures from listed values was is some cases as high as 50% I, II *etc* refer to the gene, *am*1-H7 *etc* to the mutant n d = not determined

Fragment	I *am*1-H7	II *am*2-H2	III *am*3-H5	IV *am*4-H38	V *am*5-H3	VI *am*6-H1	VII *am*7-H2	VIII *am*8-H1	VII R-68
Hap-A	0	0	0	**222**	1	0	1	0	0
Hap-B	**269**	0	0	0	1	**116**	**105**	**177**	**59**
Hap-C	2	0	0	1	2	0	1	0	0
Hap-D	1	**308**	1	0	1	1	0	0	0
Hap-E	1	1	2	0	0	2	1	0	0
Hap-F	0	1	1	2	2	0	0	1	1
Hap-G	1	0	2	0	2	0	1	2	1
Hap-H	0	1	**23**	1	3	2	0	1	0
Hap-I	0	0	1	0	**26**	0	1	0	0
Hap-J	1	1	0	0	0	1	1	1	0
Hap-A(*am*4)	0	0	1	2	0	2	0	1	n d
Hap-B_1	**257**	1	1	0	0	**179**	4	6	2
Hap-B_2	1	1	0	0	1	0	**185**	**205**	**68**
Hap-I_1	n d	n d	0	2	**16**	2	0	2	0
Hap-I_2	n d	n d	1	0	3	2	1	0	0

Table 3 *Biological activity recovered from partial* H aphrophilus *digest fragments and the corresponding terminal digest fragments of M13 replicative form I DNA*
Cell suspensions of $CaCl_2$-treated *E coli* C89(*su*) cells were infected with DNA hybrids constructed from 0 025 pmol of wild-type M13 *Hap* fragments and 0 25 pmol of circular amber mutant phage DNA Infection mixtures were plated together with *E coli* C89(*su*) as indicator strain and the plates were scored for infective centers The partial *Hap*-digest fragments were prepared and isolated as described in the previous article [18] The terminal fragments *Hap*-B and *Hap*-I are composed of the doublets B_1 and B_2 and I_1 and I_2, respectively I, II *etc* refer to the genes, *am*1-H7 *etc* to the mutants

Hap fragment	I *am*1-H7	II *am*2-H2	III *am*3-H5	IV *am*4-H38	V *am*5-H3	VI *am*6-H1	VII *am*7-H2	VIII *am*8-H1	VII R-68
B_1 H	**257**	1	**96**	1	0	**205**	0	1	0
B_2 I_1	1	1	0	1	**88**	1	**85**	**145**	**78**
B	**245**	0	0	0	1	**162**	**145**	**215**	**68**
H	0	1	**18**	0	0	0	1	0	0
I	0	0	0	1	**21**	1	2	0	1

to the proper amber-mutant plus strand, the number of infective centers was rather low One has to consider, however, that the size of both fragments is quite small, namely 170 and 140 base pairs [18] Since actual annealing of the fragment to the mutant strand is a prerequisite for efficient salvaging of wild-type markers [20], one might expect, therefore, that the annealing efficiency of such fragments, under the standard conditions used, is lower as compared to the larger DNA fragments Although this might be the case, it seems also possible that the annealed complexes formed with small fragments in particular, are more susceptible to interfering events in the cell To circumvent conclusions, which in fact are based on a rather low infectivity due to fragment size, we isolated two larger fragments in which *Hap*-H and *Hap*-I_1 are an integral part, *i e* the fragments *Hap*-B_1H and *Hap*-B_2I_1, produced by partial digestion of M13 RF I with *Hap* II endonuclease [18] The salvaging activity obtained with both partial fragments and their corresponding limit fragments are given in Table 3 The data confirm the low infectivity values found with the small DNA fragments *Hap*-H and *Hap*-I_1

On the other hand, activity in rescuing markers is markedly increased with the partial digestion fragments, in which the genetic markers concerned are integrated into larger fragment size We conclude from this experiment that the specified genetic markers in gene III and gene V are contained in the limit digestion fragments *Hap*-H and *Hap*-I_1, respectively

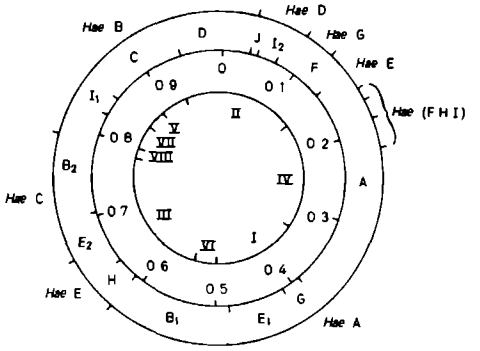

Fig 1 *Correlation of the physical map to the genetic map* The genetic map was aligned to the physical maps by genetic marker rescue with wild type M13 RF I fragments produced by *Hap* II endonuclease (inner circle) and *Hae* III endonuclease (outer circle) The sizes of genes were obtained by converting the molecular weights of the gene products to map unit length Genes VI and VII, the products of which are unknown, are represented by 0 04 map unit each One map unit equals 6400 nucleotides and map units are given as distance from *Hin* dII cleavage site per length of M13 phage DNA

Since *Hap*-B_2 is contiguous to *Hap*-I_1 which, in turn, is part of gene V, one can predict also that gene V is distal to gene VII and VIII

In the preceding article [18] the *Hap* II fragments of M13 RF I have been ordered into a physical map Since the genetic markers of the eight genes of M13 have also been allocated into the *Hap* fragments, a genetic map can be constructed by placing the genetic markers in the order corresponding to the physical map (Fig 1) The order of genes which can be deduced so far, is IV−(I, VI)−III−(VIII, VII)−V−II

Our results show that the genetic map of M13 and f1, a bacteriophage which is closely related to M13, are very similar Only one striking difference is apparent, namely the assignement of the position of gene VII, which in contrast to the findings of Lyons and Zinder [22] is between gene V and gene VIII This conclusion is based on the fact that fragment *Hap*-I_1, which contains part of gene V, is physically located between *Hap*-B_2 and *Hap*-D As fragment *Hap*-B_2 appears to contain gene VII and VIII and fragment *Hap*-D the marker for gene II, one has to conclude that gene V is positioned between gene VII (and gene VIII) on one side and gene II on the other

This discrepancy in both maps is rather unexpected, as it is well known that mutants of both phages can be used interchangeably, thus indicating that their genetic maps are most probably identical To clarify the position of gene VII, we tested the viral DNA derived from bacteriophage f1 bearing an amber mutation in gene VII (R68) Annealing of this marked f1 plus strand with the individual *Hap* fragments derived from M13 RF I and subsequent testing of the duplexes formed clearly demonstrated once more that fragment *Hap*-B_2 is capable of salvaging wild-type alleles to *am*-VII markers into infective phage particles (Table 2 and 3) This argues very strongly that gene VII constitutes a structural part of this DNA fragment Hence, we conclude that gene VII must be aligned between gene V and VIII

In the preceding article [18] we also determined the physical order of M13 RF I fragments, produced by a second restriction endonuclease, namely *Hae* III from *H aegyptius* This map, superimposed onto the *Hap* II enzyme cleavage map together with the positions of their respective cleavage sites, is shown in Fig 1

In analogy, the limit *Hae* III fragments were assayed for their capacity in rescuing markers in each of the M13 genes in order to determine their genetic information At the same time, the results of these analyses support the order of fragments and genes, deduced by analysis of the *Hap* II fragments As shown in Table 4, the results obtained are straightforward and completely consistent with the order of genes already deduced The assignement of the position of some mutant markers used can now be given more accurately For instance, the genetic markers *am*7-H2 (gene VII) and *am*8-H1 (gene VIII) are located in a fragment of about 300 nucleotide pairs, namely fragment *Hae*-B *Hap*-B_2, which is the region common in fragment *Hae*-B and *Hap*-B_2 The *am*4-H38 (gene IV) marker is present in the exterior quarter of fragment *Hap*-A, *i e* in fragment *Hae*-F

Although a gross positional pattern of M13 genes has been obtained from marker rescue of both series of restriction fragments, the mutual positions of both gene I and VI and gene VII and VIII are still ambiguous Firm support however for the order of genes being VIII−VII−V could be obtained from our protein-synthesis studies Protein synthesis *in vitro*, programmed by the individual restriction fragments [17,29], has shown that fragment *Hap* B_2 directs the synthesis of genes-VIII protein only This suggests that the entire information to code for this protein is retained in this fragment On the other hand, fragment *Hae*-B gives rise to the synthesis of gene-V protein, while no synthesis of gene-VIII protein is apparent, despite the fact that this fragment contains among others the genetic markers for gene V, VII and VIII This observation argues for a *Hae*-cleavage site within gene VIII, in such a way that the entire gene VII and gene V but only part of gene VIII is preserved in fragment *Hae*-B Although one should

Table 4. *Biological activity recovered from* H aegyptius *fragments of M13 replicative form I DNA*
A cell suspension of $CaCl_2$-treated *E coli* C89(*su*$^-$) cells was infected with DNA hybrid constructed from 0 025 pmol of wild-type *Hae* III fragment of M13 RF I and 0 25 pmol of circular amber-mutant phage DNA Infection mixtures were plated together with *E coli* C89 as indicator strain and the plates were scored for infective centers The figures listed are the mean value of results compiled from at least two assays I, II *etc* refer to the genes, *am*1-H7 *etc* to the mutants

Hae fragment	I *am*1-H7	II *am*2-H2	III *am*3-H5	IV *am*4-H38	V *am*5-H3	VI *am*6-H1	VII *am*7-H2	VIII *am*8-H1
Hae-A	**172**	0	0	0	1	**87**	0	0
Hae-B	1	**135**	1	2	**128**	0	**57**	**35**
Hae-C	0	1	1	0	0	1	0	0
Hae-D	0	0	2	2	2	0	0	1
Hae-E	0	0	**10**	2	1	0	1	1
Hae-F	1	1	2	**14**	0	0	0	0
Hae-G	2	1	0	1	0	2	0	0
Hae-H	1	2	0	0	0	0	0	0
Hae-I	0	0	1	0	0	0	0	0

expect that fragment *Hae*-B should also give rise to the synthesis of gene-VII protein, the latter is absent or at an undetectable level. As a matter of fact, gene-VII-protein synthesis has never been detected yet *in vivo* [12, 14] or *in vitro* [13, 15 – 17].

Although our knowledge concerning exact number and size of phage genes and intercistronic regions is still lacking, an effort has been made to quantify distances between genes and to correlate gene sizes with respect to the physical maps. The relative sizes of genes are probably best estimated by assuming that the circular genetic map corresponds to the entire circular DNA of the phage which, in turn, has to code for about 220000 daltons of protein. The sizes of six M13 (and f1) gene products are now available [12 – 17]. The proteins encoded by gene V and gene VIII are 87 and 54 amino acids long [1, 30, 31] (*cf.* [17]). This corresponds to molecular weights of 9688 and 5800, respectively. The estimated molecular weights of the products of genes I, II, III and IV formed *in vitro* were found to be 36000, 46000, 59000 and 48000, respectively [17] (*cf.* [13]). The protein products of gene VI and gene VII are still unknown. If one assumes that the molecular weights of the products are proportional to the sizes of genes, one can calculate and express their approximate lengths in nucleotides or map units [18]. The map shown in Fig. 1 is a schematic summary of our results concerning the order of M13 genes. In addition, it accounts for what we know about gene length and their approximate positions in relation to the *Hap* II and *Hae* III cleavage maps.

DISCUSSION

It has already been demonstrated that RF I of phage M13 can be dissected into 10 and 13 fragments with the *Hae* III and *Hap* II restriction endonuclease, respectively. Each set of restriction fragments has been ordered into an enzyme cleavage map [18]. Undoubtedly, such cleavage maps are valuable tools for analysing structure and function of particular parts of the phage genome. Since each restriction fragment represents a unique part of the genome, such physical maps can also serve as a reference for mapping genes.

The methodology we have used to map the M13 genes is taken directly from prior work with phage ΦX-174 [20] in which specific fragments of RF were assayed for their activity in rescuing individual phage *ts* mutants. We have applied this technique to eight amber mutants of M13 and as a result from this the mutational sites could be localized to specific restriction fragments and consequently to specific segments of the M13 genome. For each amber mutant analysed there was a unique fragment which consistently showed a higher activity in correcting the mutational defect than any other, both in the case of *Hap* and *Hae* fragments. Moreover, when the results are compared in the light of the relationship between *Hap* and *Hae* fragments, it can be seen that each *Hae* fragment encompassing a biologically active *Hap* fragment does reveal a marker rescue activity which corresponds to the active *Hap* fragment, reinforcing the inference that the mutant site is present in a segment of the genome corresponding to the active fragment.

The order of M13 genes which can be deduced from the alignment of the amber-mutant sites along the physical map appears to be ambiguous at two positions. First, gene VI has been positioned between III and I, although the specified markers of both gene VI and gene I have been detected on a single fragment only and, therefore, are interchangeable.

The position of gene VI as given in Fig 1 is inferred primarily from its relationship with genes III and I It has been shown that these three genes form a weak complementation group [23], and the polarity of complementation suggests the order III–VI–I Recently such an order of genes has been demonstrated from marker rescue experiments similar to ours, using RF fragments of phage fd (Seeburg and Schaller, personal communication) Since fd is very closely related to M13 and the DNA of both phages show nearly identical enzyme cleavage maps, we felt it reasonable to assume that the gene order of both phages are identical The second point is the interchangeable position of genes VII and VIII for which a similar argument is applicable As gene V and gene VII mutant markers, however, complement weakly with each other [22], an adjacent position of both genes is more likely and argues for the positions of these genes as given in Fig 1 More direct evidence for a gene order VIII–VII–V could be deduced from protein-synthesis studies in a DNA-dependent cell-free system, programmed with the restriction fragments *Hap*-B_2 and *Hae*-B [17,29]

In constructing a map as shown in Fig 1 the assumption was made that the molecular weights of the gene product are proportional to gene lengths Lack of information concerning two gene products, together with the inherent inaccuracy in determining molecular weights of gene products on the basis of their relative mobility in polyacrylamide gels, makes *a priori* a proper fitting of gene length within the physical map less accurate However, a good congruency between both maps is obtained, especially in the region of genes V, VII and VIII For instance, the genetic markers *am*7-H2 and *am*8-H1 were rescued by the overlap fragment *Hae*-B *Hap* B_2 This fragment, encompassing 300 nucleotides only, is located at 0 79–0 83 unit on the physical map which is also the region which contains gene VII and (part of) gene VIII The small gene V is at 0 84–0 88 unit and overlaps the small fragment *Hap*-I_1, containing the gene V marker, to a great extent Firm support for the correct alignment of both maps can further be deduced from the positions of some defined markers within the genetic map Protein synthesis *in vitro* specified by RF I, prepared from cells infected with M13 phage carrying the amber mutation *am*2 H2, *am*3-H5, *am*4-H38 and *am*1 H7, respectively, gave rise to abortive termination of protein synthesis and subsequent formation of "amber protein fragments' The molecular weights of the protein fragments were 22000, 51000, 38000 and less than 1000 respectively [17] On the assumption that the map positions of the M13 genes, as shown in Fig 1, are correct and taking into account that transcription of M13 RF I occurs in a counter-clockwise direction of the map [17,29], the genetic markers mentioned have to be positioned at about 0 02, 0 58, 0 17 and 0 49 map unit Since the position of these markers, as calculated from the genetic map, coincide with or are in direct vicinity of the physical DNA fragments from which those genetic markers could be salvaged, we feel confident that the alignment of both the genetic and physical maps is quite accurate

It is worth mentioning in this respect that one half of the M13 genes namely genes I through IV, occupy more than 85% of the whole genome The small genes V and VIII occupy another 6–7%, leaving less than 600 nucleotides for the remaining genes VI and VII Though the products of the latter genes are unknown, we assume that the sizes of both genes are rather small Therefore, each of these two genes is represented in Fig 1 by 250 nucleotides Whether this postulated size approaches the exact gene length depends primarily on the accuracy of the molecular weight value estimated for the M13 DNA genome If the accepted best value of 6400 nucleotides deviates about 15%, *i e* 950 nucleotides, the sizes of genes VI and VII would be at least three times as large as the suggested size That this, however, is very unlikely as far as gene VII is concerned, can be inferred from our results which showed that the entire gene VII and part of genes VIII and V as well are confined to the region encompassing fragment *Hap*-I_1 and *Hap*-B_2 *Hae*-B (*i e* the region common in *Hap*-B_2 and *Hae*-B) As this region is only 420 ± 15% base pairs in length [18], we feel confident that the actual size approximates the suggested size

The proper location of genes VI and VII within the genetic map and the availability of defined DNA fragments in which these genes constitute a structural part, enables further studies to elucidate their exact length and biological function Alternatively, it might soon be possible from nucleotide sequence studies, currently under way, to trace their gene length

The authors would like to thank Dr D Pratt and Dr J Woolford for providing us with the amber mutants used in this study and for their generous gifts of strains Thanks are also due to Dr W Fiers for his gift of samples of *Hae* III endonuclease to Ms R Matze for expert technical assistance and Drs D Pratt and H Schaller for making manuscripts available prior to publication

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REGULATION OF GENE ACTIVITY IN BACTERIOPHAGE M13 DNA: COUPLED TRANSCRIPTION AND TRANSLATION OF PURIFIED GENES AND GENE-FRAGMENTS

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Regulation of Gene Activity in Bacteriophage M13 DNA: Coupled Transcription and Translation of Purified Genes and Gene-Fragments

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Specific DNA fragments, obtained after cleavage of M13 replicative form DNA with restriction endonucleases from *Haemophilus aphrophilus* (endo R.*Hap*.II), *H. parainfluenzae* (endo R.*Hpa*.II) and *H. aegyptus* (endo R.*Hae* III), have been used to direct transcription and translation in the cell-free system of *Escherichia coli*. Analyses of the *in vitro* products on sodium dodecyl sulfate–polyacrylamide gels revealed that several restriction fragments directed the synthesis of authentic phage specific proteins. 1) Fragment *Hap* A directs the synthesis of gene IV protein, 2) fragment *Hap* B_2, of gene-VIII protein, and 3) fragment *Hae* B, of gene-V protein. In addition, fragments *Hap* A and *Hae* A direct the synthesis of prematurely terminated translation products of gene IV. It is suggested that the polypeptide designated X-protein (MW, 12,000) and encoded by fragments *Hap*.C and *Hae*.B is the product of a hitherto unidentified gene. Furthermore, it is concluded that replicative form DNA is transcribed in a counterclockwise direction along the genetic map published by C. A. van den Hondel, A. Weijers, R. N. H. Konings, and J. G. G. Schoenmakers ((1975) *Eur. J. Biochem.*, **53**, 559–567). It is proposed that promoter sites are located on the genetic map immediately proximal to the 5'-ends of genes IV and VII and also immediately proximal to the 5'-end of the "gene" coding for X-protein.

1. INTRODUCTION

M13 is an F-specific filamentous coliphage, closely related to the phages f1 and fd (for a review, see Marvin and Hohn (1969)). After infection of an *Escherichia coli* cell with this phage not all phage-specific proteins are synthesized in equimolar amounts (Henry and Pratt, 1969). Some proteins, such as the "DNA unwinding protein" encoded by gene V and the major capsid protein encoded by gene VIII, are synthesized in much larger quantities than the other phage-specific proteins. These two proteins are also the major products synthesized in M13 DNA-directed reactions *in vitro* (Konings, 1973; Model and Zinder, 1974). To explain these noticeable differences in the level of gene expression, several regulatory mechanisms have been proposed (Konings, 1973). The experimental data available (Okamoto *et al.*, 1969; Takanami and Okamoto, 1973; Jacob *et al.*, 1970; Edens *et al.*, manuscript in preparation) favour the model according to which replicative form DNA[1] is transcribed into several distinct polycistronic mRNAs, which are initiated at different promoter sites (RNA initiation sites) but are all terminated at the same unique termination signal.

In order to obtain more detailed information about this regulatory mechanism and

[1] Abbreviations used: SS, single-stranded phage DNA; RF, double-stranded replicative form DNA; RF-I, RF in which both strands are covalently closed circles; RF-II, circular RF containing one or more single-strand breaks; I-P, II P etc. refer to the proteins encoded by gene I, gene II etc., respectively; *Hap*, *Hpa*, and *Hae* refer to the fragments obtained after cleavage of an RF molecule with the restriction endonucleases from *Haemophilus aphrophilus* (endo R.*Hap* II), *H. parainfluenzae* (endo R.*Hpa*.II) and *H. aegyptus* (endo R.*Hae* III), respectively.

in particular about the location of promoter sites, the ability of specific M13 DNA fragments (restriction fragments) to direct the *in vitro* synthesis of M13-specific proteins has been studied The distribution of promoter sites along the genetic map will be discussed in relation to the model of transcription mentioned

Preliminary results of these studies were already summarized by Konings (1974) and cited by van den Hondel *et al* (1975)

2 MATERIALS AND METHODS

a Cell-free protein synthesis All of the materials and methods used for the growth of bacteria, isolation of purified phage, isolation of replicative form DNA, preparation of the cell-free extract and the conditions of *in vitro* protein synthesis have been described previously (Konings, 1973, Konings *et al*, 1973, Konings *et al*, 1975) Also, the conditions for electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels and autoradiography have been reported (Konings *et al*, 1975)

b Isolation and purification of restriction fragments The methods for cleavage of replicative form DNA with the restriction endonucleases R *Hind* II and R *Hap* II, purified from *Haemophilus influenzae* and from *Haemophilus aphrophilus*, have been described (van den Hondel and Schoenmakers, 1973, 1975) The digestions of M13 replicative form DNA with the restriction endonucleases R.*Hpa* II and R *Hae* III, isolated from *Haemophilus parainfluenzae* and from *Haemophilus aegyptius*, were kindly performed by Dr Bernard Allet, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY After digestion, the fragments were separated by polyacrylamide-gel electrophoresis and further purified as described by van den Hondel *et al* (1975)

3 RESULTS

Previously it has been demonstrated that, *in vitro*, M13 replicative DNA directs the synthesis of a discrete number of polypeptides ranging in molecular weight from 5,000 to 65,000 (Fig 2l, Konings, 1973, Konings *et al*, 1975) The sum of the molecular weights of these polypeptides is larger than one would expect on the basis of the size of the M13 genome (MW of RF-I DNA, ca 4×10^6) Some of these polypeptides, therefore, cannot represent complete, authentic, viral proteins With the aid of RF preparations having defined amber mutations, six of these polypeptides have been identified as being the products of genes I-V and of gene VIII (Fig 2l) Furthermore, a number other polypeptides, of which IV'-P was the most predominant (Fig 2l), have been identified as being the result of premature termination of polypeptide synthesis For reasons, still unknown, with such amber RF preparations, no gene-protein relationship could be demonstrated for genes VI and VII (Konings *et al*, 1975, Konings, unpublished results)

3 1 Analyses of the Polypeptides Synthesized in Vitro Under the Direction of Hap Fragments

Both the restriction endonuclease R *Hap* II as well as the restriction endonuclease R *Hpa* II cleave M13 replicative form DNA into 13 discrete fragments which can readily be separated by polyacrylamide-gel electrophoresis (van den Hondel and Schoenmakers, 1975, van den Hondel, Schoenmakers, and Konings, unpublished results) The sizes of these fragments, numbered *Hap* A–*Hap* J in decreasing order of fragment size, range from ca 1,530 to ca 50 base pairs Recently, these *Hap* fragments have been ordered in an enzyme cleavage map (van den Hondel and Schoenmakers, 1975) This cleavage map, indicating the various positions of cleavage sites and the location of eight genetic markers (amber mutations), each corresponding to a gene (van den Hondel *et al*, 1975), is illustrated in Fig 1

When the DNA dependent cell-free system was programmed with each of the individual *Hap* fragments, analyses on SDS–polyacrylamide gels revealed that only under the direction of the restriction fragments *Hap* A, *Hap* B_2, and *Hap* C (Figs 2a–c) polypeptides are made which are clearly distinguishable from the polypeptides synthesized in the endogenous system (Fig 2k) Identical results were

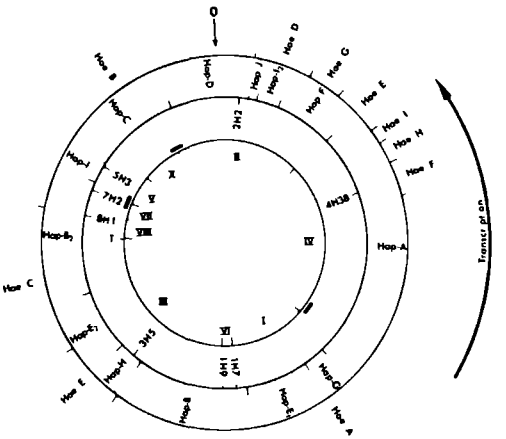

FIG 1 Genetic and physical map of bacteriophage M13 The inner circle represents the genetic map (van den Hondel *et al*, 1975) The middle and outer circles show the locations of the *Hap* II and *Hae* III fragments, respectively The arrow indicates the cleavage site of RF DNA by the restriction endonuclease R.*Hind* II from *Haemophilus influenzae* The size of the genes was estimated from the molecular weights of the *in vitro* products (Koninga *et al*, 1975), with the exception of genes VI and VII whose definite size is unknown The location of eight amber mutations, each corresponding with a gene, is indicated in the middle circle The direction of transcription is counterclockwise around the genetic map The black bars indicate the locations of the promoter sites deduced from our *in vitro* protein synthesis studies The approximate position of the central terminator for RNA synthesis is indicated (T)

obtained when the individual restriction fragments obtained after cleavage of M13 RF DNA with the restriction endonuclease from *Haemophilus parainfluenzae* (R.*Hpa*.II) were added to the cell-free system. These results together with the observations mentioned above support the findings that the restriction endonucleases R.*Hap*.II and R.*Hpa*.II do have the same cleavage-site specificity (Sugisaki and Takanami, 1973, Garfin and Goodman, 1974) In the following sections the identification of the polypeptides synthesized under the direction of the *Hap* fragments will be described

1 *Fragment Hap.A* Recently it has been shown that amber mutations in gene IV of phage M13 can be rescued by fragment *Hap* A (van den Hondel *et al*., 1975). Furthermore it has been demonstrated (Konings *et al*, 1975) that this restriction fragment (ca 1530 base pairs long) directs in the coupled system the synthesis of gene-IV protein (Figs 2a and l, IV-P, MW, 48,000) The minimum number of nucleotides required to code for a polypeptide of this size is approximately 1450 This size is almost identical to the size of fragment *Hap*.A. The conclusion therefore seems to be justified that fragment *Hap*.A consists of almost pure gene IV Furthermore, the observation that this gene can be expressed does strongly suggest that this fragment contains a promoter which must be located immediately proximal to its 5'-end (Fig 1)

As shown in Fig 2a, fragment *Hap* A directs also the *in vitro* synthesis of a number of other "smaller polypeptides," of which IV'-P (MW, 24,000) is the most predominant The minimum number of nucleotides required to code for IV'-P is approximately 700 The room left on fragment *Hap*.A, however, for genes other than gene IV is only about 100 nucleotides Therefore, it is reasonable to assume that the smaller polypeptides are also products

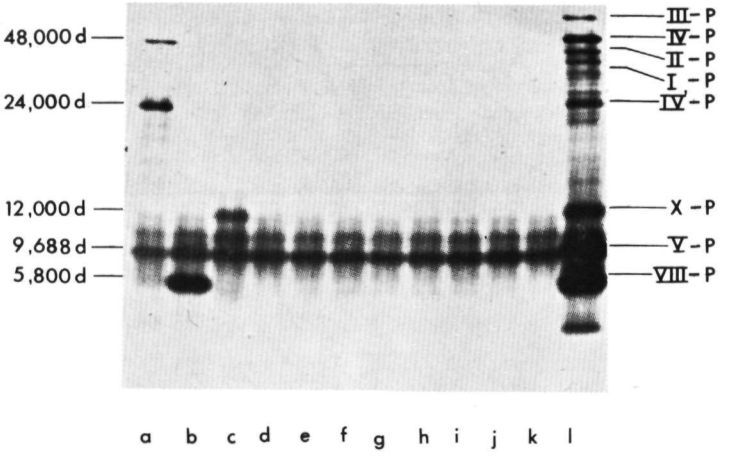

FIG. 2. Autoradiogram of [^{35}S]methionine-labeled polypeptides synthesized *in vitro* in the presence and absence of the *Hap* fragments of phage M13 replicative form DNA. For comparison the gel electropherogram of the polypeptides synthesized *in vitro* in the presence of wild-type replicative form DNA (RF-I) has also been given. (a), Products synthesized in the presence of fragment *Hap*.A; (b), products synthesized in the presence of either the doublet fragment *Hap*.B (composed of a mixture of equimolar amounts of fragment *Hap*.B_1 and *Hap*.B_3) or fragment *Hap*.B_3; (c), products synthesized in the presence of fragment *Hap*.C, (d), products synthesized in the presence of fragment *Hap*.B_2 having an amber mutation in gene VIII (M13 *am*8-H1); (e), products synthesized in the presence of fragment *Hap*.D; (f), products synthesized in the presence of the doublet fragment *Hap*.E (*Hap*.E_1 and *Hap*.E_2); (g), products synthesized in the presence of fragment *Hap*.F; (h), products synthesized in the presence of fragment *Hap*.G; (i), products synthesized in the presence of fragment *Hap*.H; (j), products synthesized in the presence of the doublet fragment *Hap*.I (*Hap*.I_1 and *Hap*.I_2) and fragment *Hap*.J; (k), products synthesized in the absence of DNA; (l), products synthesized in the presence of wild-type replicative form DNA. Protein synthesis *in vitro* was carried out as described (Konings *et al.*, 1975). Of each DNA fragment, an amount equivalent to 1 μg of replicative form DNA was added per 0.025-ml reaction mixture. The *in vitro* synthesized polypeptides were analyzed on 15% SDS–Tris–glycine gels (Konings *et al.*, 1975). The identification and characterization of the products synthesized *in vitro* under the direction of wild-type replicative form DNA have been described (Konings *et al.*, 1975). I-P, II-P etc. refer, respectively, to the position of migration of the products of gene I, gene II, etc. The arabic numerals indicate the approximate molecular weights of these polypeptides. The molecular weight of gene V protein (MWt, 9,688) is deduced from its amino acid sequence (Cuypers *et al.*, 1974).

of gene IV. Several possibilities exist concerning the origin of these smaller polypeptides: 1) The polypeptides concerned are products produced by proteolytic cleavage of precursor molecules; 2) they are the result of translational reinitiation as has been shown to occur for instance in the *lac i* gene (Files *et al.*, 1974) and in the A-protein gene of phage ϕX174 (Linney and Hayashi, 1973); 3) the smaller polypeptides are the result of premature termination of polypeptide synthesis.

Explanation 1) is unlikely since the gel pattern of the *in vitro*-synthesized polypeptides did not change after prolonged incubation (150 min) of the cell-free system at 37°. Furthermore, no differences in the autoradiograms could be observed when protein synthesis was studied in the presence of potent protease inhibitors (Konings *et al.*, 1975). Explanation 2) seems unlikely because, in the case of the closely related bacteriophage f1, it has been shown that no synthesis of IV′-P could be demon-

strated when protein synthesis was studied under the direction of an RF preparation having an amber mutation located proximal to the 5'-end of gene IV On the contrary, the smaller polypeptides are still made when protein synthesis is studied under the direction of an *Hap*.A fragment carrying an amber mutation in the distal quarter of gene IV (M13 *am*4-H38, Konings *et al.*, 1975) The sole explanation 3) which thus remains is that IV'-P and the other smaller polypeptides are the result of premature termination of polypeptide synthesis This explanation is completely consistent with the observed data

ii. Fragment Hap.B_2 With the aid of marker rescue experiments it has been demonstrated that fragment *Hap*.B_2 contains genetic markers for both genes VII and VIII (Fig 1, van den Hondel *et al*, 1975) Addition of the latter fragment to the cell-free system resulted in the synthesis of a single polypeptide having an electrophoretic mobility that was identical to that of the *in vitro* product of gene VIII, a gene coding for the major capsid protein (Figs 2b and 1, VIII-P, MW, 5800) Although fragment *Hap* B_2 contains genetic markers for both genes VII and VIII, and probably for gene III as well (van den Hondel, *et al*, 1975), no *in vitro* synthesis of other polypeptides could be demonstrated Similar results have been obtained when, instead of fragment *Hap* B_2, fragment *Hap*.B, composed of equimolar amounts of fragment *Hap*.B_1 and fragment *Hap* B_2 (van den Hondel and Schoenmakers, 1975), was added to the cell-free system (Fig 2b)

In order to obtain evidence for whether the products encoded by fragment *Hap*.B_2 and gene VIII are identical, we have studied *in vitro* protein synthesis under the direction of an *Hap* B_2 fragment which contains an amber mutation in gene VIII (M13 *am*8-H1, Fig 2d) In addition protein synthesis was studied in the presence of [^{14}C]arginine (not shown), an amino acid not present in gene-VIII protein (Konings, 1973, Beyreuther, 1968) In both instances the synthesis of a polypeptide comigrating with gene VIII protein (VIII-P) could not be demonstrated The conclusion therefore seems to be justified that the complete gene VIII is located on fragment *Hap*.B_2, which can be expressed because of the presence of a promoter upstream from the 5' end of this gene

iii. Fragment Hap.C. Although direct experimental data concerning the true genetic content of fragment *Hap* C are not yet available, both our genetic as well our *in vitro* protein synthesis studies (van den Hondel *et al*, 1975, Konings *et al*, 1975) indicate that this fragment consists of parts of genes II and V (Fig 1)

Addition of fragment *Hap* C to the coupled system resulted in the synthesis of a polypeptide having an electrophoretic mobility that was identical to that of X protein (X P, MW, 12,000, Fig 2c), a polypeptide for which no gene–protein relationship has been established The synthesis of the latter polypeptide has not (yet) been demonstrated *in vivo* (Henry and Pratt, 1969) However, its synthesis can clearly be demonstrated *in vitro* when polypeptide synthesis is studied under the direction of replicative form DNA (Fig 2l, Konings, 1973, Model and Zinder, 1974)

The observation that a polypeptide with a molecular weight of 12,000 is not synthesized under the direction of the other *Hap* fragments (Fig 2) suggests that X protein is not an *in vitro* artifact but the product of a unique region of the M13 genome As will be shown in Section *3 2.ii*, a polypeptide with the same electrophoretic mobility as X-protein is also synthesized under the direction of fragment *Hae*.B, a fragment that encompasses the complete nucleotide sequence of fragment *Hap*.C (Fig 1, van den Hondel *et al.*, 1975) The meaning of these results with respect to the genetic origin of X protein will be discussed

3.2 Analyses of the Polypeptides Synthesized in Vitro Under the Direction of Hae Fragments

The restriction endonuclease R.*Hae* III cleaves M13 replicative form DNA into ten discrete fragments, ranging in size from ca 2500 to ca 70 base pairs As in the case of the *Hap* fragments, the physical order of these restriction fragments has been estab-

lished (Fig. 1; van den Hondel and Schoenmakers, 1975). Furthermore the location of eight genetic markers (amber mutations), each corresponding to a gene, has also been described (Fig. 1; van den Hondel *et al.*, 1975).

Analyses on polyacrylamide gels of the polypeptides synthesized in the DNA-dependent system under the direction of the individual *Hae* fragments revealed that only the electropherograms of the polypeptides synthesized in the presence of fragments *Hae*.A and *Hae*.B (Figs. 3c, c', d and d') were clearly distinguishable from

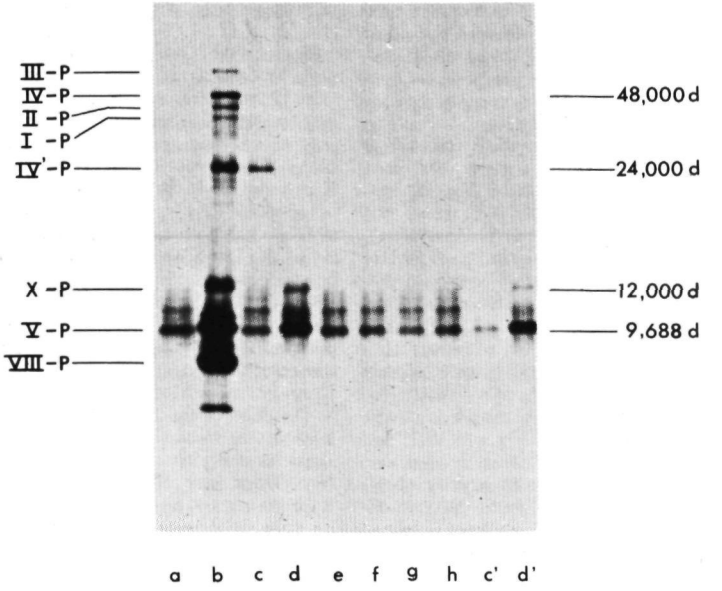

FIG. 3. Autoradiogram of the polypeptides synthesized *in vitro* in the presence and absence of the *Hae* fragments of phage M13 replicative form DNA. For comparison the gel electropherogram of the polypeptides synthesized *in vitro* in the presence of wild-type replicative form DNA (RF-I) has also been given. (a), Products synthesized in the absence of exogenous DNA; (b), products synthesized in the presence of wild-type replicative form DNA; (c,c'), products synthesized in the presence of fragment *Hae*.A; duplicate experiments performed with different preparations of restriction fragments; (d,d'), products synthesized in the presence of fragment *Hae*.B; duplicate experiments performed with different preparations of restriction fragments; (e), products synthesized in the presence of fragment *Hae*.C; (f), products synthesized in the presence of fragment *Hae*.D; (g), products synthesized in the presence of the doublet fragment *Hae*.E (*Hae*.E_1 and *Hae*.E_2); (h), products synthesized in the presence of fragments *Hae*.F, *Hae*.G, *Hae*.H, and *Hae*.I. Protein synthesis *in vitro* was carried out as described (Konings *et al.*, 1975). Of each DNA fragment an amount equivalent to 1 μg of replicative form DNA was added per 0.025-ml reaction mixture. The *in vitro* synthesized polypeptides were analyzed on 15% SDS-Tris-glycine gels (Konings *et al.*, 1975). The identification and characterization of the products synthesized *in vitro* under the direction of wild-type replicative form DNA has been described (Konings *et al.*, 1975). I-P, II-P etc. refer, respectively, to the position of migration of the products of gene I, gene II etc. The arabic numerals indicate the approximate molecular weights of these polypeptides. The molecular weight of gene V protein is deduced from its primary structure (Cuypers *et al.*, 1974).

the electropherogram of the polypeptides synthesized in the control experiment (Fig 3a) These results will be described in succession

i Fragment Hae A Genetic and physical analyses have demonstrated that fragment *Hae* A consists of gene I, part of gene IV and at least part of gene VI (van den Hondel and Schoenmakers, 1975, van den Hondel *et al*, 1975) The order of these genes on the genetic map is VI–I–IV (Fig 1)

As shown in Fig 3c and c', fragment *Hae* A *in vitro* directs primarily the synthesis of a polypeptide having an electrophoretic mobility that is identical to that of the prematurely terminated product of gene IV (IV'-P, MW, 24,000, cf Section *3 1 i*)

Several observations indicate that this polypeptide is a prematurely terminated product of gene IV and not a product of the other genes (genes I and VI) located on this restriction fragment 1) The molecular weight of this polypeptide is different from that encoded by gene I (MW, 36,000, Konings *et al*, 1975) Its molecular weight must also be different from that of the polypeptide encoded by gene VI, since genetic studies and *in vitro* protein synthesis studies have indicated that the size of gene VI, and consequently the size of its encoded product, must be small, i e , not larger than 400 nucleotides (van den Hondel *et al*, 1975, Konings *et al*, 1975, Model and Zinder, 1974) 2) Genetic studies have indicated that a polarity exists among genes III, VI, and I in such a way that the expression of genes VI and I is (completely ?) dependent on the expression of gene III (Pratt *et al*, 1967) Gene III, however, which is located next to gene VI on the genetic map (Fig 1) is not located on this restriction fragment 3) The sole explanation which remains is that the polypeptide encoded by fragment *Hae* A is a prematurely terminated translation product of gene IV This conclusion is supported by the fact that a sequence of about 1000 nucleotides of gene IV is located on this restriction fragment (van den Hondel and Schoenmakers, 1975) This gene IV fragment, which potentially can be transcribed due to the presence of promoter immediately proximal to the 5 -end of gene IV (Section *3 1 i*), is large enough to code for a (prematurely terminated) polypeptide with a molecular weight of 24,000 The expression of this gene IV fragment has become apparent by the fortunate circumstance that, within the transcript of gene IV, regions are present where, for reasons still unknown, premature termination of polypeptide synthesis preferentially occurs (Konings *et al*, 1975) From the results described it can furthermore be deduced that the 5 end and not the 3 end of gene IV is located on restriction fragment *Hae* A. This together with the observation that the order of the genes on fragment *Hae* A is VI–I–IV (Fig 1) suggests that these genes are transcribed counterclockwise around the genetic map illustrated in Fig 1 The fact that phage M13 mRNA is only transcribed from the "nonviral" strand of replicative form DNA (Jacob and Hofschneider, 1969, Sugiura *et al*, 1969, unpublished results) implies that all other genes are transcribed in the same direction This direction of transcription is consistent with the observed polarity relationship among genes III, VI and I (Pratt *et al*, 1967) Similar conclusions have recently been reached from alternative deductions by Model and Zinder (1974) from their studies on phage f1, a close relative of phage M13

ii Fragment Hae B Fragment *Hae* B consists of genes V and VII and at least parts of genes II and VIII (Fig 1) The order of these genes on the genetic map is II–V–VII–VIII (Fig 1, van den Hondel *et al*, 1975) Comparison of the autoradiogram of the polypeptides synthesized under the direction of fragment *Hae* B (Figs 3d and d) with that of the polypeptides made in the endogenous system (Fig 3a) revealed that under the direction of fragment *Hae* B two polypeptides are made One of these polypeptides comigrated with gene-V protein (V P, MW, 9688) while the other comigrated with X protein (X-P, MW, 12,000), a polypeptide whose gene–protein relationship has not yet been established (Konings *et al*, 1975)

The observations that no synthesis of the former polypeptide could be demonstrated

when protein synthesis was studied either under the direction of an *Hae*.B fragment having an a amber mutation in gene V (M13 *am*5-H3) or in the presence of [^{14}C]tryptophan, an amino acid not present in gene V protein (Konings, 1973, Cuypers *et al*, 1974) indicate that this polypeptide is a product of gene V

Just as fragment *Hap* C (Section *3.1.iii*) directs the *in vitro* synthesis of a polypeptide that comigrates with X-protein so does fragment *Hae* B. The latter polypeptide is not synthesized when the other *Hae* fragments are added to the DNA-dependent cell free system (Fig 3) As the complete genetic content of fragment *Hap*.C is located on fragment *Hae* B (Fig 1, van den Hondel *et al*, 1975), these results suggest that X-protein is not an *in vitro* artifact but a polypeptide encoded by a unique region of the M13 genome

4 DISCUSSION

The mechanism that regulates the expression of the genes located on the circular M13 genome can best be explained by a model according to which replicative form DNA is transcribed into a discrete number of polycistronic mRNAs which have, at their 3'-end, the coding information for at least one protein in common (gene VIII protein, Edens *et al.*, manuscript in preparation) This is the consequence of a transcription process in which mRNA synthesis starts at different promoter sites (RNA initiation sites) but terminates at the same unique termination signal (Fig 1) This termination site is most likely located immediately after gene VIII (Konings *et al*, manuscript in preparation)

One of the main objectives of the present study was to localize promoter sites on the M13 genome For this reason we have investigated whether the individual M13 genes retain their ability to direct transcription and translation after disconnection (with restriction endonucleases) from the intact genome From the results obtained it became evident that only the restriction fragments *Hap* A, *Hap* B_2, *Hap* C, *Hae* A, and *Hae* B were able to direct the synthesis of phage-specific proteins Based on the assumption that the ability of a particular fragment to direct the *in vitro* synthesis of a polypeptide reflects the presence of a promoter, we conclude that the latter fragments contain a promoter for RNA initiation and transcription (cf Allet *et al*, 1973)

Since fragment *Hap*.A consists of almost pure gene IV, the promoter present on this restriction fragment must be located immediately proximal to the 5'-end of this gene (Fig 1) Also, this promoter is most probably responsible for the expression of the 5'-end of gene IV located on restriction fragment *Hae* A (Fig 1) The observation that the latter fragment infrequently also directs the synthesis of a polypeptide comigrating with gene I protein suggests that a (weak) promoter is also located immediately proximal to the 5' end of gene I The reason for this weak expression is unknown, but it should be mentioned here that in the case of the intact M13 genome the expression of gene I is also weak and variable (Konings *et al*, 1975, Model and Zinder, 1974)

Although fragment *Hae* A and fragment *Hae* B encompass the complete genes VI and VII respectively, we have not (yet) been able to demonstrate the synthesis of their encoded proteins Three hypothesis could account for this 1) Due to the absence of a promoter immediately proximal to the 5' ends of these genes, they are not transcribed, 2) genes VI and VII are transcribed but the frequency of translation of their transcript is very low, 3) genes VI and VII are transcribed but due to the presence of nucleases and proteases in the *E coli* extract the synthesis of their encoded proteins cannot be demonstrated Which of these possibilities is valid awaits further investigation In this context it is worthwhile to mention that, in contrast to the other gene products of phage M13, we also have not yet been able to demonstrate the *in vitro* synthesis of gene VI and gene VII protein under the direction of replicative form DNA (Konings *et al*, 1975, Model and Zinder, 1974)

Besides gene VIII, the complete nucleotide sequence of gene VII is most likely located on fragment *Hap* B_2 (Fig 1) For this reason and for reasons presented in the

previous paragraph, the question whether the promoter, which is responsible for the expression of gene VIII, is located in the vicinity of the 5'-end of this gene remains unanswered In this connection it is interesting to note that *in vitro* transcription studies on fragment *Hap* B_2 revealed that the promoter responsible for the expression of gene VIII is located immediately proximal to the 5'-end of this restriction fragment (Fig 1) and the size of the mRNA (360 nucleotides) transcribed from this promoter is an order of magnitude larger than the size of the mRNA required to code for gene-VIII protein (MW, 5800, L Edens *et al*, manuscript in preparation) These observations together with the findings that all amber mutations, tested so far, in gene VII are rescued by fragment *Hap*.B_2 suggest that the promoter responsible for the expression of gene VIII is located immediately proximal to the 5'-end of gene VII (Fig 1). The transcription studies suggest furthermore that the central terminator for RNA transcription is located immediately after the 3'-end of gene VIII (Fig. 1).

Among all the restriction fragments tested, only fragments *Hap*.C and *Hae*.B were able to direct the *in vitro* synthesis of X-protein, a polypeptide for which no gene-protein relationship has yet been established. Since fragment *Hae*.B encompasses the complete nucleotide sequence of fragment *Hap* C, these results strongly suggest that X-protein is a unique phage specific protein By emphasizing the location of these fragments on the genetic map (Fig 1) it is speculative to postulate that X-protein is the product of a gene hitherto unidentified and that is located on the genetic map between genes II and V (Fig 1). In this connection it is interesting to note that Linney and Hayashi (1974) have found that gene A of phage ϕX174, a gene whose product has the same function in the process of DNA replication as the product of gene II of phage M13, directs the synthesis of two (biologically active ?) polypeptides Since the exact location of gene II with respect to genes IV and V is not yet known, by analogy to the situation with ϕX174, an alternative hypothesis concerning the genetic origin of X-protein therefore is that it is a product of an mRNA whose promoter is located within gene II The main objection against this hypothesis, however, can be deduced from nucleotide-sequence studies which indicate that the nucleotide sequence at the 5'-end of the transcript of fragment *Hap*.C contains, irrespective of the reading frame chosen, one or more termination codons (Sugimoto *et al*, 1975)

From the results described it is clear that a promoter is located immediately proximal to the 5'-end of the "gene" coding for X-protein Due to the fact that we could only demonstrate the synthesis of gene-V protein on a fragment which also codes for X-protein, we are not yet able to answer the question whether there is a promoter located immediately proximal to the 5'-end of gene V It is possible, however, that the genes coding for X-protein and for gene-V protein share the same promoter

Cleavage of replicative form DNA with the restriction enzymes R.*Hap* II and R.*Hae*.III, respectively, did not result in fragments that contained the complete nucleotide sequences of genes II and III (van den Hondel *et al*, 1975) Hence, at present evidence cannot be given as to whether a promotor is located immediately proximal to the 5'-ends of either one of these genes DNA fragments obtained after cleavage of replicative form DNA with other restriction endonucleases may answer this question

Apart from the three promoters already localized in the present study, the existence of a promoter in front of gene III has been evidenced by genetic studies (Pratt *et al*, 1967), *in vitro* protein synthesis studies (Konings *et al*., manuscript in preparation), and transcription studies on restriction fragments (Takanami and Okamoto, 1973) Recently, promoter sites on the genome of phage fd, a close relative of phage M13, have been localized, with the aid of *in vitro* transcription and RNA polymerase-binding studies, on the restriction fragments *Hpa*.A, *Hpa* B_1 *Hpa* B_2 *Hpa*.C, *Hpa* E_1 and *Hpa* F, respectively (M Takanami, P H Seeburg and H Schaller, personal communications) Since

it is known from the present and previous studies (Sugisaki and Takanami, 1973; Garfin and Goodman, 1974) that the restriction endonucleases R.*Hpa*.II and R.*Hap*.II do have the same cleavage-site specificity, these results are, therefore, in excellent agreement with the location of the promoter sites deduced from our *in vitro* protein synthesis studies.

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Note added in proof After submission of the manuscript we were able to demonstrate that gene V protein, synthesized under the direction of restriction fragment *Hae* B is biologically active and binds tightly and selectively to single-stranded but not to double-stranded DNA (cf Konings *et al*, 1973) In addition, from comparative studies on several M13 mutant phages bearing an amber mutation in gene II, the position of the genetic marker *am*2 H2 was localized at map position 0 96

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RESTRICTION ENZYME CLEAVAGE MAPS OF BACTERIOPHAGE M13: EXISTENCE OF AN INTERGENIC REGION ON THE M13 GENOME

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SUMMARY

Replicative form DNA of bacteriophage M13 was cleaved into specific fragments by an endonuclease isolated from *Haemophilus aegyptius* (endoR.*Hae* II) and an endonuclease from *Arthrobacter luteus* (endoR.*Alu* I). The fragments were ordered so as to construct a circular map of the phage M13 genome by: (1) using each fragment as a primer for the *in vitro* synthesis of its respective neighbour; and (2) digesting the isolated fragments with the *Haemophilus aegyptius* enzyme endoR.*Hae* III or the *Haemophilus aphirophilus* enzyme endoR.*Hap* II and subsequent analysis of the overlapping sets of fragments. The resulting physical map was correlated with the M13 genetic map by marker rescue experiments with amber mutant phage DNAs and purified wild type fragments.

From the results of these analyses it has been concluded that gene II and gene V are contiguous on the genetic map. Evidence is provided that there is an internal start of RNA synthesis within the C-terminal region of gene II which then ultimately leads to the *in vitro* synthesis of X-protein. Furthermore, we conclude that there is an intergenic space of considerable length (450-500 base-pairs) which is located between gene II and gene IV on the M13 genome. The function of this intergenic region as the origin site for phage DNA replication is discussed.

INTRODUCTION

The genome of the small filamentous bacteriophage M13 consists of a circular, single-stranded DNA (M_r = 1.9 x 10^6) which, upon infection, is converted into a double-stranded, circular replicative form (RF) molecule (for review see 1, 2). Genetic analyses have demonstrated the existence of eight genes (3), which have been ordered on a circular map (4-6).

The functions of only four of these genes are known: gene II which is required for the replication of both RF and single-stranded viral DNA; gene V which codes for a DNA-binding protein that is required for the synthesis of progeny viral DNA; gene III and gene VIII which code for the precursors of the minor and major coat proteins, respectively. With exception of gene VI and gene VII, all the protein products encoded by the M13 genes have been characterized according to their different molecular weights (7-12).

Restriction enzyme cleavage maps of M13 RF, related to the genetic map, have contributed significantly to a better understanding of the mechanism by which the transcription and translation of the M13 genome are regulated (4, 13-18). Protein synthesis studies, carried out in a "coupled" transcription-translation system in which various restriction fragments were used as a template, have revealed the synthesis of several M13-specific proteins (6, 9, 13). One of these proteins, although M13-specified, could not yet been assigned to an M13 gene and it was therefore referred to as "X-protein" (9, 10, 13). The DNA fragments which code for this unknown protein were found to be located proximal to gene V (13). To accord with the genetic map, a new gene coding for X-protein and which is located between gene II and gene V has been inferred. On the other hand, an internal start of RNA synthesis within the C-terminal part of gene II itself may also be considered to be responsible for the occurrence of X-protein (13).

In order to test this supposition, a more accurate determination of the position of gene II on the genetic map was essential. For this reason we constructed additional restriction enzyme cleavage maps of M13, *i.e.* the endoR.*Hae* II and the endoR.*Alu* I cleavage map. The physical map so obtained was correlated to the genetic map by marker rescue experiments. From this correlation, additional information was obtained concerning the localization of the M13 genes, from which the existence of an intergenic region between gene II and gene IV has been deduced. Moreover, evidence is provided that due to an internal start of RNA synthesis, gene II generates two proteins, one of which is X-protein.

MATERIALS AND METHODS

Bacteria and phages

Arthrobacter luteus was obtained from the American Type Culture Collection (ATCC 21606). *Haemophilus influenzae* strain Rd was kindly supplied by Dr. H. O. Smith; *Haemophilus aphirophilus* was a gift of Dr. M. Takanami, Kyoto; *Haemophilus aegyptius* was a gift of Dr. W. Fiers, Gent. *E.coli* K37 (Su-1) and K143 (Su-3), the permissive host for M13 and f1 amber mutant phages and *E.coli* K38, the non-permissive host, were donated by Dr. D. Pratt, Davis.

Phage M13 (wt) was supplied by Dr. P. Hofschneider, Munich; the M13 nonsense mutants *am*1-H7 (gene I), *am*2-H2 (gene II), *am*3-H1, *am*3-H4, *am*3-H5 (gene III), *am*6-H1, *am*6-H2, *am*6-H6, *am*6-H7 (gene VI), and *am*7-H1, *am*7-H3 (gene VII), were provided by Dr. D. Pratt. The f1 nonsense mutants R86 and R124 (amber in gene II) were kindly supplied by Dr. N. D. Zinder, New York.

EndoR.*Hind* II was isolated as described previously (19). EndoR.*Alu* I was isolated as follows: 20 g of *A.luteus* cells, which were grown in Tryptone broth (20) and harvested at mid-exponential phase, were suspended in 200 ml of 30 mM Tris-HCl, pH 8.4, 2 mM dithiothreitol, 5% glycerol. The cells were disrupted by sonication and the sonicate was centrifuged at 15,000 revs/min for 30 min. The resulting supernatant was brought to a concentration of 4 mM $MgCl_2$ and 0.3 M NaCl and then centrifuged for 3 h at 50,000 revs/min. Two ammonium sulphate cuts were made by adding 26 g, and an additional 23 g of solid ammonium sulphate per initial 100 ml of supernatant. After stirring for 30 min, the precipitates were collected by centrifugation for 30 min at 10,000 revs/min. The second ammonium sulphate precipitate, which contained most of the enzyme activity, was dissolved in 50 ml of buffer A (20 mM Tris-HCl, pH 8.4, 14 mM mercaptoethanol, 5% glycerol) and dialysed against buffer A containing 0.3 M KCl. The dialysed material was passed through a DEAE-cellulose column (20 x 2 cm) which had previously been equilibrated with buffer A containing 0.3 M KCl. The flow-through fractions were pooled and dialysed against buffer B (20 mM potassium phosphate, pH 7.6, 14 mM mercaptoethanol, 5% glycerol). Thereafter, the dialysed material was applied to a phosphocellulose (P11) column (20 x 2 cm) that had been equilibrated with buffer B. This was followed by a linear gradient, ranging from 0 - 1.0 M KCl in buffer B. The total gradient volume applied was 300 ml. The fractions obtained were assayed by incubation at 37°C for 1 h in a reaction mixture (0.05 ml) containing 7 mM Tris-HCl, pH 7.4, 7 mM $MgCl_2$, 7 mM mercapto-ethanol and 3 µg of T_7 DNA. After incubation, the samples were analysed electrophoretically on a 3% polyacrylamide slab gel, as described previously (21). The assay run indicated that the endoR.*Alu* I activity eluted at 0.45 - 0.70 M KCl and was preceded by a potent exonuclease activity which came off the column at about 0.40 M KCl. The fractions which yielded a characteristic pattern of T_7 DNA fragments were collected, dialysed against buffer B and were subsequently rechromatographed on a second phosphocellulose column (15 x 2 cm) under further identical conditions. The active fractions were collected, dialysed against 20 mM potassium phosphate, pH 7.6, 14 mM mercaptoethanol, 50% glycerol and stored at -20°C. The final enzyme fraction contained approximately 450 enzyme units per ml (4) and did not contain any detectable exonuclease activity.

EndoR.*Hae* II, endoR.*Hae* III and endoR.*Hap* II were isolated according to the procedure described above, except that each enzyme fraction, obtained

after phosphocellulose chromatography, was further purified by rechromatography on a DEAE-cellulose column equilibrated in buffer B. *E.coli* DNA polymerase I was a generous gift of Dr. H. L. Heyneker. Endonuclease S_1 was a kind gift of Dr. J. Sanders, Amsterdam.

Preparation of DNA

The methods for the preparation of phage M13 RF and ^{32}P-labeled M13 RF have been described (21, 25). Propagation and purification of M13 and f1 amber mutant phages were performed as described previously (4). The isolation of viral DNA from M13 wild type and M13 amber mutant phages was carried out as described by Marvin and Schaller (20).

Cleavage of M13 by restriction endonucleases and polyacrylamide gel electrophoresis of DNA fragments

For the preparation of complete endoR.*Alu* I digests, ^{32}P -labeled M13 RF-I supplemented with unlabeled M13 RF (1 µg) was incubated with 4 µl of endoR.*Alu* I in a final reaction volume of 0.05 ml containing 10 mM Tris-HCl, pH 7.6, 7 mM $MgCl_2$, 7 mM mercaptoethanol and 0.1 mM EDTA. After incubation for 2 h at 37°C, the reaction was terminated by the addition of EDTA to a final concentration of 20 mM. Digestion of M13 RF with endoR.*Hind* II, endoR.*Hap* II, endoR.*Hae* II and endoR.*Hae* III was performed under the same reaction conditions as applied for the endoR.*Alu* I enzyme. EndoR.*Hae* II digests, however, were extracted with phenol and the DNA was precipitated with 2 volumes of ethanol. After dissolution, the DNA fragments were subjected to a second digestion with enzyme in order to achieve complete fragmentation.

Electrophoresis of DNA fragments was carried out on a discontinuous slab gel (10 cm x 20 cm x 0.2 cm) consisting of a 3% polyacrylamide gel (26 cm high) above a 10% polyacrylamide gel layer (10 cm high). In the case of analysis of endoR.*Alu* I fragments of M13 RF, a discontinuous slab gel of 3%, 10% and 20% polyacrylamide was used. The gels were prepared in an electrophoresis buffer consisting of 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.8. After electrophoresis, the wet gels were wrapped in Saran wrap and used for radioautography. The conditions for electrophoresis and radioautography have been described previously (21).

For preparative purposes, the relevant portions of the gel, as determined from the radioautographs, were cut out, crushed and extracted twice at room

temperature by shaking with 2 ml of 0.1 x SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate). The combined extracts were centrifuged and the DNA in the supernatant was precipitated with ethanol. The precipitate was recovered by centrifugation (15 min at 30.000 revs/min) and then dissolved in the appropriate incubation buffer or stored at $-20^{o}C$.

The chain lengths of M13 restriction fragments, produced by either endoR. *Hae* II or endoR.*Alu* I, were deduced from radioactivity analyses. ^{32}P-labeled M13 RF was digested with the appropriate enzyme and the fragments were fractionated on a 3% discontinuous polyacrylamide slab gel. The portions of the gel containing the fragments were localized by autoradiography, excised, dissolved in 30% H_2O_2 (27) and counted with Triton X-100-toluene scintillation fluid. The chain lengths were calculated from the percent total radioactivity assuming a molecular weight of 4×10^6 for M13 RF (1).

In case of analysis of the double-digest fragments of M13 RF, the chain lengths were calculated from their relative electrophoretic mobilities by means of a plot relating log molecular size to the distance migrated in the polyacrylamide gel. The chain lengths of the endoR.*Hap* II and endoR.*Hae* III fragments of M13 RF, the sizes of which have been determined previously (21), were used to standardize this plot.

Hybridization of RF DNA fragments to M13 DNA viral strands

Hybridization mixtures (50 µl) contained 0.02 M Tris-HCl, pH 7.6, 0.2 mM EDTA, 0.1 M NaCl, 1 pmole of purified wild type M13 RF DNA fragment and 3 pmoles of circular single-stranded M13 viral DNA. The mixture was heated to $100^{o}C$ for 8 min and incubated for 1 h at $56^{o}C$, followed by an incubation for 1 h at $46^{o}C$.

Fragment-primed DNA synthesis

DNA synthesis was carried out in 0.05 M Tris-HCl, pH 7.6, 0.1 M NaCl, 0.01 M $MgCl_2$ and 0.5 mM EDTA. A standard reaction mixture (100 µl) contained in addition: 80 pmoles of ^{32}P-labeled dCTP (6×10^6 cpm), 3 nmoles each of unlabeled dATP, dGTP, TTP, 0.2 pmole of primer template DNA hybrid and 0.4 units of DNA polymerase I. Label incorporation was allowed to proceed at $20^{o}C$ for 20 min. Thereafter, 3 nmoles of unlabeled dCTP (5 µl) was added to the mixture and the incubation continued for 10-80 min at $33^{o}C$. Synthesis was terminated by the addition of 200 µl phenol which was saturated with 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.6 and 1 mM EDTA. The reaction mixture was extracted twice with phenol and the DNA was precipitated with ethanol.

The DNA precipitate was dissolved in 200 µl of nuclease S_1 buffer consisting of 30 mM sodium acetate, pH 4.6, 40 mM NaCl, 1 mM $ZnSO_4$, 5% glycerol. Unlabeled M13 RF-I (3 µg) was added as an internal marker and subsequently the single-stranded DNA present in the reaction mixture was digested with 1 µl of nuclease S_1 for 30 min at 45°C. (Under the conditions described 1 µl of nuclease S_1 converted 2 µg of M13 viral DNA to an acid-soluble form). The reaction was terminated by the addition of phenol. This was followed by two extractions with phenol, precipitation with ethanol and dissolution of the DNA-synthesis products in 100 µl of enzyme digestion buffer. The products were digested by addition of 20 µl of endoR.*Alu* I and incubation for 2 h at 37°C. The reaction was stopped by the addition of EDTA to a final concentration of 20 mM and the digestion products were further analysed by polyacrylamide gel electrophoresis. After electrophoresis, the gel was stained with ethidiumbromide (2 µg/ml), as described by Sharp *et al.* (23) and then exposed to U.V.-light. The visible bands were cut out of the gel and the radioactivity was measured by Cerenkov-radiation.

Transfection

DNA heteroduplexes for transfection experiments were prepared as described previously (4). Transfection was carried out, using $CaCl_2$-treated cells of *E.coli* C89 (Su^-) following the procedure described by Taketo (24).

RESULTS

Cleavage of M13 RF by endonuclease R.*Alu* I

^{32}P -labeled M13 RF was incubated with varying amounts of endoR.*Alu* I and the digestion products were analysed by electrophoresis on polyacrylamide gels. Autoradiography of these gels showed 14 distinct bands, designated Alu I-A through Alu I-N in order of decreasing fragment size (Fig. 1). Increased enzyme concentration or prolonged incubation did not change this pattern, which indicates that the fragments shown in Fig. 1 represent terminal digestion products.

Comparison of the ^{32}P-label content of each band *versus* the electrophoretic mobility indicated the presence of only one fragment in each band except for band J and N. Band J contained two fragments of nearly equal size, further designated as Alu I-J_1 and Alu I-J_2, while the very small band N contained in fact four fragments. Cleavage of M13 RF by the *Alu* I restriction endonuclease, therefore, yields 18 specific limit fragments.

Table 1. *Size of M13 RF fragments produced by cleavage with endoR.*Alu *I and endoR.*Hae *II*

Alu I fragments	Relative size (% of RF)	Chain length in basepairs	Hae II fragments	Relative size (% of RF)	Chain length in basepairs
A	22.3 ± 0.8	1430	A	56 ± 3	3600
B	21.2 ± 0.7	1360	B	39 ± 1.4	2500
C	9.3 ± 0.4	600	C	5 ± 0.5	320
D	8.9 ± 0.4	570			
E	7.5 ± 0.4	480			
F	5.1 ± 0.3	330			
G	4.7 ± 0.3	305			
H	4.0 ± 0.3	260			
I	3.6 ± 0.2	230			
$J_{1,2}$	6.0 ± 0.4	190			
K	2.5 ± 0.2	160			
L	2.0 ± 0.2	130			
M	1.8 ± 0.3	115			
N*	1.2 ± 0.3	20			

* Band N is actually composed of 4 fragments of about equal size.

^{32}P-labeled M13 RF-I was digested with either endoR.*Alu* I or endoR.*Hae* II as described under Methods, and the digest products were separated on a 3% polyacrylamide discontinuous slab gel. Individual bands were excised and the radioactivity determined (27). Relative sizes were evaluated by expressing the radioactivity values of each band as a fraction of total summed radioactivity. Chain lengths are given in basepairs and were calculated from the percent total radioactivity recovered from the respective gel bands by assuming a total of 6400 basepairs for M13 RF. Figures represent average values obtained from analysis of 4 endoR.*Alu* I and 2 endoR.*Hae* II-fragmented RF preparations.

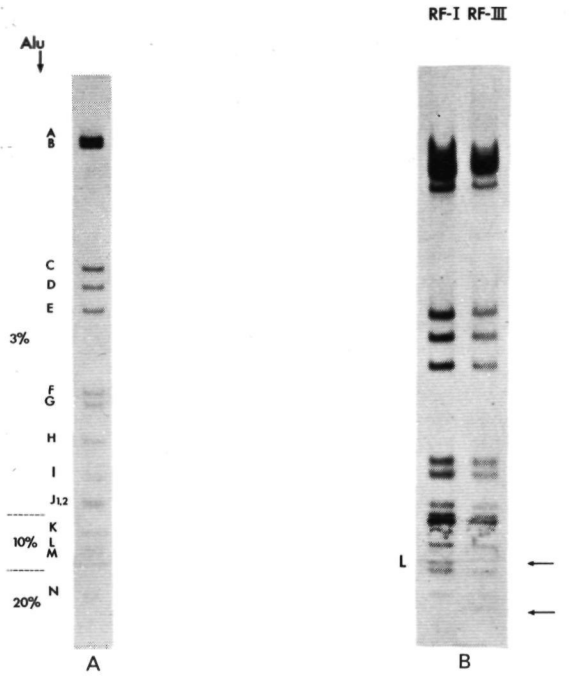

Figure 1A.: *Autoradiograph of the digestion products of ^{32}P-labeled M13 RF, produced by cleavage with endonuclease R.*Alu *I.* ^{32}P-labeled RF (0.04 µg ; 40,000 counts min^{-1}), supplemented with 1.0 µg of unlabeled RF, was incubated for 2 h at 37°C with 2.0 units of endoR.*Alu* I in a final volume of 0.05 ml of 10 mM Tris-HCl, pH 7.6, 7 mM $MgCl_2$, 7 mM mercaptoethanol and 0.1 mM EDTA. Thereafter, the digest was subjected to electrophoresis on a discontinuous polyacrylamide slab gel, containing a layer of 3%, 10% and 20% acrylamide concentration.

Figure 1B.: *Autoradiograph of the endoR.*Alu *I digestion products of covalently closed circular M13 RF-I and of M13 RF, previously split into full-length linear RF-III by predigestion with endoR.*Hind *II.* For preparation of RF digest, ^{32}P-labeled RF (0.07 µg ; 55,000 counts min^{-1}) supplemented with 1.0 µg of carrier RF was incubated for 60 min at 37°C with 2.5 units of endoR.*Hind* II in a volume of 0.05 ml of 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 7 mM $MgCl_2$, 7 mM mercaptoethanol and 0.1 mM EDTA. Thereafter, endoR.*Alu* I was added in excess and the incubation continued for 2 h at 37°C. The endoR.*Alu* I digest of M13 RF-I was prepared as described in Fig. 1A.

The size of the individual endoR.*Alu* I cleavage products of M13 RF was calculated on the basis of the relative ^{32}P-yield of each fragment from uniformly ^{32}P -labeled RF (Table 1). Assuming a total of 6400 basepairs per M13 RF molecule (1), a range of 1430 basepairs for the largest fragment Alu I-A upto about 20 basepairs for the smallest fragments Alu I-N is obtained.

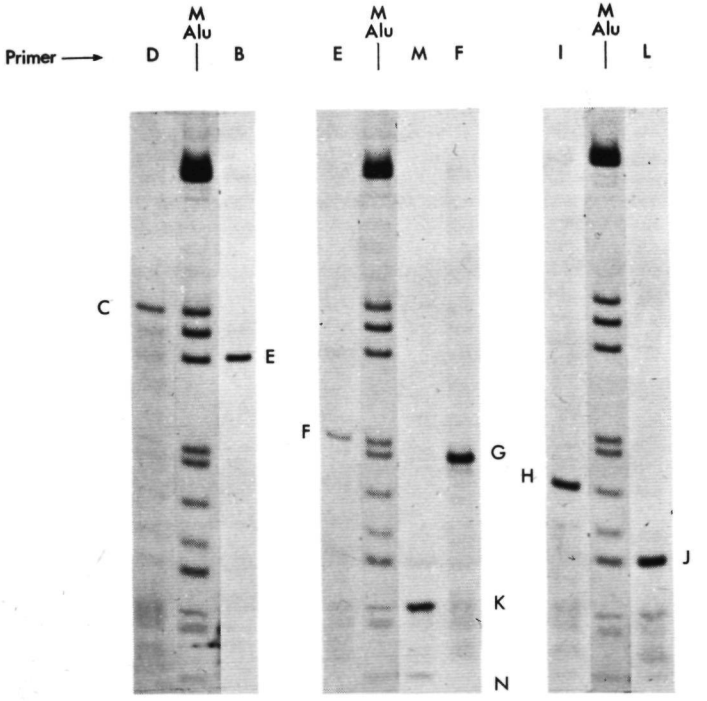

Figure 2.: *Polyacrylamide gel electrophoresis of products of fragment-primed DNA synthesis in which Alu I fragments of M13 RF were used as primers.* DNA synthesis was carried out in 50 mM Tris-HCl, pH 7.6, 0.1 M KCl, 10 mM $MgCl_2$ and 0.5 mM EDTA. Reaction mixtures (0.1 ml) contained in addition: 80 pmoles of (α-^{32}P)-labeled dCTP (40 Ci/mmole), 3 nmoles each of unlabeled dGTP, dATP, TTP, 0.4 units of DNA polymerase I, and primer-template DNA constructed from 0.2 pmole of denatured Alu I fragment which was hybridized to 3 pmoles of circular viral M13 DNA. Label incorporation was allowed to proceed at 20°C for 20 min. Thereafter, 3 nmoles of unlabeled dCTP was added and the incubation continued for 10-80 min at 33°C. Synthesis was terminated by addition of 0.2 ml of phenol and the DNA was recovered as described under Methods. Prior to digestion with endoR.*Alu* I, the DNA was digested with endonuclease S_1 in order to eliminate excess of single-stranded viral DNA. The conditions for digestion with endonuclease S_1 and subsequent digestion with endoR.*Alu* I are described under Methods. The endoR.*Alu* I digestion products were fractionated on discontinuous 3% polyacrylamide slab gels, together with a complete digest of M13 RF, run in parallel, as a marker.

The general approach for ordering the DNA fragments into a physical map was the determination of the neighbour of each DNA fragment using fragment-primed DNA synthesis directed by *E.coli* DNA polymerase I. From this relation among the M13 RF fragments the physical order of fragments could be deduced.

To determine the neighbour of each endoR.*Alu* I fragment,a template-primer DNA hybrid was constructed by denaturing and reannealing each purified fragment in the presence of circular M13 DNA viral strands. By offering ^{32}P -labeled deoxyribonucleoside triphosphates for short pulse-periods, followed by a chase of unlabeled deoxyribonucleoside triphosphates, DNA polymerase was made to synthesize specifically-started DNA chains which were ^{32}P-labeled only in the regions vicinal to the primer. In order to eliminate the excess of single-stranded DNA, which was observed to be strongly inhibitory to endoR.*Alu* I action, the products of DNA synthesis were first digested with nuclease S_1. This was followed by digestion with endoR.*Alu* I enzyme. The digestion products were then subjected to electrophoresis on polyacrylamide gels and analysed by radioautography. The fragments produced from unlabeled M13 RF,which was added to the digest mixture,and ^{32}P-labeled M13 RF DNA fragments, run in parallel, served as internal and external fragment markers, respectively. After radioautography, the gel was stained with ethidium bromide, the appropriate bands were excised and the radioactivity of the newly synthesized neighbour fragments was determined.

Several examples of radioautographic analyses of the newly synthesized neighbour fragments are presented in Figure 2. ^{32}P-labeled DNA, which was primed by fragment Alu I-B, delivered after digestion with endoR.*Alu* I and subsequent separation on polyacrylamide gel a radioactive fragment which has the same mobility as marker fragment Alu I-E. This strongly suggests that Alu-E is the fragment which is preferentially labelled by Alu-B primed DNA synthesis and, hence, argues for a contiguous position of both fragments on the M13 genome. Such sets of contiguous fragments were also obtained by priming with other M13 RF DNA fragments. As is shown in Fig. 2, no radioactivity could be detected in the primer fragment itself. This indicates that the fragment primers used were rather intact and apparently did not contain single-stranded breaks which might cause incorporation of deoxyribonucleoside monophosphates into the primer regions. Nevertheless, a low level of background radioactivity was apparent within each band, especially in the smaller fragment region. This is most probably caused by the relatively high level of ^{32}P-precursor still present after chase which results in a random incorporation of label into newly synthesized DNA chains.

Table 2. *Relative percentage of ^{32}P-radioactivity in endoR.*Alu *I fragments after Alu I fragment-primed DNA synthesis*

Primer	Alu-A	Alu-B	Alu-C	Alu-D	Alu-E	Alu-F	Alu-G	Alu-H	Alu-I	Alu-J	Alu-K	Alu-L	Alu-M	Alu-N
Fragment	% total													
Alu-A	9.3	4.1	4.2	7.0	2.7	3.2	16.3	1.1	1.1	3.2	0.3	1.3	0.3	1.7
Alu-B	6.9	5.8	3.8	6.0	2.6	2.1	5.0	1.2	3.4	10.6	1.2	2.1	1.0	4.7
Alu-C	5.6	3.6	7.2	23.6	5.9	3.9	9.5	2.6	4.5	5.3	2.7	3.5	2.6	6.2
Alu-D	21.2	5.5	5.0	9.4	5.3	3.6	9.4	2.3	3.6	5.1	2.0	2.8	2.3	14.4
Alu-E	18.5	56.6	3.4	8.1	7.2	3.2	7.5	2.7	3.3	5.9	2.5	2.4	2.0	5.7
Alu-F	3.6	3.2	3.5	6.7	26.8	3.7	8.2	1.7	3.6	5.1	2.4	2.6	1.4	6.0
Alu-G	3.3	3.7	4.5	5.7	6.3	44.1	8.9	3.5	4.4	5.6	5.6	3.8	3.5	5.3
Alu-H	2.9	2.4	3.4	5.6	5.2	4.4	8.2	2.8	39.2	6.9	2.6	2.7	2.4	5.3
Alu-I	2.0	2.9	4.2	3.4	6.5	3.8	9.9	5.5	8.6	15.6	2.4	4.0	3.1	12.5
Alu-J	3.5	2.9	3.9	7.2	8.0	5.9	9.7	57.5	5.8	5.6	13.7	48.5	5.1	4.2
Alu-K	1.7	2.2	3.4	5.2	8.7	7.0	2.8	8.1	7.3	7.0	6.8	7.9	51.6	13.8
Alu-L	1.4	2.2	4.0	4.6	7.1	8.5	3.0	4.2	6.4	6.5	37.4	6.9	4.5	10.7
Alu-M	1.6	2.7	44.2	4.5	5.8	4.9	0.9	4.9	6.1	6.4	5.4	5.8	4.2	3.7
Alu-N	18.3	1.7	5.2	2.8	2.3	1.6	0.5	1.6	2.7	11.2	14.9	5.3	15.6	2.4

Individual Alu I fragments were annealed to circular M13 viral DNA strands and the hybrids obtained were used as template-primers for DNA synthesis under the direction of *E.coli* DNA polymerase I in the presence of ^{32}P-labeled deoxyribonucleoside triphosphates. The products of DNA synthesis were cleaved with endoR.*Alu* I and the cleavage products were fractionated on 3% discontinuous polyacrylamide slab gels. The conditions of fragment-primed DNA synthesis, subsequent digestion with endoR.*Alu* I and electrophoretic fractionation of the digestion products are described under Methods. After electrophoresis and autoradiography, the gels were stained with ethidiumbromide (23), the visible bands were excised and the radioactivity content of each band was determined. Relative percentages of ^{32}P-radioactivity were evaluated by expressing the radio-activity of each band as a fraction of the total summed radioactivity.

In order to establish the correct arrangement of the fragments within each set, the respective gel bands were quantitated by calculating the relative percentage of ^{32}P-radioactivity present in each fragment. The radioactivity values determined within each fragment are presented in Table 2. The radioactivity present in fragment Alu I-E after priming with Alu I-B is several orders of magnitude higher than the radioactivity present in the other M13 RF fragments, thereby confirming the results already obtained by autoradiographic analysis. In the same way, priming with Alu I-E leads to a distribution of radioactivity which points to Alu I-F as the fragment which is exclusively labeled by Alu I-E primed DNA synthesis (Fig. 2 and Table 2). Thus, Alu I-F is generated by Alu I-E primed synthesis and both fragments, therefore, encompass a contiguous part of the M13 genome. In the same manner, the contiguous positions of fragments within the other sets could be deduced unambiguously.

Since fragment Alu I-F is generated by Alu I-E primed synthesis, and fragment Alu I-E, in turn, is labeled exclusively after priming with fragment Alu I-B, one has to conclude that the order of fragments is Alu I-B → Alu I-E → Alu I-F in the 5' - 3' direction of the complementary strand. In the same way, all the endoR.*Alu* I fragments of M13 RF could be uniquely arranged and the results are shown in Figure 3. In some cases, ambiguities arose when high percentage values of radioactivity were obtained for two or more fragments in the same slab gel. As is shown in Fig. 2, Alu I-M primed synthesis, for instance, leads to the appearance of two radioactive fragments, namely Alu I-K and Alu I-N. The distribution of radioactivity among the fragments was found to accord with the radioautographic analysis (Table 2). Such ambiguities, however, could be eliminated by taking into account the size of the individual restriction fragments. The relative value of the radioactivity content per fragment size, as calculated for fragment Alu I-K (160 basepairs) and Alu I-N (20 basepairs), is highest for fragment N. This suggests that fragment Alu I-N is completely and fragment Alu I-K is only partially synthesized during the pulse period of Alu I-M primed DNA synthesis. On the basis of such a decline in ^{32}P-incorporation values with increasing distance of the fragments from their respective primer, not only the fragments Alu I-K and Alu I-N but also Alu I-D and N, Alu I-L and N, and Alu I-I and N could be ordered correctly. That this deduced arrangement of the four Alu I-N fragments is correct, is demonstrated by the fact that Alu I-N primed synthesis gives rise to an exclusive labeling of the endoR.*Alu* I fragments D, I, K and L, *i.e.* those fragments which are contiguous to the Alu I-N fragments (Table 2). A second ambiguity is apparent in case of Alu I-A primed DNA synthesis, in which a high level of ^{32}P-incorporation is observed also in fragment Alu I-E (Table 2). The latter synthesis, however, does

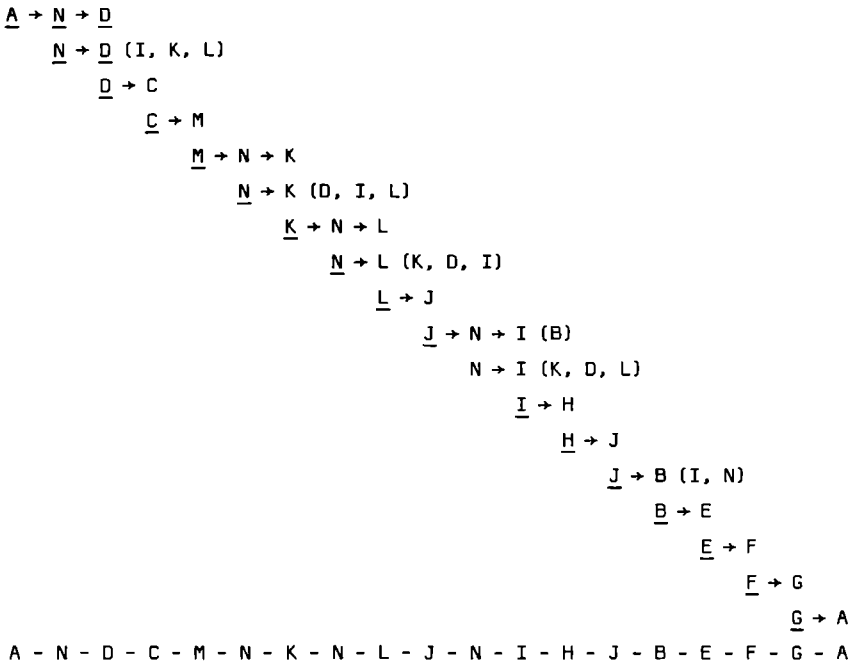

Figure 3.: *Physical order of Alu I fragments of M13 RF as deduced from fragment-primed DNA synthesis.* The order within each group is deduced from autoradiographic analyses and is based further on the decline of the percentage of ^{32}P-label calculated for these fragments (Table 2). The primer-fragments are underlined. Arrows indicate the direction of DNA synthesis (5' - 3' of the complementary strand). Fragment symbols in brackets and separated by a comma denote alternative fragments or alternative fragment orders. The fragment groups have been arranged in overlapping order. The resulting arrangement of all Alu I fragments is shown at the bottom.

not originate from an Alu I-A primed reaction, but is caused by the incomplete separation of fragment Alu I-A and Alu I-B on the preparative gels (see Fig. 1). Primer-templates constructed from such incompletely separated Alu I-A fragments give rise to an additional priming of Alu I-B and, hence, to the appearance of Alu I-E in the Alu I-A primed DNA synthesis. From such results, together with the radioautographic analysis and the data summarized in Table 2, we conclude that the physical order of endoR.*Alu* I fragments of M13 RF is as given in Figure 3.

Physical order of endoR.*Hae* II fragments

We have demonstrated elsewhere (25), that endoR.*Hae* II cleaves M13 RF at three specific sites to yield three fragments which have been named Hae II-A,

Hae II-B and Hae II-C. The size of these fragments was calculated on the basis of the relative ^{32}P-yield of each fragment from uniformly ^{32}P -labeled RF (Table 1). In order to determine the physical order of these endoR.*Hae* II fragments, use was made of the previously constructed endoR.*Hap* II and endoR.*Hae* III maps of M13 RF (21). Such maps served not only as a guide for ordering such fragments but also for the determination of the positions of the respective cleavage sites in the RF molecule. The location of these sites, together with those determined for endoR. *Alu* I, are discussed together in the following section.

Correlation between the endoR.*Alu* I, endoR.*Hae* II, endoR.*Hae* III and endoR.*Hap* II cleavage maps

The restriction enzyme endoR.*Hind* II produces one specific double-stranded break in RF-I of bacteriophage M13, fd and f1 (14, 16, 21, 25, 26). For this reason, the unique Hind II site is generally used as a reference (zero) point on the circular genome. In order to determine this cleavage site within the order of endoR.*Alu* I fragments, M13 RF was digested with the restriction endonuclease and the digest was compared with a similar endoR.*Alu* I digest of RF-I which had been predigested with endoR.*Hind* II. As is demonstrated in Fig. 1, the sole difference between both digests was the absence of fragment Alu I-L in the double-digest mixture and the appearance of a new band which is located beneath Alu I-M. The ^{32}P-content of this new band was twice as high as one should expect from the relationship between single fragments *versus* their electrophoretic mobility. The new band, therefore, is composed of two DNA fragments of equal size. The cleavage site of endoR.*Hind* II, *i.e.* the zero point of the physical map, must be positioned just in the middle of fragment Alu I-L.

A similar approach was used for the location of the zero point within the endoR.*Hae* II cleavage map. From electrophoretic analysis (data not shown) it could be concluded that the Hind II site is located in fragment Hae II-A which then gives rise to the appearance of two new fragments of about 800 and 2800 basepairs in size. Exact positioning of the zero point within the endoR.*Alu* I and endoR. *Hae* II map should theoretically be sufficient for correlating both maps with the other restriction enzyme cleavage maps of M13 RF. On the other hand, the correctness of such a correlation is only proven to be valid if the respective cleavage sites on one map, superimposed on the other (and vice versa), are found indeed in the positions as theoretically expected. Moreover, the results of such analyses will give additional support to the physical order of fragments as deduced from fragment-primed DNA synthesis. In order to verify the endoR.*Alu* I cleavage sites

Table 3. *Redigestion of Hap II fragments and of Hae III fragments of M13 RF with endonuclease R.*Alu *I*

Hap II fragment	size*	products with Alu I	product size	Alu I fragment	size
A	1530	Δ J	150		
		B	1360	B	1360
		Δ E	65		
G	200	Δ E	200	E	490
E_1	450	Δ E	225		
		Δ F	225	F	325
B_1	820	Δ F	100		
		G	305	G	305
		Δ A	430		
H	170	Δ A	170	A	1435
E_2	450	Δ A	450		
B_2	800	Δ A	385		
		N	20	N	20
		Δ D	385		
I_1	140	Δ D	140	D	540
C	650	Δ D	< 20		
		C	600	C	600
		Δ M	45	M	110
D	560	Δ M	65		
		N	20	N	20
		K	160	K	160
		N	20	N	20
		L	130	L	130
		Δ J	155	J	195
J	50	Δ J	40		
		Δ N	< 20	N	20
I_2	140	Δ N	< 20		
		Δ I	130	I	230
F	410	Δ I	100		
		H	260	H	260
		Δ J	55	J	205

Hae III fragment	size*	products with Alu I	product size	Alu I fragment	size
I	70	Δ J	< 20		
		Δ B	50		
H	120	Δ B	120	B	1320
F	190	Δ B	190		
A	2500	Δ B	960		
		E	480	E	480
		F	330	F	330
		G	305	G	305
		Δ A	430		
E_1	290	Δ A	290	A	1420
C	820	Δ A	700		
		N	20	N	20
		Δ D	100	D	550
B	1630	Δ D	450		
		C	600	C	600
		M	115	M	115
		N	20	N	20
		K	160	K	160
		N	20	N	20
		L	130	L	130
		Δ J	155	J	210
D	310	Δ J	55		
		N	20	N	20
		I	230	I	230
G	160	Δ H	160	H	260
E_2	290	Δ H	100		
		Δ J	195	J	210

Purified Hap II or Hae III fragments of ^{32}P-labeled M13 RF were digested with endoR.*Alu* I in excess, and each digest mixture was analysed by electrophoresis on a discontinuous slab gel containing layers of 3%, 10% and 20% acrylamide concentration. Alu I fragments and either Hap II or Hae III fragments, run in parallel, were used as external markers. After radioautography, the sizes of the digestion products were estimated from their relative electrophoretic mobilities by means of a plot relating log molecular size *versus* the distance migrated in the polyacrylamide gel, in which the sizes of the Hap II, Hae III and Alu I fragments were used as a reference. A part of an Alu I fragment is denoted by Δ, *i.e.* Δ E means part of Alu I-E fragment. The sizes denoted with an asterisk have been determined previously and are taken from reference (21).

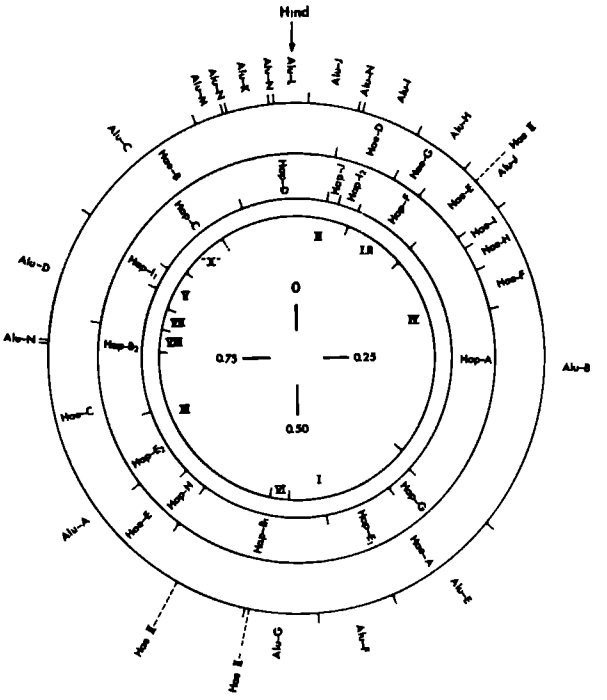

Figure 4.: *Physical maps and genetic map of bacteriophage M13.* The outer circle shows the location of Alu I fragments and the cleavage sites of endonuclease R.*Hind* II (an arrow marked Hind) and endoR.*Hae* II (the broken lines on the outside of the circle). The two middle circles represent the locations of the Hap II and Hae III fragments of M13 RF, the order of which has been determined previously (4). The inner circle represents the M13 genetic map. The order of the genes (designated with Roman numerals) was determined by van den Hondel *et al.* (4) and has been confirmed by Vovis *et al.* (6). The sizes of the genes were estimated from the molecular weight of the *in vitro* gene products (8, 9, 10) with the exception of gene VI and gene VII, whose definite size is unknown. The direction of transcription and translation is counterclockwise (9). The location of gene II, which was still ambiguous (13, 18, 26), has now been determined accurately on the genetic map, as is described in the text. From this position, it is concluded that gene II and gene V are contiguous and that "X" represents a part of gene II in which an internal start of RNA synthesis takes place which then leads to the synthesis of "X-protein". From the position of gene II and the location of gene IV, as determined previously (13), the existence of an intergenic region (indicated I.R.) of about 450 basepairs between gene II and IV is indicated and is so shown in the figure. The I.R. contains the initiation site for replication of M13 parental RF (van den Hondel and Schoenmakers, unpublished results). Both maps are divided into four equal map units with the endoR.*Hind* II cleavage site as a (zero) reference point.

relative to the *Hap* II and *Hae* III cleavage sites, ^{32}P -labeled M13 RF was digested with restriction enzyme, and the respective endoR.*Hap* II fragments and endoR.*Hae* III fragments were isolated by polyacrylamide gel electrophoresis. Each purified fragment was then digested with endoR.*Alu* I and the digest mixture was analysed on the discontinuous polyacrylamide slab gel. From the estimated lengths of the various double-digest fragments on the radioautographs, the positions of the endoR.*Alu* I cleavage sites relative to the other enzyme cleavage siteswere determined. The results of these analyses are summarized in Table 3. From these data it is concluded that, upon digestion with endoR.*Alu* I, fragment Hap II-A is split up into three further fragments, one of which is identical to Alu I-B. Hence, the other two fragments, the sizes of which have been estimated to be 150 and 65 basepairs, respectively, constitute overlaps with the respective neighbours of fragment Alu I-B, *i.e.* Alu I-J and Alu I-E (Fig. 3). The fragment which is contiguous to Hap II-A, namely Hap II-G, is not split by endoR.*Alu* I, but the next fragment, Hap II-E_1, is cleaved into two fragments of 225 basepairs long (Table 3). Therefore, the endoR.*Alu* I cleavage site which gives rise to the formation of Alu I-E, *i.e.* the fragment which is contiguous to Alu I-B, must be positioned in fragment Hap II-E_1, 225 basepairs distant from the *Hap* II cleavage site between fragment Hap II-E_1 and Hap II-G.

In the same way, the positions of the other endoR.*Alu* I cleavage sites have been determined. From this, a correlation of the endoR.*Alu* I map of M13 RF with the endoR.*Hap* II map could be deduced, which is shown in Fig. 4.

Analyses were also made of the products which were formed after digestion of the individual endoR.*Hae* III fragments with endoR.*Alu* I (Table 3). The data obtained confirmed not only the physical order of M13 RF fragments as found by primed DNA synthesis but also gave additional support for the correct positions of the endoR.*Alu* I cleavage sites within the Hap II and the Hae III cleavage maps of M13 RF (Fig. 4).

Positioning of the three endoR.*Hae* II cleavage sites in M13 RF was achieved using a similar approach. The individual endoR.*Hae* II fragments were digested with either endoR.*Hap* II or endoR.*Hae* III and the double-digest products were analysed on polyacrylamide gels. The results of such analyses are shown in Fig. 5 and are summarized in Table 4. Upon treatment with endoR.*Hap* II, the smallest fragment Hae II-C (320 basepairs) is not split. Hae II-B produced four fragments, namely Hap II-E_1, Hap II-G and two new fragments, the sizes of which are 1500 and 320 basepairs, respectively. The largest new fragment must be derived from fragment Hap II-A (1530 basepairs). This follows from the fact that Hap II-A is contiguous to Hap II-G and this large fragment is the only one which can produce a double-

Table 4. *Redigestion of Hae II fragments of M13 RF with endoR.* Hap *II and endoR.* Hae *III*

Hae II fragment	size	Hap II fragments detected	sum of Hap II fragments	overlap fragments
A	3600	B_2,C,D,E_2,F,H,I_1,I_2,J.	3380	Δ A (170)
				Δ A (30)
B	2500	E_1,G.	660	Δ B (1500)
				Δ B (320)
C	320	no cleavage		

Hae II fragment	size	Hae III fragments detected	sum of Hae III fragments	overlap fragments
A	3600	B,C,D,E,G.	3210	Δ A (190)
				Δ A (190)
B	2500	F,H,I.	380	Δ B (2000)
				Δ B (170)
C	320	no cleavage		

Purified Hae II fragments of ^{32}P-labeled M13 RF were digested with the appropriate enzyme, and the digestion mixture was analysed on a 3% discontinuous polyacrylamide gel. The conditions for isolation of fragments, digestion and electrophoresis are described under Methods. The presence of overlap fragments was detected by autoradiographic comparison of the double-digest products of the Hae II fragments with an endoR.*Hap* II-or endoR.*Hae* III-fragmented RF preparation as markers. The size of the overlap fragments, expressed in basepairs, were estimated graphically from a plot relating log molecular size *versus* mobility, in which the Hap II or Hae III fragments were used to standardize this plot. Overlap fragments were denoted as part of Hae II fragments, *i.e.* Δ A means part of Hae II-A fragment. The sizes of Hap II, Hae III and Hae II fragments were taken from reference (21) and Table 1.

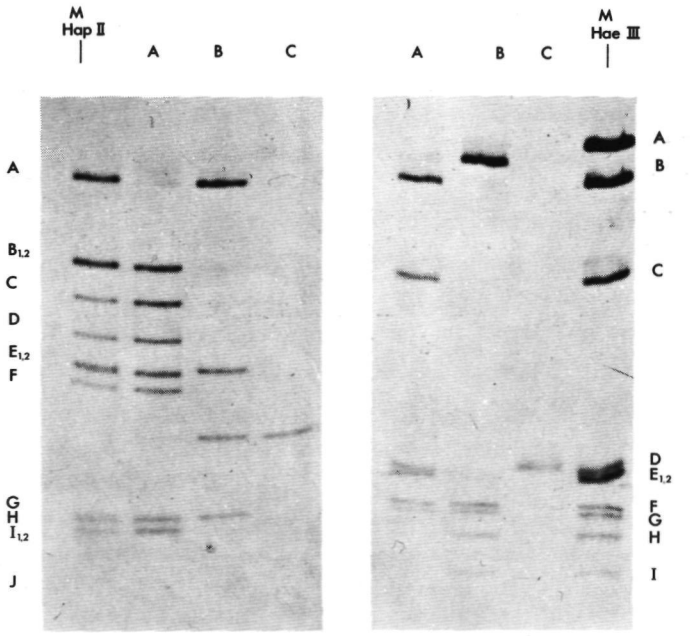

Figure 5.: *Electrophoretic analysis of the cleavage products of Hae II fragments after digestion with endonuclease R.* Hap *II and endonuclease R.* Hae *III.* Individual Hae II fragments of ^{32}P-labeled M13 RF were isolated from polyacrylamide gels and subsequently digested in 0.05 ml of 10 mM Tris-HCl, pH 7.6, 7 mM $MgCl_2$, 7 mM mercaptoethanol with either 1.5 units of endoR.*Hap* II or 0.8 units of endoR.*Hae* III for 120 min at 37°C. In both types of analysis, the digest products were electrophoresed in a 3% discontinuous polyacrylamide gel with either a complete endoR.*Hap* II digest ($M_{Hap\ II}$) or an endoR.*Hae* III digest ($M_{Hae\ III}$) of M13 RF as fragment markers. Each Hae II fragment digested is designated on top of this figure, the Hap II and Hae III fragments are indicated vertically along the autoradiograph.

digest product of 1500 basepairs. Therefore, one endoR.*Hae* II cleavage site must be present in Hap II-A at 30 nucleotides distant from its terminal end. A second cleavage site must be located in Hap II-B_1, *i.e.* the fragment which is contiguous to Hap II-E_1, at a distance of 320 basepairs from its terminal end. As is shown in Figure 5, the large fragment Hae II-A gives rise to the Hap II fragments B_2, C, D, E_2, F, H, I_1, I_2 and J, and an overlap fragment of about 30 basepairs which is apparent as a faint band just beneath the position of Hap II-J. We have concluded that the latter product is the small terminal fragment which arises upon endoR. *Hap* II cleavage of fragment Hae II-A. The ^{32}P-radioactivity content of the band containing fragment Hap II-H was found about twice as high as one should expect on the basis of a single fragment-containing band. Apparently, this band contained fragment Hap II-H and an overlap fragment, the size of which is 170 basepairs.

Table 5. *Rescue of amber mutations by restriction fragments of M13 RF*

Fragment	I	II			III			VI				VII	
	1H7	2H2	R86	R124	3H1	3H4	3H5	6H1	6H2	6H6	6H7	7H1	7H3
Alu I-A	0	0	0	0	46	73	47	0	0	0	0	0	0
Alu I-D	0	0	0	0	0	0	0	0	0	0	0	35	42
Alu I-G	35	0	0	0	0	0	0	55	23	27	24	0	0
Alu I-K	0	0	25	0	0	0	0	0	0	0	0	0	0
Alu I-L	0	0	0	17	0	0	0	0	0	0	0	0	0
Alu I-M	0	0	0	0	0	0	0	0	0	0	0	0	0
Hap II-B1	118	-	-	-	0	0	0	110	135	117	209	-	-
Hap II-B2	-	-	-	-	0	0	0	-	-	-	-	37	53
Hap II-E2	-	-	-	-	23	37	0	-	-	-	-	-	-
Hap II-H	-	-	-	-	0	0	27	0	0	0	0	-	-
Hap II-D.Hin1	-	42	47	0	-	-	-	-	-	-	-	-	-
Hap II-D.Hin2	-	0	0	33	-	-	-	-	-	-	-	-	-
Hae III-A	135	-	-	-	0	0	0	153	167	212	193	-	-
Hae III-B	-	83	92	102	-	-	-	-	-	-	-	39	67
Hae III-C	-	-	-	-	17	21	0	-	-	-	-	0	0
Hae III-E	-	-	-	-	0	0	36	0	0	0	0	-	-

A suspension of $CaCl_2$-treated cells of *E.coli* C89 (Su^-) was transfected with amber mutant viral DNA to which denaturated wild type M13 RF fragments were hybridized. The transfection mixtures were plated together with *E.coli* C89 as indicator strain and the plates were scored for infective centers. Approximately 1.5×10^{10} molecules of DNA fragments were used per plate. Figures represent the number of plaques scored per plate. The Roman numerals refer to the M13 genes in which the amber mutation is located, 1H7 etc. to the mutant.

Since Hae II-C (320 basepairs) is not split by endoR.*Hap* II, and the sum of the latter and two overlap fragments equals to the size of fragment Hap II-B_1 (Table 4), the most simple explanation is that Hae II-C is positioned in Hap II-B_1 between one overlap fragment of 170 basepairs on one side and a 320 basepairs-long fragment on the other.

An identical conclusion could be drawn from the analyses of the digestion products of Hae II fragments by endoR.*Hae* III (Figure 5). The small Hae II-C fragment was found to encompassed by fragment Hae III-A, whereas fragment Hae II-A and Hae II-B covered the Hae III fragments as summarized in Table 4. Fragment Hae III-A and one of the doublet fragments Hae III-E were the only fragments which were split. This confirmed the presence of two endoR.*Hae* II cleavage sites in fragments Hae III-A and a third one in Hae III-E between the endoR.*Hae* II fragments A and B. The endoR.*Hae* II cleavage map of M13 RF which has been constructed from these analyses, is presented in Fig. 4.

Marker rescue of amber mutations

Previously, we have determined which of the individual Hap II and Hae III fragments are capable of rescuing genetic markers in the eight genes of phage M13 (4). In this study, the individual endoR.*Alu* I fragments were tested for salvaging activity. In addition, a number of additional amber mutants of M13 and f1 were used now for such experiments.

In order to determine which genetic sites are contained in each fragment, the endoR.*Alu* I fragments obtained from wild type M13 RF were isolated from a preparative discontinuous polyacrylamide slab gel. After extraction and further purification (4), each fragment was denatured by heating together with single-stranded viral DNA of various amber mutants and subsequently annealed to form partial duplex DNA molecules. These partial duplex molecules were then used to transfect Su^-*E.coli* cells which were made permeable to exogenous DNA by pretreatment with $CaCl_2$ (24). One can expect production of wild type phage and, hence, formation of plaques on the Su^- bacterial lawn, only when the fragment covers the site of the amber mutation in the viral DNA strand (28, 29).

As shown in Table 5 the M13 mutant 1H7, which is an amber mutation in gene I, is rescued not only by fragment Hae III-A and Hap II-B_1 as was found previously (4), but is also rescued by fragment Alu I-G. Interestingly, the amber mutants 6H1, 6H2, 6H6 and 6H7 (all mutants in gene VI) are rescued by fragment Alu I-G also. From this, the assignment of the position of these genetic markers in gene I and gene VI can now been given more accurately.

The M13 amber mutant 2H2 and the f1 mutants R86 and R124 (all mutants of gene II) were rescued by fragment Hap II-D and, more precisely, the mutants 2H2 and R86 by fragment Hap II-D.Hind-1, and the mutant R124 by fragment Hap II-D.Hind-2. The latter fragments are the Hind II cleavage products of Hap II-D which are located to the left-hand and right-hand side of the Hind II cleavage site (see Fig. 4). Mutant R86 is also rescued by fragment Alu I-K, whereas R124 is covered by fragment Alu I-L. Amber mutant 2H2, however, was not rescued by either fragment Alu I-M, Alu I-K or Alu I-L although one should expect that this particular region of Alu I fragments should cover this mutation. Since two small Alu I-N fragments of only 20 basepairs long are positioned between the fragments Alu I-K and L and Alu I-K and M, it is very probable that one of these small N-fragments contains the wild type allele of the amber mutation in 2H2.

It has to be emphasized that R124 is covered not only by Hap II-D.Hind-2 but also by Alu I-L, *i.e.* that endoR.*Alu* I fragment which contains the Hind II cleavage site. From these combined results, we now conclude that the R124 mutation is located within a fragment only 65 bases in length, namely in fragment Alu I-L.Hind-2. This accurate pinpointing of some genetic markers in gene II, and especially R124, enables us now to localize gene II quite accurately within the genetic map. The molecular weight of the polypeptide encoded by gene II is 46,000 (9, 10). RF, derived from R124 mutant phage, when added as a template in a "coupled" transcription-translation system, gives rise to the synthesis of an "amber protein fragment", the molecular weight of which is 11,000 (10). Emphasizing that the order of transcription and translation is counterclockwise around the genetic map, and given these estimations, together with the position of the R124 mutant marker on the physical maps, leads to an exact positioning of gene II as indicated in Fig. 4. The existence of an intergenic region located between gene II and gene IV, which is concluded from this, will be discussed later in the context of the origin of M13 RF replication.

As shown in Table 5, the M13 amber mutants 3H1, 3H4 and 3H5 (mutations in gene III) were all covered by fragment Alu I-A. A more precise location of these amber mutants was derived from marker rescue experiments of Hap II fragments and Hae III fragments which are encompassed by fragment Alu I-A. The mutants 3H1 and 3H4 were covered both by Hap II-E_2 and Hae III-C, which pinpoints these mutant sites between 0.63-0.70 map units on the physical maps (see Fig. 4). Mutant 3H5 was rescued by both fragments Hae III-E and Hap II-H. This limits the position of this mutant to a region corresponding to map position 0.61-0.63. By analogy, genetic markers of gene VII were found to be covered only by those fragments which have the 0.78-0.82 region (*i.e.* the overlap between Hap II-B_2

and Hae III-B) in common. Mutants of gene IV, V and VIII have not been tested in this study. The positions of these genes have already been determined, rather accurately, on the basis of the results of marker rescue experiments and from protein biosynthesis studies in which restriction fragments were used as templates (4, 9, 13).

Correlating these results with the endoR.*Alu* map shown in Fig. 4 and the endoR.*Hae* III and endoR.*Hap* II restriction mapping and transfection experiments, reported earlier (4, 21), one can deduce the M13 genetic map as shown in Fig. 4. In constructing this map, use was made of the fact that the molecular weights of six M13 gene products are known yet (7-12). In addition, the molecular weights of the protein fragments which are formed by premature termination at the amber mutant sites have been determined (9, 10). If one assumes that the molecular weights of the products are proportional to the sizes of genes, and that the sizes of the amber-protein fragments are a justified estimate of the distance between the mutant site and the N-terminal end of the gene, then one can calculate their approximate lengths in nucleotides or map units. The genetic map shown in Fig. 4 is a schematic summary of our results and accounts for our understanding of gene lengths and their positions in relation to the various M13 RF cleavage maps.

DISCUSSION

The present paper presents a physical map of the genome of bacteriophage M13, constructed of fragments produced by two restriction endonucleases, namely endoR.*Hae* II from *H.aegyptius* and endoR.*Alu* I from *A.luteus*. The methodology we have applied for ordering the endoR.*Alu* I fragments is based on DNA synthesis in which the fragments were used as primers for the synthesis of their respective neighbours. Each fragment applied in this way gave rise to a pronounced incorporation of ^{32}P-radioactivity which was found to be present only in regions vicinal to the primer. When the products of DNA synthesis were cleaved by endoR. *Alu* I and subsequently analysed on polyacrylamide gels, there was in each case a unique fragment which consistently showed a higher specific ^{32}P-radioactivity than any other fragment. On the basis of such an analysis the neighbourhood relationships among the M13 RF fragments could be established from which, in turn, the physical order of fragments could be constructed. Once the physical order had been determined, a correlation was made between the Alu I map and several other cleavage maps of M13 RF on the basis of overlapping sets of fragments arising upon digestion of the individual DNA fragments with a second restriction endo-

nuclease. The latter principle was followed also for ordering the endoR.*Hae* II fragments of M13 RF into a physical map.

The order of endoR.*Hae* II fragments as reported in this study, appears to be identical to the one reported by Horiuchi *et al.* (26) for phage f1. We recently observed that this given order of fragments is also valid for phage ZJ/2 (25), but not for fd (25, *cf.* 16). In addition, we have demonstrated that the physical order of endoR.*Alu* I fragments of M13, f1 and ZJ/2 is identical, but deviates from the one found for fd (25).

The physical dissection and mapping of the M13 genome, as presented in Fig.4, is an extremely useful tool for detailed studies of DNA replication, gene expression and base sequencing of this phage. Recent studies in our laboratory, in which the individual Hap II fragments and Hae III fragments were used as template for an *in vitro* transcription-translation system, have shown that Hap II-A directs the synthesis of gene IV protein, that Hap II-C directs the synthesis of X-protein and that Hap II-B_2 directs the synthesis of the protein encoded by gene VIII. Fragment Hae III-B directs the synthesis of both gene V protein and X-protein but not of gene VIII protein (9, 13 *cf.* 6). These results are consistent with the map shown in Fig. 4. Moreover, the capacity of the fragments in functioning as proper templates in the DNA-dependent protein-synthesizing system accords with the existence of an RNA promoter on these fragments as recently deduced from our *in vitro* transcription studies (17, 30, *cf.* 14, 15, 18).

An interesting aspect is the existence of X-protein. Previously, we demonstrated (13) that the DNA fragments which code for this hitherto unknown protein were located proximally to gene V (*i.e.* on Hap II-C). To accord with the genetic map, a position of the "X-protein gene" between gene V and gene II was suggested. On the other hand, the occurrence of this protein as the result of an internal start of RNA synthesis within gene II could not be excluded. To clarify this point, we have made use now of the physical order of endoR.*Alu* I fragments of M13 RF as reported in this study. As is shown in Fig. 4, the vast majority of small endoR.*Alu* I fragments are grouped around the unique Hind II site. Previously we have demonstrated, both by marker rescue experiments (4) and by protein synthesis studies under the direction of the linear RF-III DNA as a template (9), that the Hind II site is located in gene II. The present data confirm this observation and have clearly shown that the Hind II site is located between the R86 and the R124 mutant sites (Table 5). The position of the R124 mutation was found immediately to the right of the Hind II site at a distance of less than about 60 basepairs from this site. Model and Zinder (10) have shown that RF-I, prepared from cells infected with phage carrying the amber mutation

R124, gives rise to the synthesis of a prematurely terminated protein fragment, the molecular weight of which is 11,000 only. On the assumption that sizes of gene products are proportional to the sizes of genes or gene sections and taking into account the position of the R124 mutation, one can calculate that the N-terminus of gene II must be located at map position 0.06 (see Fig. 4). An identical starting position for gene II was estimated on the basis of the results obtained with the R86 and 2H2 mutant markers. Their approximate positions on the cleavage maps (Table 5), together with the estimated length of their corresponding amber-protein fragments of 18,000 (10) and 22,000 (9), respectively, point as well to the same N-terminal position already deduced from the results obtained with R124.

If it is emphasized that the entire gene II codes for a protein, the molecular weight of which is 46,000 (9, 10), one finds the C-terminal end of gene II at map position 0.84. As a consequence of this, it can be concluded that gene II and gene V are contiguous. The latter conclusion is additionally supported by nucleotide sequence analysis. The nucleotide sequence of the region encompassing the endoR.*Hap* II site at map position 0.81 has been determined recently (Takanami, personal communication). Since the nucleotide sequence at this particular site corresponds exactly with the known amino acid sequence of the C-terminal end of gene V protein, we conclude that the 5' end of gene V itself is very near to this endoR.*Hap* II cleavage site. Gene V protein is only 87 amino acids long (12) and is not synthesized in a precursor form as has been shown to occur with the proteins encoded by gene III and gene VIII (9, Konings, unpublished results). Therefore, the starting position of gene V is fixed at around position 0.85, *i.e.* at the C-terminal end of gene II. The existence of a new M13 gene which is located between gene II and gene V can therefore be excluded.

Taking this into account, one has to conclude inevitably that X-protein is a translational product of gene II. One of the mechanisms which have been considered to be responsible for the occurrence of X-protein, is a translational re-initiation event during the synthesis of gene II protein. Such a phenomenon, although reported to occur in the mRNA of the *lac i* gene (31) and of the A-protein gene of phage ϕX-174 (32, 33), is not in accordance with our results obtained from *in vitro* transcription and translation studies. The mere fact that only a part of gene II, namely fragment Hap II-C, is able to direct the synthesis of X-protein already excludes a re-initiation phenomenon at the level of translation. A more plausible explanation, therefore, is the occurrence of a second transcriptional initiation within the gene II backbone, giving rise to a mRNA

which codes for the C-terminal part of gene II protein. Our recent observations of the presence of two RNA-initiation sites in fragment Hap II-C, namely one in front of gene V and the other within gene II (17, 30), are in favour for such an intergenic transcriptional initiation phenomenon. Strong support was also obtained from the results of our *in vitro* transcription studies in which M13 RF-I was used as a template. Among the RNA species synthesized, there is one mRNA which has the coding capacity for the synthesis of gene VIII-, gene V- and X-protein but not for the synthesis of gene II protein (Edens *et al.*, unpublished data). Whether this intragenic transcription reflects particular function(s) of this gene II region remains to be ascertained.

A second point of interest is biased to the region which is located proximally to gene II. Previously we have demonstrated that fragment Hap II-A contains the entire information required to code for gene IV protein (9, 13). From the estimated molecular weight of this protein, namely 48,000, and the size of fragment Hap II-A (1530 basepairs), it is calculated that the C-terminal end of gene IV is located at the extreme end of this fragment, namely at about map position 0.13. Since the starting position of the next gene, *i.e.* gene II, is at about 0.05 map units, we conclude from this that there exists within the M13 genome an intergenic space of considerable length (about 450-500 basepairs) between gene II and gene IV. During the course of this work, a similar intergenic region has been noted to occur in phage f1 (6).

The function of such a region is not yet clear but evidence is accumulating that this region is involved in DNA replication. Tabak *et al.* (34) and Griffith and Kornberg (35) have produced strong evidence that the origin of conversion of single-stranded M13 DNA into RF *in vitro* is located from 0.05 to 0.10 map units from the Hind II locus. An identical conclusion, though reached by a different approach, was obtained recently by Schaller and co-workers (personal communication). These authors have shown clearly that in the presence of *E.coli* unwinding protein the binding of RNA polymerase to single-stranded fd viral DNA is confined to a region encompassing fragment Hap II-F only. Both these data confirm a position of the origin of ssDNA → RF within this intergenic region.

We now have strong evidence that the same region also contains the origin of replication of parental RF to progeny RF. Using a methodology already applied by Danna and Nathans on Simian virus-40 DNA (36), we have demonstrated that short-time pulse labeling experiments, carried out on M13-infected cells after removal of chloramphenicol, resulted in ^{3}H-labeled RF-I molecules which carried the label only in regions which were vicinal to or part of the intergenic region (van den Hondel and Schoenmakers, unpublished data).

The exact location of the origin(s) of phage DNA replication, together with the availability of DNA restriction fragments containing this regulatory region, will afford further studies on DNA replication in relation to DNA structure.

ABBREVIATIONS AND ENZYMES

Abbreviations : Hap II, Hind II, Hae II, Hae III and Alu I refer to DNA fragments produced by the restriction endonucleases from *Haemophilus aphirophilus*, *Haemophilus influenzae* Rd, *Haemophilus aegyptius* and *Arthrobacter luteus*. RF, replicative form DNA; RF-I, double-stranded circular replicative form I with both strands covalently closed.

Enzymes : DNA polymerase from *Escherichia coli* (EC 2.7.7.7). Nuclease S1, single-stranded nucleate endonuclease from *Aspergillus oryzae* (EC 3.1.4.21).

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CHAPTER VI

ORIGIN AND DIRECTION OF THE COMPLEMENTARY STRAND SYNTHESIS OF M13 DNA REPLICATION

SUMMARY

Replicative form I DNA of phage M13, isolated shortly after the start of the replicative form DNA replication, is primarily labeled in its complementary (non-viral) strand. Analysis of the distribution of label in the fragments, produced by the digestion of this replicative form (RF-I) DNA with the restriction endonucleases from *Haemophilus aphrophilus* (endoR.*Hap* II) and *Haemophilus aegyptius* (endoR.*Hae* III), reveals that the synthesis of the complementary strand is unidirectional and clockwise around the genetic map published by C.A.van den Hondel, A. Weyers, R. N. H. Konings, and J. G. G. Schoenmakers ({1975} Eur. J. Biochem., *53*, 559-567). Furthermore from these data, it could be concluded that the origin of the complementary strand synthesis during replicative form DNA replication is located in the same region on the M13 genome as the origin of the *in vitro* synthesis of the complementary strand of the parental replicative form DNA. Both replication origins are located in the intergenic region between gene IV and II.

INTRODUCTION

The F-specific filamentous coliphage M13 contains a circular single-stranded (SS) DNA genome (mol. wt. ca. $2x10^6$) (for a review, see {1, 2}). Upon infection, this SS DNA is rapidly converted into a double-stranded "parental" replicative form (pRF-I) molecule. For this process an unknown number of host cell functions in addition to the M13-specific protein encoded by gene III are required (3, 4).

The replication of the parental RF-I DNA, which is initiated by gene II action, is likely to occur by a "rolling circle" mechanism proposed by Gilbert and Dressler (5-9). According to this model, a 3' end is generated in the viral strand by a specific endonucleolytic nick. This 3' end is elongated resulting in the displacement of the "parental" viral strand. When a "tail" of genome length of the "parental" viral strand is exposed, the synthesis of the complementary strand is initiated. Finally the linear double-stranded "tail" is cut from the "rolling circle" intermediate, circularized and converted into RF-I DNA. In this manner a pool of progeny RF-I molecules is formed.

About 20 min after phage infection double-stranded DNA synthesis ceases almost completely, due to the action of the phage specific protein encoded by

gene V (10-14). This protein, a DNA-binding protein, prevents the conversion of the newly formed SS DNA molecules into RF molecules by binding co-operatively to the nascent single-stranded "tails" and thereby preventing the initiation of the synthesis of the complementary strand (15-20).

In vitro studies have indicated that the origin for the conversion of M13 viral SS DNA into pRF DNA is located at a unique site on the M13 genome (23, 24). This site is most probably located within the "intergenic region" between gene IV and gene II (25). This region, which possesses the characteristics of double-stranded DNA, codes for the RNA primer required for the initiation of the complementary strand synthesis (21-24). The *in vivo* origin of both the pRF formation and the RF replication is not known.

In the present study we have examined double-stranded DNA synthesis and have determined the origin of the complementary strand synthesis by using the method applied by Danna and Nathans for the determination of the origin of replication of the virus SV40 (26): DNA molecules are pulse labeled during DNA replication, followed by isolation of DNA molecules which have been completed during the labeling time. Digestion of these molecules with restriction endonucleases and subsequent determination of the relative specific radioactivity of the restriction fragments, make it possible to determine the origin and direction of DNA replication from the gradient of relative specific activity in the DNA fragments. By following this strategy,we have found that the origin of the complementary strand synthesis during double-stranded M13 DNA replication is located in the "intergenic region" between gene IV and gene II. Furthermore, evidence was obtained indicating that the direction of the complementary strand synthesis is unidirectional and clockwise around the M13 genetic map (see Fig. 5).

MATERIALS AND METHODS

Bacteria and bacteriophages

Escherichia coli C59 (F^+, thy^-, met^-, his^-, RC^{rel}) was obtained from Dr. H. van der Putten (Rijswijk). *E.coli* K37 (Su-1), the permissive host for M13 amber mutant phages, and *E.coli* K38, the non-permissive host, were donated by Dr. D. Pratt.

Phage M13 (wt) was obtained from Dr. P. Hofschneider. The M13 amber mutant *am*2-H2, the isolation and characteristics of which have been described (27-29), was a gift from Dr. D. Pratt.

Medium

Cells were grown under aeration at 37°C in TPA-medium supplemented with 1 µg/ml thymine (30).

Isolation of labeled {^{3}H} RF-I DNA

To label RF-I molecules immediately after the onset of the RF replication, *E.coli* C59 was grown in 300 ml TPA-medium at 37°C to a density of $5x10^8$ cells/ml, then 150 µg/ml chloramphenicol (Sigma) was added and 5 min later the cells were infected with M13 (wt)(m.o.i.=100). 25 min after infection, the cells were harvested by centrifugation, washed three times with ice-cold TPA-medium lacking glucose and thymine and suspended in 300 ml of the same medium at 18°C. Subsequently 2.4 ml 50% glucose and 0.25 ml thymine (1 mg/ml) were added, immediately followed by addition of 5 mCi {^{3}H} thymidine (17 Ci/mmole). 1, 3 or 5 min later 100-ml samples were removed and poured quickly into a 300 ml Erlenmeyer flask placed in a water-ice-bath and containing crushed ice and 9 ml of 0.5 M KCN, 3 mM sodium azide. By shaking the Erlenmeyer flask vigorously the temperature was reduced below 2°C in less than 30 sec. The cells were harvested by centrifugation (15,000 revs/min in an I.E.C. B20 rotor), washed once with cold KCN-buffer (0.05 M Tris-HCl, pH 8.0, 0.1 M NaCl, 0.005 M EDTA, 0.02 M KCN) and suspended in 16 ml KCN-buffer at 0°C. After the addition of lysozyme (200 µg/ml) the mixture was incubated at 0°C for 60 min. The subsequent sodium dodecyl sulfate precipitation, phenol extraction and RNAse treatment were performed as before, whereafter RF-I and RF-II were separated by CsCl/ethidiumbromide centrifugation as described previously (31, 35).

To determine the amount of synthesis of pRF DNA in cells after chloramphenicol treatment, *E.coli* C59 cells, infected with M13 (wt) or M13 *am*2-H2, were labeled in the same way as described above. After harvesting and washing, the cells were lysed according to a modification of the lysozyme-sarkosyl method (11): the cells were suspended in 4 ml KCN-buffer and incubated with lysozyme (200 µg/ml) for 60 min at 0°C. Sarkosyl was then added to a concentration of 0.5% and the mixture was incubated for 15 min at 37°C. The viscous lysate was layered on a 30 ml 5% to 20% sucrose gradient (in 1.0 M NaCl, 0.01 M Tris-HCl, pH 7.6, 0.001 M EDTA), made on a 2 ml pad of 65% sucrose and centrifuged in an I.E.C. SB 110 rotor at 24,000 revs/min at 2°C for 19 h. The gradient was fractionated by pumping 70% sucrose into the bottom of the tube and collecting 1 ml fractions from the top of the gradient. The fractions were assayed for radioactivity by counting in Triton X-100-toluene scintillation fluid in a Packard liquid scintillation counter.

Preparation of DNA

The methods for the preparation of uniformly-labeled {^{3}H} or {^{32}P} RF-I DNA have been described (31, 32). Propagation and purification of M13 (wt) and M13 *am*2 phages were performed as before (33). The methods for the isolation of viral DNA and of {^{32}P}-labeled restriction fragments have been described (33, 34).

Restriction endonucleases

EndoR.*Hind* II/III, endoR.*Hae* III and endoR.*Hap* II from *Haemophilus influenzae* strain Rd, *Haemophilus aegyptius* and *Haemophilus aphrophilus* respectively were isolated as described before (32, 35).

Digestion of M13 RF-I DNA with restriction endonucleases and polyacrylamide gel electrophoresis of the DNA fragments

The methods for cleavage of RF-I DNA with the restriction endonucleases endoR.*Hind* II, endoR.*Hap* II and endoR.*Hae* III have been described (31). After digestion, the DNA fragments were separated by polyacrylamide gel electrophoresis (31).

For quantitation of the radioactivity in each DNA fragment, the {^{3}H}-labeled DNA bands were first located by fluorography (36). The segments corresponding to these bands were excised and the {^{3}H}-content of each band was determined by combusting the gel slices in a Packard Model 300 Tri-Carb Sample Oxidizer. The samples were then counted in Triton X-100-toluene scintillation fluid. Control experiments with uniformly-labeled {^{3}H} RF-I DNA have shown that with this method 95 - 100% recovery of the {^{3}H}-counts was obtained.

Base analysis of purified {^{32}P}-labeled complementary strands of M13 restriction fragments

To isolate {^{32}P}-labeled complementary strands of M13 restriction fragments, each {^{32}P}-labeled fragment was separately mixed with a 50-fold excess of unlabeled M13 viral SS DNA. Subsequently the complementary strand was annealed to M13 SS DNA by heating the mixture for 10 min at 100°C, followed by incubation for 2 h at 56°C. Annealing was carried out in 0.05 M Tris-HCl, pH 7.6, 0.1 M NaCl, 0.001 M EDTA. The hybridized complementary strands were then separated from the non-hybridized strands by sedimentation on a 5% to 20% neutral sucrose gradient (1 M NaCl, 0.01

M Tris-HCl, pH 7.6, 0.001 M EDTA) in a Spinco SW56 rotor at 56,000 revs/min at 5°C for 3 h.

The base composition of the {^{32}P}-labeled complementary strands was determined by hydrolysing the DNA to 5'-deoxyribonucleotides with pancreatic deoxyribonuclease and snake venom phosphodiesterase, followed by high voltage paper electrophoresis and quantitation of the amount of radioactivity label in each deoxyribonucleotide (37, 38).

Alkaline equilibrium sedimentation

To separate the viral and the complementary strand of labeled M13 RF-I DNA, RF-I was first cleaved into linear double-stranded DNA molecules by digestion with endoR.*Hind* II (32). The digest (0.05 ml) was mixed with 5.0 g CsCl in a polypropylene tube and 50 mM glycine-EDTA, pH 12.5, was added to a final volume of 5.0 ml. After mixing, the tube was filled with mineral oil and centrifuged in a Spinco R65 rotor at 36,000 revs/min at 20°C for 40 h. A total of 60 fractions were collected from the bottom of the tube directly on filter paper disks and assayed for radioactivity.

RESULTS

As outlined in the introduction, it should be possible to determine the origin and direction of the double-stranded DNA synthesis of bacteriophage M13 by pulse labeling the M13 DNA during RF replication and analysing the label distribution in the newly synthesized RF-I molecules. However, due to the low adsorption rate of phage M13 to the host cell, the conversion of SS DNA to pRF DNA interferes with this analysis. To separate these two processes, cells were infected in the presence of chloramphenicol. This drug prevents the replication of double-stranded phage M13 DNA by inhibiting the synthesis of the phage specific protein encoded by gene II, which is required for the initiation of RF replication (5-7, 14). Control experiments have shown that, upon infection, an incubation period of 25 min with this drug is sufficient to complete the conversion of SS DNA into pRF (data not shown). To initiate RF replication, chloramphenicol was removed and the infected cells were immediately labeled with {^{3}H} thymidine for 1, 3 or 5 min at 18°C. The newly synthesized RF-I molecules were isolated and purified by equilibrium centrifugation in caesium chloride/ ethidium bromide gradients (Materials and Methods).

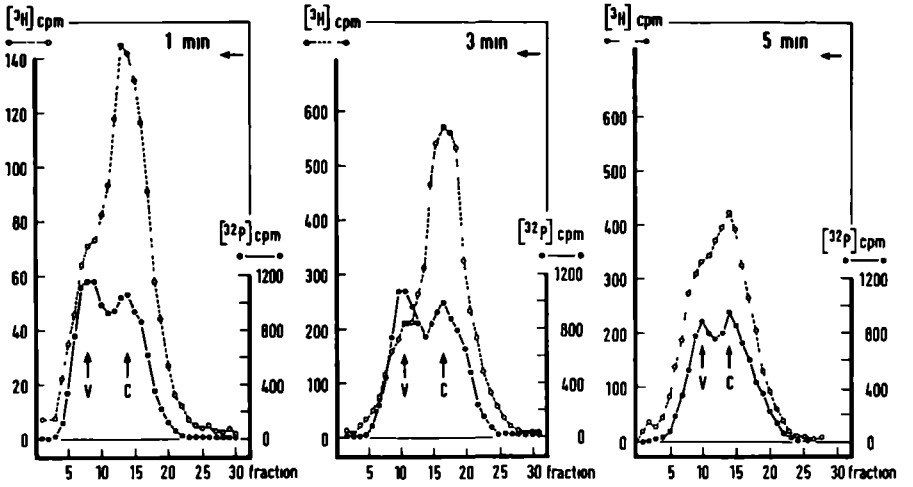

Fig. 1.: *Distribution of label between the viral and the complementary strand of {^{3}H} RF-I DNA, isolated 1, 3 or 5 min after the addition of {^{3}H} thymidine. E.coli* C59 was grown in 300 ml TPA-medium at 37°C to 5x10^8 cells/ml, treated with chloramphenicol (150 µg/ml) for 5 min and infected with M13 at a m.o.i. of 100. After 25 min the infected cells were harvested by centrifugation at 0°C, washed three times with ice-cold TPA-medium without glucose and thymine and resuspended in 300 ml of the same medium at 18°C. Subsequently 2.4 ml of 50% glucose and 0.25 ml of thymine (1 mg/ml) were added, immediately followed by addition of 5 mCi {^{3}H} thymidine. A 100 ml-sample was taken 1, 3 or 5 min later and added to 9 ml 0.5 M KCN, 3 mM sodium azide and crushed ice. Cells were lysed and {^{3}H} RF-I DNA was isolated as described in Materials and Methods. {^{3}H} RF-I DNA (appr. 0.1 µg; 2x10^3 ± 1x10^4 counts min^{-1}) mixed with uniformly-labeled {^{32}P}RF-I DNA (0.01 µg; 1x10^4 counts min^{-1}) and with 1.0 µg of carrier RF-I DNA was converted into linear RF DNA by incubation with 2.5 units of endoR.*Hind* II for 60 min at 37°C in a volume of 0.1 ml of 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 7 mM $MgCl_2$, 7 mM mercaptoethanol and 0.1 mM EDTA. The strands were separated by centrifugation on an alkaline CsCl gradient as described in Materials and Methods. Centrifugation was performed in a Spinco Ti 65 rotor at 36,000 revs/min for 36 h at 20°C. Fractions were collected from the bottom of the tube directly on filter paper disks and assayed for radioactivity. The last 30 drops did not contain any radioactivity and are not shown. The horizontal arrow indicates the direction of sedimentation. V and C refer to the density position of the viral and the complementary single strand respectively.
--o--o--, {^{3}H} RF-I DNA; —●—●—, {^{32}P} RF-I DNA Marker.

To analyse the label distribution between the viral and the complementary strand in the newly synthesized RF-I molecules, {^{3}H} RF-I DNA was digested with the restriction endonuclease endoR.*Hind* II. The latter enzyme creates a unique double-stranded nick into the RF-I molecules (32). The linear RF molecules formed

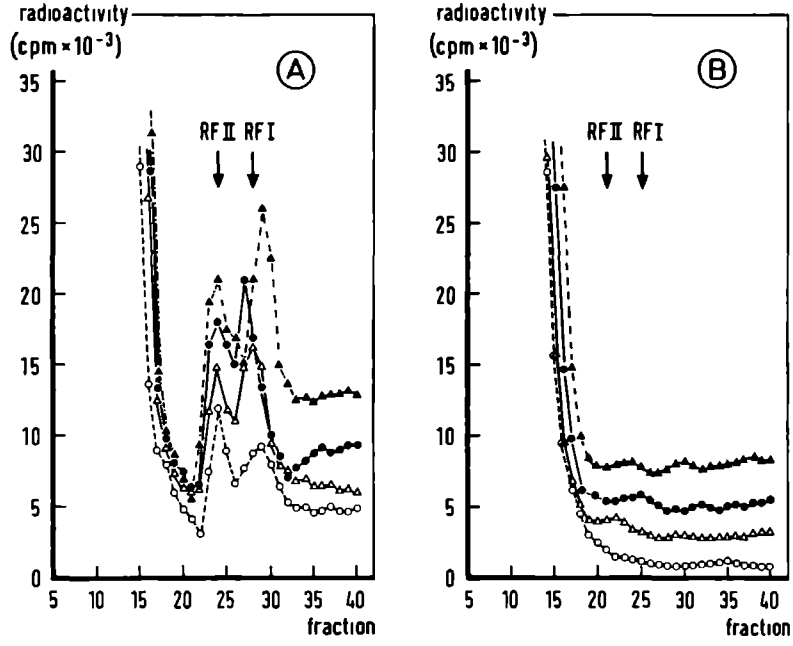

Fig. 2.: *Sedimentation analysis of {^{3}H}-labeled M13 RF DNA from lysates of cells infected with wt M13 and M13* am2-H2. *E.coli* C59 was grown in 800 ml of TPA-medium at 37°C to a cell density of 5x10^8 cells/ml, treated with chloramphenicol (150 µg/ml) for 5 min and divided into 2 equal portions. One portion was infected with wt M13 and the other with M13 *am*2-H2 (m.o.i.=100). After 25 min the infected cells of each culture were harvested by centrifugation at 0°C, washed three times with ice-cold TPA-medium without glucose and thymine and resuspended in 400 ml of the same medium at 18°C. Subsequently 3.2 ml of 50% glucose and 0.32 ml of thymine (1 mg/ml) were added, immediately followed by addition of 6.5 mCi {^{3}H} thymidine. 100 ml-samples were removed 2, 4, 6 or 8 min later and added to 9 ml 0.5 M KCN, 3 mM sodium azide and crushed ice. The cells of each sample were lysed as described in Materials and Methods and the lysate was sedimented on a 30 ml 5% to 20% sucrose gradient (in 1M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.6) at 24,000 revs/min for 19 h at 2°C. Fractions of about 0.9 ml each were collected and assayed for radio-activity. The sedimentation positions of RF-I and RF-II are indicated by vertical arrows. Direction of sedimentation is from left to right.
A. wt M13 infected cells; B. M13 *am*2-H2 infected cells.
—o—o—, 2-min labeling; —Δ—Δ—, 4-min labeling;
—●—●—, 6-min labeling; —▲—▲—, 8-min labeling.

were subjected to equilibrium centrifugation in alkaline caesium chloride gradients. Under these conditions the viral and the complementary strand band at different densities because of their difference in guanine-plus-thymine content (10). As shown in Fig. 1, {^{3}H} RF-I DNA, isolated after labeling periods of 1 or 3 min respectively, is almost exclusively labeled in the complementary (non-viral) strand. After correction for differences in thymine content between both strands,

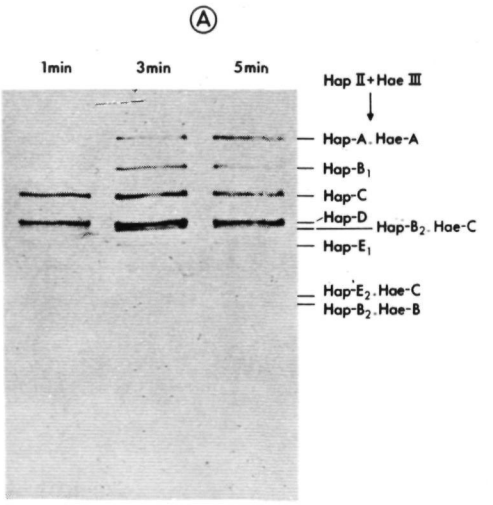

Fig. 3A.: *Fluorogram of pulse labeled* {^{3}H} *RF-I DNA, digested with a mixture of endoR.*Hap *II and endoR.*Hae *III.* {^{3}H} RF-I DNA was isolated after labeling periods of 1, 3 or 5 min as described in the legend of Figure 1. {^{3}H} RF-I DNA (appr. 0.1 µg; 5×10^3 - 1×10^4 counts min^{-1}) was mixed with 1.0 µg of carrier RF-I DNA and digested with endoR.*Hap* II and endoR.*Hae* III in excess (2.5 units) for 2 h at 37°C. The reaction was terminated by the addition of 15 µl of a solution containing 0.2 M EDTA, pH 7.6, 70% sucrose and 0.1% bromophenol blue. The DNA fragments were separated on a discontinuous polyacrylamide slabgel (20 by 20 by 0.2 cm) consisting of a 3% polyacrylamide and a 10% polyacrylamide gel layer. The gels were prepared in electrophoresis buffer consisting of 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.8. Electrophoresis was for 16 h at 120 V. The {^{3}H}-labeled fragments were visualized by fluorography (36). The positions of the double-digest fragments on the gel have been deduced from the results of the digestion of Hap II fragments with endoR.*Hae* III and vice versa (31) and are given on the right hand sight of Fig. 3A. Fragments, caused by endoR.*Hap* II cleavage, are denotated as Hap fragments, whereas fragments, caused by both endoR.*Hap* II and endoR.*Hae* III cleavage, are denotated as Hap.Hae fragments.

the estimated percentage of label in the viral strands is about 10%. RF-I DNA, isolated 5 min after the addition of {^{3}H} thymidine contains, besides label in the complementary strand (65-75%), also label in the viral strand (25-35%).

To exclude the possibility that the initial complementary strand label originates from a residual conversion of SS DNA into pRF DNA, cells were infected under the same conditions as described for wt phages with a M13 gene II amber mutant (M13 *am2*-H2). Gene II mutants cannot replicate pRF DNA under non-permissive conditions since their gene II product is nonfunctional (14). As shown in Fig. 2, no labeled

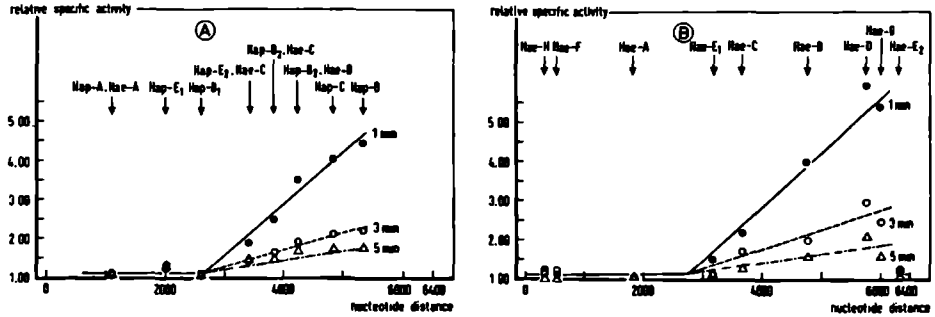

Fig. 4.: *Plot of the relative specific activity of each fragment, obtained after digestion of* {^{3}H} *RF-I DNA with a mixture of endoR.*Hap *II and endoR.*Hae *III or endoR.*Hae *III separately, against its position on the physical map.* {^{3}H} RF-I DNA, isolated after 1, 3 or 5 min of labeling respectively, was digested with either endoR.*Hap* II and endoR.*Hae* III or with endoR.*Hae* III. The fragments were isolated, their radioactivity content determined and the relative specific activity was calculated as described in the legend of Table 1. The relative specific activities were plotted against the position of the corresponding fragments on the linear M13 physical map. The endoR.*Hap* II cleavage site between the fragments Hap-A and Hap-F (see Fig. 5) was taken as zero point. The nucleotide distance is measured clockwise from the left most end of fragment Hap-A (0 basepairs) to the right most end of fragment Hap-F (6400 basepairs), assuming the total length of the genome to be 6400 basepairs. The vertical arrows refer to the midpoints of the different restriction fragments. A. Plot of the relative specific activity of the fragments, produced after digestion of {^{3}H} RF-I DNA with a mixture of endoR.*Hap* II and endoR.*Hae* III. B. Plot of the relative specific activity of the endoR.*Hae* III fragments. —●—●—, 1-min labeling; --O--O--, 3-min labeling; --Δ--Δ--, 5-min labeling.

RF DNA is found after chloramphenicol treatment of cells infected with this amber mutant (Fig. 2B), whereas in cells infected with wild type phage the RF DNA is clearly labeled (Fig. 2A). From the results of these experiments we have concluded that the length of the chloramphenicol treatment was indeed sufficient to allow a complete conversion of SS DNA to pRF DNA. The label found in the complementary strand after infection of the cells with wild type phages therefore must have arisen from replication of pRF DNA.

To determine the distribution of label in the complementary strand of the newly synthesized RF-I molecules, {^{3}H} RF-I DNA was digested simultaneously with the restriction endonucleases endoR.*Hap* II and endoR.*Hae* III. The double-digestion products were separated by polyacrylamide gel electrophoresis. The position of the fragments on the gel was visualized by fluorography (36). After a labeling period of one minute, the label in {^{3}H} RF-I DNA is mainly located in the restriction

Table 1. *Relative specific activity of the fragments obtained after digestion of pulse labeled {^3H} RF-I DNA with a mixture of endoR.*Hap *II and endoR.*Hae *III or endoR.*Hae *III separately (see Fig. 3)*

Hap.Hae fragments	%T*	Relative specific activity		
		1-min	3-min	5-min
Hap-A.Hae-A	28.4	1.08	0.98	0.97
Hap-B_1	27.2	1.00	1.00	1.00
Hap-C	26.5	4.04	2.08	1.72
Hap-D	30.8	4.44	2.20	1.75
Hap-B_2.Hae-C	27.9	2.49	1.65	1.50
Hap-E_1	26.6	1.22	1.25	1.31
Hap-E_2.Hae-C	27.6	1.89	1.34	1.47
Hap-B_2.Hae-B	24.1	3.49	1.89	1.70

Hae fragments	Relative specific activity		
	1-min	3-min	5-min
Hae-A	1.00	1.00	1.00
Hae-B	4.02	1.99	1.58
Hae-C	2.17	1.69	1.30
Hae-D	5.98	2.99	2.11
Hae-$E_{1,2}$	1.30	1.03	0.98
Hae-F	1.30	1.13	1.02
Hae-G	5.52	2.44	1.58
Hae-H	1.10	1.24	1.07

DNA fragments, obtained after digestion of {^3H} RF-I DNA with a mixture of endoR.*Hap* II and endoR.*Hae* III or with endoR.*Hae* III alone, were separated on polyacrylamide gels and their {^3H}-radioactivity content was determined as described in Materials and Methods. Their relative specific activities were calculated by expressing the {^3H}-radioactivity per unit length and normalizing to 1 for fragment Hap-B_1 for the double-digest fragments. The Hae fragments were normalized to fragment Hae-A. The relative specific activities of the double-digest fragments are corrected for the differences in thymine content of the complementary strands of these fragments.

* The values for %T in the complementary strand used, are based on duplicate determinations for each fragment (see Methods). The base composition of uniformly-labeled {^3H} RF-I DNA determined at the same time, was 58.7% A+T, which is in agreement with the reported values (1).

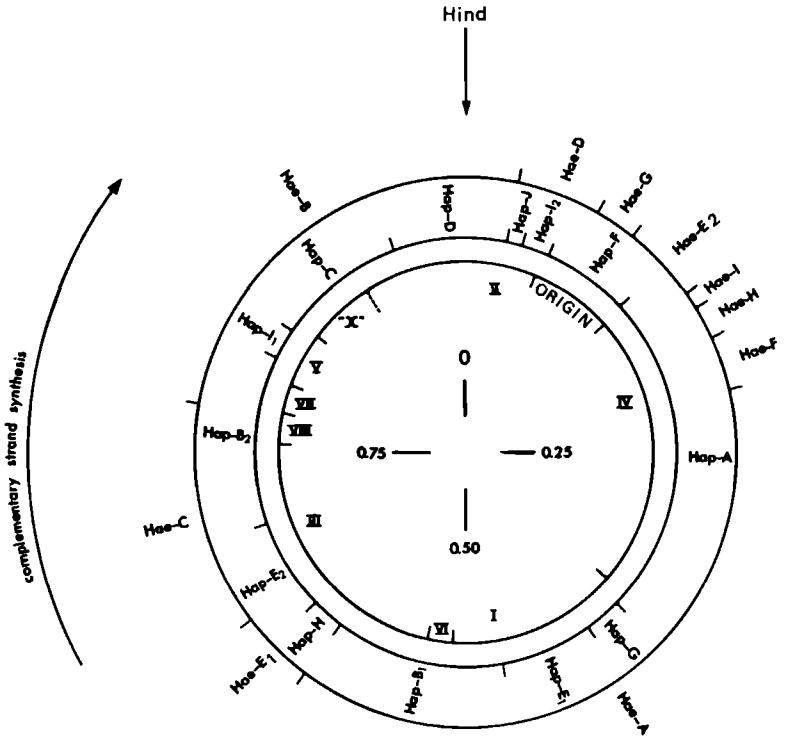

Fig. 5.: *Physical and genetic map of bacteriophage M13.* The outer circle shows the location of the fragments obtained after digestion of M13 RF with endoR. *Hind* II (arrow) and endoR.*Hae* III. The middle circle represents the locations of the endoR.*Hap* II fragments. The order of these fragments has been determined previously (31). The inner circle represents the M13 genetic map (genes are designated with Roman numerals) (25, 33). The sizes of the genes were estimated from the molecular weight of the *in vitro* gene products with the exception of gene VI and gene VII, whose definite sizes are unknown (39, 41). The direction of transcription is counterclockwise (46). "X" refers to the polypeptide encoded by the C-terminal end of gene II (25). The direction of complementary strand synthesis during RF replication is clockwise around the genetic map. The origin of complementary strand synthesis (indicated ORIGIN) is located in fragment Hae-G, the position of which is in the intergenic region between gene IV and gene II (25).

fragments Hap-C and Hap-D, while after a labeling period of three or five minutes all the restriction fragments of {^{3}H} RF-I DNA are labeled (Fig. 3A). Comparison of the intensities of the label in the bands indicates,however,that there is relatively more label in the fragments Hap-C and Hap-D than in the other fragments.

To quantify the distribution of radioactivity in the different fragments of the RF-I molecules, the relative specific activities of the eight major fragments of the double digest were calculated. For this purpose the {^{3}H}-content of each fragment was estimated and corrected for the difference in the thymine content (Table 1). The values obtained were divided by the fragment length and then normalized to one for fragment Hap-B_1. The relative specific activities of the eight largest fragments were plotted against their positions on the M13 physical map. As shown in Fig. 4A, the relative specific activities of the fragments Hap-A.Hae-A through Hap-B_1 are identical, whereas those of the fragments Hap-E_2.Hae-C through Hap-D increase linearly. The highest relative specific activity is found in fragment Hap-D. Since we are only examining completed RF molecules, the last part of the circular DNA replicated should contain the highest specific activity. Of all the restriction fragments studied, Hap-D contains the highest relative specific activity and, therefore, the replication must terminate beyond the end of this fragment. Furthermore, since a higher relative specific activity of a fragment indicates that it is synthesized later during the replication cycle, it is very likely from these data that the start of the complementary strand synthesis is located in the region between the fragments Hap-D and Hap-A.Hae-A and that synthesis proceeds in one direction clockwise around the genetic map (Fig. 5).

The low relative specific activity of the fragments Hap-A.Hae-A through Hap-B_1 is probably the result of initiation of the complementary strand synthesis before {^{3}H} thymidine was available for incorporation. This is illustrated in a diagram of the theoretical radioactivity distribution in labeled RF molecules, in which the first completed RF-I molecules are only partially labeled (Fig. 6). Clearly, at short labeling times (1t), only the second half of the molecules will be labeled and the relative specific activity of anyone place within this labeled part increases linearly with the distance from the origin of replication. As the labeling time increases (2t and 3t),the molecules will be more uniformly-labeled and the gradient of relative specific activity will decrease in slope.

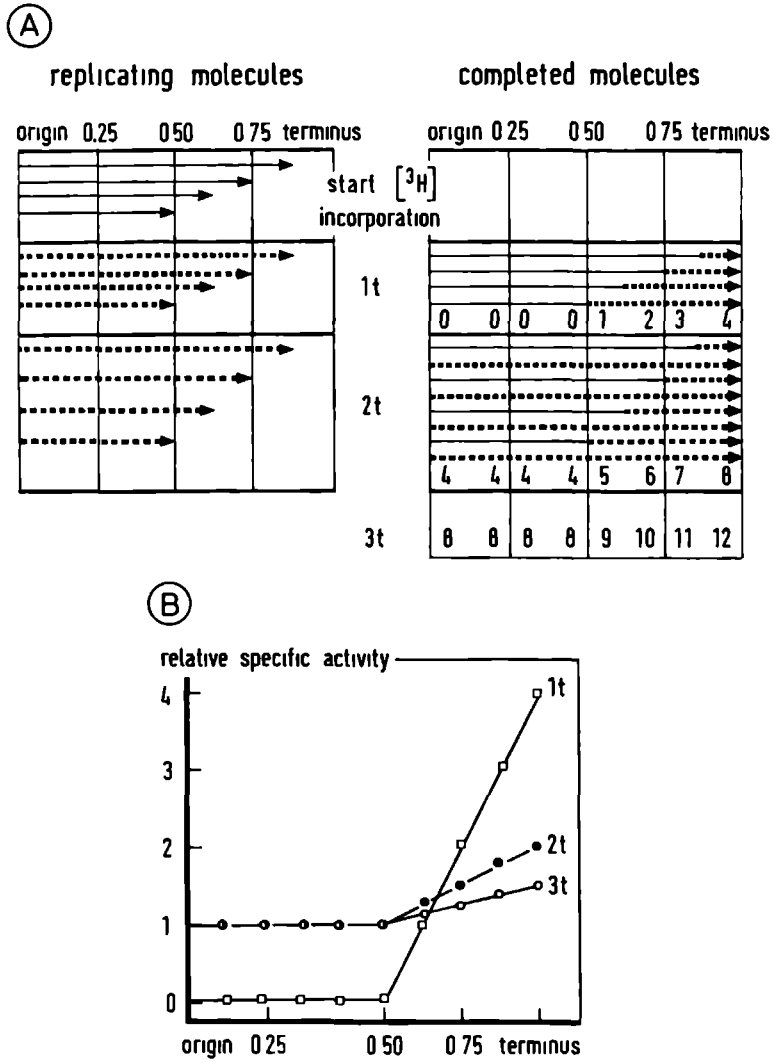

Fig. 6.: *Diagram of a theoretical distribution of {^3H} thymidine in replicating and completed DNA molecules after different labeling periods.* It is assumed that more than half of each replicating molecule has been synthesized at the time of the start of the label incorporation. The origin of replication of the linear representation of the molecules is on the left and the termination is on the right, while the molecules are divided into eight equal parts. The time to replicate the whole molecule is taken to be 1t. The distribution of label accumulated in the population of completed molecules at 1t to 3t is given in Fig. 6A. The figures in each panel, which represent one-fourth of the molecule, indicate the total amount of label (in arbitrary units) accumulated in that position during the labeling period denoted at the left side of these panels. These figures are replotted graphically in Fig. 6B. The unlabeled regions are drawn as solid lines and the labeled regions are drawn as broken lines.

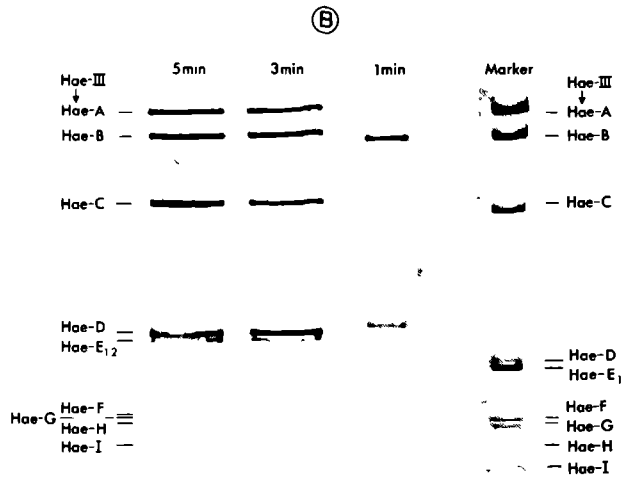

Fig. 3B.: *Fluorogram of pulse labeled {^3H} RF-I DNA digested with endoR.* Hae *III.* The isolation of {^{3}H} RF-I DNA and the subsequent digestion with endoR.*Hae* III were carried out as described in the legend of Figure 3A. The DNA fragments were separated on a discontinuous polyacrylamide slabgel (20 by 20 by 0.2 cm) consisting of a 3% polyacrylamide and a 10% polyacrylamide gel layer. The preparation of the gels and the condition of electrophoresis were the same as described in Fig. 3A. The positions of the Hae fragments are indicated on the left hand side of Fig. 3B. An autoradiograph of all ^{32}P -labeled Hae fragments of M13 RF is shown on a separate gel, designated as marker, on the right hand side of this figure.

To determine the place of termination of the complementary strand synthesis more precisely, pulse labeled {^{3}H} RF-I was digested with endoR.*Hae* III (32). The latter enzyme introduces a number of cuts within or near the region encompassing the termination point (Fig. 5). The fluorogram of the digestion products produced by this endonuclease shows that after a one minute labeling time only the fragments Hae-B and Hae-D are labeled (Fig. 3B). After a labeling period of three or five minutes, label could be detected in several other Hae fragments. Determination of the radioactivity content of each Hae fragment and comparison of the relative specific activities of these fragments with their positions on the physical map (Fig. 4B) clearly show , that, since the highest relative specific activity is found in the fragments Hae-D and Hae-G, whereas the activity present in fragment Hae-E_2 is near unity, the complementary strand synthesis terminates within the region defined by fragment Hae-D and Hae-G.

It should be noted that the relative specific activity of fragment Hae-G is slightly lower than the activity of its proximal fragment Hae-D. This difference has repeatedly been observed in each of the different labeling periods used (Fig. 4B and Table 1). This suggests that the complementary strand synthesis terminates within or near the end of fragment Hae-G.

From the observation that the M13 complementary strand synthesis is unidirectional together with the observation that its synthesis terminates within or near the end of fragment Hae-G, we infer that also the origin of the complementary DNA synthesis is located near the end of fragment Hae-G.

DISCUSSION

The *in vitro* conversion of M13 single-stranded DNA into the parental replicative form has been studied extensively during the past few years (21, 22). It has been shown that this process starts at a unique site on the genetic map which is most probably located within the intergenic region between gene IV and gene II (23-25, 44). The location of the origin of double-stranded DNA replication (pRF → RF) is unknown. To determine where this origin is located on the genetic map, we have studied the kinetics of labeling of restriction fragments of replicating DNA molecules under conditions which only permit replication of the parental replicative form, namely after pretreatment of infected cells with chloramphenicol. This pretreatment allows the conversion of SS DNA to pRF DNA but not the replication of pRF DNA (14).

Analysis of the distribution of label in completed RF-I molecules, pulse labeled during the first minutes after the start of the replicative form DNA replication, revealed that virtually only the non-viral (complementary) strand was labeled (Fig. 1). This could be due to a residual conversion of parental SS DNA into replicative form DNA instead of replicative form DNA replication. This is unlikely since control experiments with a M13 mutant phage, M13 *am*2-H2, carrying an amber mutation in gene II, a gene whose product is absolutely required for the RF replication (5-7), indicated that no conversion of parental SS DNA molecules into the replicative form DNA occurred during the labeling period. Therefore, the incorporation of label in the complementary strand is the result of a replication process in which only the complementary strands of the newly synthesized RF-I molecules are labeled.

Label distribution analysis has indicated that the restriction fragments located in one half of the pulse labeled RF-I molecules are uniformly-labeled,

while the label per unit length in the fragments of the other part of the molecules increased linearly (Fig. 4). This observation might be explained by the fact that the replication of the parental replicative form molecule is already initiated before the incorporation of label occurs (*cf.* Fig. 6). Consequently only the "3' ends" of the newly synthesized RF-I molecules are labeled.

From the distribution of label found and from the observation that during the first minutes after chloramphenicol treatment only the complementary strands of the newly synthesized RF-I molecules are labeled, one may conclude that the synthesis of the complementary strand starts near the end of fragment Hae-G and proceeds clockwise around the genetic map towards fragment Hae-G (Figs. 4 and 5). In other words, the results suggest that both the origin and the termination site for the synthesis of the complementary strand of RF DNA are located within the intergenic region between gene IV and gene II (Fig. 5). Kornberg and co-workers (23, 44) have found that the origin for the *in vitro* conversion of single-stranded phage DNA into parental replicative form DNA is also located in this region. This together with the finding that in both cases an RNA-dependent step is obligatory for the synthesis of the complementary strand (15, 21, 22, 24, 42, 43), suggests that the origin of synthesis of the complementary strand during pRF formation is identical to the origin of the complementary strand synthesis during RF replication. Studies on M13 deletion mutants (miniphages), containing 20% to 50% of the M13 genome, have indicated that in miniphages the intergenic region is conserved (14, van den Hondel, C.A., R. N. H. Konings, and J. G. G. Schoenmakers, unpublished results). This observation suggests that this region is of functional importance in the replication process of M13 RF DNA which is consistent with the results we have obtained.

Phage M13 messenger RNA is only transcribed from the complementary strand in a direction which is counterclockwise around the genetic map (45, 46). This means that the 5' → 3' polarity of the complementary strand is clockwise. This together with the observation that the gradient of label of the pulse labeled RF-I molecules increased in a clockwise direction, implies that the synthesis of the complementary strand occurs in a continuous fashion and there is no need to assume a discontinuous synthesis. From this observation we may furthermore conclude that at least a "tail" of one genome length of the viral strand must be formed on the replicating molecule before the synthesis of the complementary strand is initiated.

Our data obtained so far fit a replication mechanism according to the rolling circle model proposed by Gilbert and Dressler for the replication of single-stranded phage DNA (8). Especially the labeling of only the complementary

strand of the first newly synthesized RF-I molecules and the initiation of the complementary strand on a "tail" of one genome length of the replicating intermediate molecules are features required by this model.

At the present no information is available about the location of the origin of the viral strand synthesis. However, since in all the DNAs of deletion mutants the intergenic region between gene II and gene IV is conserved (44), this region may contain the common initiation site for the three stages of M13 DNA replication, including the viral strand synthesis.

Abbreviations : SS, single-stranded phage DNA; RF, circular double-stranded replicative form DNA; RF-I, RF DNA in which both strands are covalently closed circles; RF-II, RF DNA containing one or more single-stranded breaks; sarkosyl, sodium lauryl sarcosinate; m.o.i., multiplicity of infection.

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CLEAVAGE MAPS OF THE FILAMENTOUS BACTERIOPHAGES M13, fd, f1 AND ZJ/2

CLEAVAGE MAPS OF THE FILAMENTOUS BACTERIOPHAGES M13, fd, f1 AND ZJ/2

ABSTRACT

The replicative form DNAs of bacteriophage M13, fd, f1 and ZJ/2 were found to be sensitive to cleavage by the restriction endonucleases endoR.*Hap* II, endoR.*Hae* II, endoR.*Hae* III, endoR.*Hind* II, endoR.*Alu* I, endoR.*Hha* and endoR.*Hinf*. With respect to M13 DNA the number of cleavage sites varied from 21 for endoR.*Hinf*, 18 for endoR.*Alu* I, 15 for endoR.*Hha*, 13 for endoR.*Hap* II, 10 for endoR.*Hae* III, 3 for endoR.*Hae* II, to only a single site for endoR.*Hind* II. In contrast to M13, fd and f1, the ZJ/2 DNA molecule was not cleaved by the endoR.*Hind* II endonuclease. No cleavage site on either phage DNA was detected for the endonucleases endoR.*Hsu*, endoR.*Eco* RI and endoR.*Sma*. When compared with M13 DNA, several differences were noted in the number and size of cleavage products obtained with DNA of phage fd, f1 and ZJ/2. From the results of these analyses, using the M13 enzyme cleavage maps as a reference, the endoR.*Hap* II, endoR.*Hae* II, endoR.*Hae* III, endoR.*Hind* II and endoR.*Alu* I maps of phage fd, f1 and ZJ/2 could be constructed. As is expected for very closely related phages, the enzyme cleavage patterns exhibit a high degree of homology. Phage f1 and ZJ/2 are most related since an identical pattern was obtained with seven different restriction endonucleases. Evidence is provided also that f1 is more similar to M13 than to fd. Furthermore, characteristic differences exist within the endoR.*Hinf* enzyme cleavage pattern of all the four phages tested. Digestion of phage DNA with this enzyme, therefore, provides a new and sensitive method of distinguishing these closely related filamentous coliphages.

INTRODUCTION

The small DNA bacterial viruses M13, fd, f1 and ZJ/2 belong to the group of F-specific filamentous coliphages. These phages are almost identical by several criteria that have been tested, including the dimension and structure of the virion, the morphology, molecular weight and base composition of the DNA molecule, the mode of infection of the bacterial host and its viral DNA replication (29).

In the last few years progress is being made toward an understanding of the molecular biology of these phages. In particular, genetic mapping (15, 17, 20, 27, 34, 44), the mechanism of genetic recombination (5, 10, 11) and susceptibility to restriction and modification (18, 19, 20, 26, 43) have been studied. In addition,

the elucidation of the mode of DNA replication is currently pursued in several laboratories (46, 47). At the same time, gene functions (1, 29, 31) and most of the gene products have been identified (12, 22-25, 30). Currently, efforts are being made to understand the mechanism by which transcription and translation are regulated (4, 16, 34, 40, 44).

In a number of these studies, restriction enzymes have been applied as a potentially useful aid in the elucidation of these problems. Restriction enzyme cleavage maps have been constructed for the genome of M13 (13, 14, 17, 34) of fd (34, 41) and f1 (20, 34, 44). Such maps have been used to order phage genes (15, 17, 34, 44) and to localize genetic sites, which confer upon DNA molecules susceptibility to *E.coli* B restriction and modification (20, 44). Restriction fragments have been used to trace the origin of single-stranded DNA synthesis *in vitro* on the M13 genome (8, 38, Schaller, personal communication). In addition, phage promotor regions of M13 (16, 34, Edens *et al.* 1975, submitted for publication) and of fd (32, 34) have been localized and the central terminator of transcription has been mapped on the circular M13 genome (4).

The great homology among the filamentous phages suggests that their restriction enzyme cleavage patterns are identical.The validity of this suggestion, however, is not warranted. For this reason we thought it useful to investigate in more detail the cleavage patterns of M13, fd, f1 and ZJ/2, which are produced after digestion with the endonucleases endoR.*Hap* II, endoR.*Hae* II, endoR.*Hae* III, endoR.*Hind* II, endoR.*Hha*, endoR.*Alu* I, endoR.*Eco* RI, endoR.*Hsu*, endoR.*Hinf* and endoR.*Sma*.

Using the M13 restriction enzyme cleavage maps as a reference (14, 17) we were able to construct several cleavage maps for f1, fd and ZJ/2 as well. Furthermore, we demonstrate that, upon digestion with a single restriction endonuclease, characteristic differences exist between the cleavage pattern of all of the four phage DNAs tested, thus providing a new and sensitive method of distinguishing these closely related coliphages.

MATERIALS AND METHODS

Bacteria and phages

Escherichia coli C89 (K12, 159F$^+$, uv^s, su$^-$) was used for the cultivation of phage M13, fd and f1 as well as for the preparation of phage replicative form I DNA. *E.coli* C3000 (F$^+$), a gift from Dr. D. Kay, Oxford, was used for the propagation of phage ZJ/2 and the preparation of ZJ/2 replicative form DNA.

Phage M13 was originally obtained from Dr. P. Hofschneider, Munich; phage fd originated from Dr. H. Schaller, Heidelberg; phage f1 from Dr. J. Woolford, Durham, and phage ZJ/2 from Dr. D. Kay.

Isolation of replicative form I DNA

E.coli C89 or *E.coli* C3000 was grown in 2 l. of Tryptone broth (28). At a cell density of about 5 x 10^8 cells/ml, the culture was infected with the appropriate phage at a multiplicity of 20. After 90 min at 37 C, the infected cells were harvested, washed once with 0.2 M NaCl, 0.025 M EDTA, pH 7.6, and resuspended in 200 ml of the same buffer. The bacterial suspension was brought to pH 12.4 with 1 M NaOH, incubated for 3 min at room temperature and then neutralized with 1 M HCl. The cell debris was centrifuged at 20,000 *g* for 30 min. The supernatant, containing the RF, was extracted twice with an equal volume of phenol which had previously been equilibrated with STE buffer (0.1 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA, pH 7.6). The phases were separated by centrifugation, and the nucleic acids were precipitated from the aqueous phase by the addition of 2 volumes of ethanol. The precipitate obtained was resuspended in 20 ml of STE buffer. Pancreatic ribonuclease (previously heated to destroy DNase activity) was added to a final concentration of 50 µg/ml and the solution was incubated for 30 min at 37 C. Thereafter, the same amount of ribonuclease was added and the incubation was continued for a second 30 min period. The mixture was then extracted with phenol and RF DNA was reprecipitated with 2 volumes of ethanol. The precipitate was dissolved in STE buffer (20 ml) and the solution was percolated through a Sephadex G100 column (25 x 5 cm), previously equilibrated with STE. The RF-containing fractions were collected and the DNA reprecipitated with 0.1 volume of 3 M sodium acetate, pH 5.6 and 2 volumes of ethanol. The material was redissolved in STE buffer and centrifuged to equilibrium in CsCl/ethidiumbromide. To 2.4 ml of RF solution, 4.24 g of CsCl and 2.0 ml of ethidiumbromide (2 mg/ml) were added. In addition, a small amount of ^{32}P-labeled M13 RF (approximately 40,000 counts/tube) was added as a marker. Centrifugation was performed at 20 C in a Spinco 65 angle rotor for 48 h at 38,000 rpm. The DNA was collected after piercing the bottom of the tube, and the lower ^{32}P-containing band, consisting exclusively of closed superhelical RF-I DNA, was collected. Ethidiumbromide was removed by 5 extractions with isoamylalcohol which had been saturated previously with 4 M CsCl. The samples were subsequently dialyzed extensively against STE buffer. The RF-I was precipitated with ethanol and finally dissolved in 0.1 M Tris-HCl, 1 mM EDTA,

pH 7.6. The preparation of ^{32}P-labeled M13 RF DNA has been described previously (14).

Restriction endonucleases

EndoR.*Hind* II/III from *Haemophilus influenzae* strain Rd was isolated as described previously (13). EndoR.*Hae* II, endoR.*Hae* III and endoR.*Hap* II were isolated from *Haemophilus aegyptius* and *Haemophilus aphirophilus*, respectively. The restriction enzymes were isolated according to the procedure of Takanami and Kojo (39), except that the nucleic acids still present in the 35-65% ammonium sulfate fraction were first removed by adsorption on DEAE-cellulose at 0.3 M KCl (2) prior to the phosphocellulose chromatography step. The preparation of endoR.*Alu* I, from *Arthrobacter luteus*, will be described elsewhere (17). EndoR.*Hha* from *Haemophilus haemolyticus* and endoR.*Sma* from *Serratia marcescens* were generous gifts of Dr. C. Montfoort, Utrecht. EndoR.*Eco* RI was a kind gift of Dr. J. Sanders, Amsterdam. EndoR.*Hsu* from *Haemophilus suis* and endoR.*Hinf* from *Haemophilus influenzae* strain Rf were generously provided by Dr. J. Sussenbach, Utrecht.

Cleavage of phage RF with restriction endonucleases

For preparation of complete digest mixtures,phage RF-I (3 µg) was incubated for 2 h at 37 C with appropriate amounts of restriction endonucleases in a reaction volume of 0.05 ml, containing 10 mM Tris-HCl, pH 7.6, 7 mM $MgCl_2$ and 7 mM mercapto-ethanol. Incubations were terminated by the addition of 15 µl of a solution, containing 0.2 M EDTA, 75% sucrose and 0.1% bromophenol blue. Identical reaction conditions were used for all of the restriction enzymes studied, except for endoR.*Sma* and endoR.*Hsu*. Digestion with the former enzyme was carried out in 30 mM Tris-HCl, pH 9.0, 150 mM KCl, 3 mM $MgCl_2$, the conditions for endoR.*Hsu* were 10 mM Tris-HCl, pH 7.6, 40 mM NaCl, 7 mM $MgCl_2$ and 7 mM mercaptoethanol.

Polyacrylamide gel electrophoresis

A discontinuous polyacrylamide slab gel was used for analysis of DNA fragments. Slab gels (20 x 20 x 0.2 cm) were composed of a 3% polyacrylamide gel (about 16 cm high) above a 10% polyacrylamide gel layer (about 4 cm high). Both gels were prepared in an electrophoresis buffer consisting of 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.8. Analyses of endoR.*Hae* II digests were performed on slab gels containing a 2.5% to 7.5% gradient of polyacrylamide which were prepared

as described by Jeppesen (21). In both cases a gel chamber similar to that described by de Wachter and Fiers (45) was used. Electrophoresis was performed at room temperature for 16 h at 20 mA (about 120 V). After electrophoresis, the gels were stained with ethidiumbromide solution (2 µg/ml) and then photographed in short UV-light using a Polaroid Camera, adapted with a Kodak 23A red filter (35).

The chain lengths of M13 restriction fragments were deduced from radioactivity analysis. ^{32}P-labeled M13 RF was digested with the appropriate endonuclease and the fragments were fractionated on a 3% polyacrylamide slab gel (40 x 20 x 0.2 cm) as described previously (14). The portions of the gel containing the fragments were localized by autoradiography, excised, and counted as described (14). The chain lengths were calculated from the percent total radioactivity assuming a molecular weight of 4 x 10^6 for full length M13 RF DNA (29) and a molecular weight of 650 for each basepair.

The chain lengths of the M13 RF fragments, produced by endoR.*Hap* II, endoR.*Hae* III and endoR.*Alu* I have been reported previously (14, 17). The chain lengths of fd, f1 and ZJ/2 RF fragments were calculated from their relative mobilities by means of a plot relating log molecular size to the distance migrated in the polyacrylamide gel. The chain lengths of the endoR.*Hap* II and endoR.*Hae* III fragments of M13 RF were used to standardize this plot.

Nomenclature

The nomenclature proposed by Smith and Nathans (36) for restriction enzymes has been used. For each phage individually, the DNA fragments that result from their action have been designated with capital letters in order of decreasing fragment size. When bands on a polyacrylamide gel contain two or more fragments of almost equal size, arabic numerals have been used as suffix to designate these fragments, *i.e.* N_1, N_2 etc.

RESULTS

Cleavage pattern of M13, fd, f1 and ZJ/2 with endonuclease R.*Hap* II

EndoR.*Hap* II, a restriction endonuclease from *H.aphirophilus*, cleaves the circular double-stranded replicative form DNA (RF) of bacteriophage M13 at thirteen specific sites to yield thirteen fragments, which have been named Hap II-A through Hap II-J in order of decreasing fragment size. The length of each fragment has been determined (14) and ranges from 1530 basepairs for Hap II-A to about

Table 1. *Size of fragments generated from M13 RF by various restriction endonucleases*

Fragments produced	Size of fragments (in basepairs) produced with						
	Hind II	*Hap* II	*Hae* II	*Hae* III	*Alu* I	*Hha*	*Hinf*
A	6400	1530	3500	2500	1430	990	1320
B		820*	2600	1630	1360	930	750
C		650	320	820	600	740	470
D		560		310	570	720*	410
E		460*		290*	480	515	340*
F		410		190	330	325	320
G		200		160	305	310	310
H		170		120	260	280	260*
I		140*		70	230	255	230*
J		50			190*	245	215*
K					160	205	190
L					130	95	155
M					115	70	115
N					20**	45	90
O							65
P							40*
TOTAL		6390	6420	6380	6430	6445	6365

The sizes of the endoR.*Hha* and endoR.*Hinf* fragments were estimated from analysis of the ^{32}P-content of each fragment produced from uniformly ^{32}P-labeled M13 RF, as described under Materials and Methods. In some instances, the size of a fragment was adjusted by comparing its mobility with that of the Hap II, Hae II, Hae III and Alu I fragments, the sizes of which have been determined previously (14, 17). A single asterisk refers to bands containing doublet fragments, a double asterisk refers to quartet fragments.

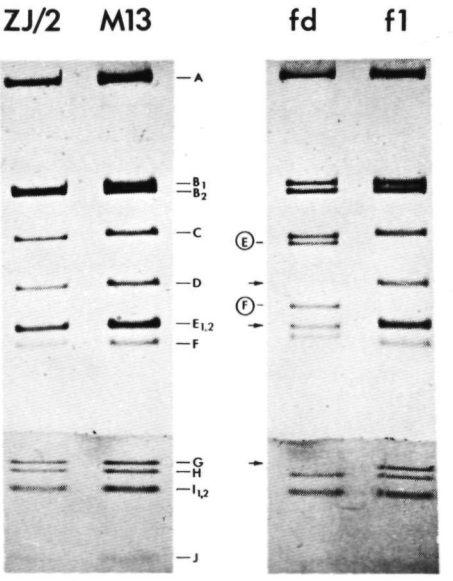

Fig. 1.: *Electrophoresis of fragments produced by the digestion of various phage RF-I with endonuclease R.*Hap *II.* Phage RF I (3 µg) was digested with endoR.*Hap* II at 37 C for 2 h in a 50 µl reaction mixture containing 10 mM Tris-hydrochloride, pH 7.4, 7 mM $MgCl_2$ and 7 mM mercaptoethanol. The reaction was terminated by adding 15 µl of a solution, consisting of 0.2 M EDTA, 70% sucrose and 0.1% bromophenol blue. The samples were layered on a discontinuous polyacrylamide slab gel (20 by 20 by 0.2 cm), consisting of a 3% polyacrylamide gel on top of a 10% polyacrylamide gel layer, which were prepared in electrophoresis buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.8). After electrophoresis for 16 h at 120 V, the gels were stained with ethidiumbromide solution (2 µg/ml) and then photographed as described by Sharp *et al.* (35). The capital letters A through J in the middle of the figure indicate, from top to bottom, the positions of the endoR.*Hap* II fragments of M13 RF-I. The arrows indicate the positions of fragments which are present in M13 RF but which are missing in the phage RF digest. The letters within circles refer to fragments, present in phage RF but which are missing in the digest of M13 RF.

50 basepairs for the smallest fragment Hap II-J (Table 1). The physical order of the M13 fragments, which has been determined previously (14), is presented in Figure 8. Subsequent to polyacrylamide gel electrophoresis and staining with ethidiumbromide, the endoR.*Hap* II digest of M13 RF gives a cleavage pattern as shown in Figure 1.

Digestion of f1 and ZJ/2 RF with endoR.*Hap* II generates the same number of DNA fragments. As judged from the respective positions of these fragments on the polyacrylamide gels it is clear that the cleavage pattern of M13 is identical to f1 and ZJ/2 (Fig. 1). This is in contrast to the pattern obtained with fd RF

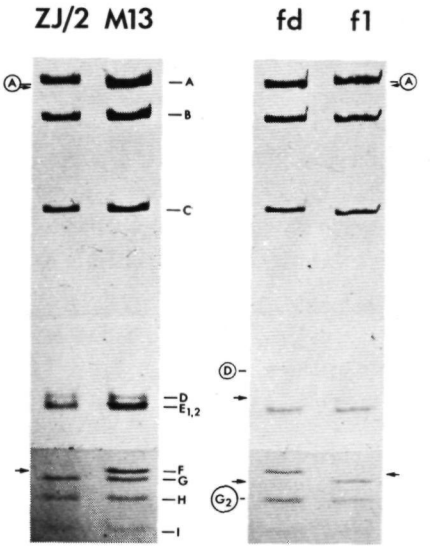

Fig. 2.: *Electrophoresis of fragments produced by the digestion of various phage RF-I with endonuclease R.*Hae *III.* The conditions for endoR.*Hae* III digestion of phage RF-I and the conditions for electrophoresis on the discontinuous polyacrylamide slab gel were identical to those described in the legend of Fig. 1. The capital letters A through I in the middle of the figure indicate the positions of the endoR.*Hae* III fragments of M13 RF. The arrows indicate the positions of fragments which are present in M13 RF, but which are missing in the phage RF digest. The letters within circles refer to fragments, present in phage RF, which are missing in the digest of M13 RF.

in which several characteristic differences can be observed. These differences arise from a missing and an additional scission in fd RF. The missing scission is the one within the M13 (f1 and ZJ/2) analogue of the fd fragment Hap II-E (630 basepairs) yielding the M13 fragments Hap II-E_1 and Hap II-G. The additional scission occurs within the fd analogue of the M13 fragment Hap II-D yielding the fd fragments Hap II-F (510 basepairs) and Hap II-M. Due to its small size (about 30 basepairs) the latter fragment can hardly be detected by ethidiumbromide staining, but is clearly recognizable by autoradiography of a ^{32}P-labeled endoR.*Hap* II digest of fd RF (van den Hondel, unpublished results). Similar differences in the cleavage pattern of fd, f1 and M13 have already been reported for the *H.parainfluenzae* enzyme endoR.*Hpa* II by Seeburg and Schaller (34), whose data for endoR.*Hpa* II fragments are completely identical with ours for endoR.*Hap* II fragments. This substantiates the conclusion made by others (6, 37) that the recognition sites of both enzymes are identical.

EndoR.*Hae* III, one of the restriction endonucleases of *H.aegyptius*, cleaves M13 RF at ten specific sites to yield 10 fragments which have been named Hae III-A through Hae III-I. The size of the individual endoR.*Hae* III fragments of M13 RF are presented in Table 1.

The physical order of these fragments, which has been determined previously (14) is given in Figure 8. After polyacrylamide gel electrophoresis, the endoR.*Hae* III digest of M13 RF exhibits a cleavage pattern which is shown in Figure 2. When compared to M13, digestion of fd RF with endoR.*Hae* III generates the same number of DNA fragments, but their cleavage patterns reveal characteristic differences. The fd analogues of the M13 fragments Hae III-D and Hae III-G are missing in the digest of fd RF, while two new fd fragments can be detected, namely Hae III-D and Hae III-G_2 (Fig. 2). We suggest that the latter fragment has the same electrophoretic mobility as the fd analogue of the M13 fragment Hae III-H and, hence, it constitutes a doublet with this fragment. Although experimental data on the ^{32}P-content of this band are lacking, the high intensity of this band after staining with ethidiumbromide, does already suggest that this is the case. Moreover, M13 fragment Hae III-D (310 basepairs) is contiguous to Hae III-G (160 basepairs) in M13 RF (Table 1 and Figure 8) whereas both their fd analogues are missing in the cleavage pattern of fd RF. Therefore, the appearance of the fd fragments Hae III-D (360 basepairs) and Hae III-G_2 (120 basepairs) is best explained by a missing endoR.*Hae* III cleavage site between the fd analogues of the M13 fragments Hae III-D and Hae III-G and an additional cleavage site in either the fd analogue of the M13 fragment Hae III-D or Hae III-G_2 (Figure 8). Whether the smallest fragment, Hae III-G_2, is adjacent to the fd fragment Hae III-B or Hae III-E_2 has not been ascertained yet.

The endoR.*Hae* III digest patterns of f1 and ZJ/2 RF appear to be identical. Nevertheless, characteristic differences can be observed between the patterns of f1 and ZJ/2 on the one hand and M13 on the other. As shown in Figure 2, the f1 (and ZJ/2) analogues of the M13 fragment Hae III-F (130 basepairs) are missing. Careful electrophoretic analysis of several endoR.*Hae* III digestion patterns have also shown that the largest fragment of f1 and ZJ/2, designated Hae III-A, has in fact a slightly smaller electrophoretic mobility than the M13 fragment Hae III-A (2500 basepairs). Since the M13 fragments Hae III-A and Hae III-F are adjacent to each other (14; *cf.* Figure 8), it is reasonably certain that in the f1 RF molecule the endoR.*Hae* III cleavage site between the f1 analogues of M13

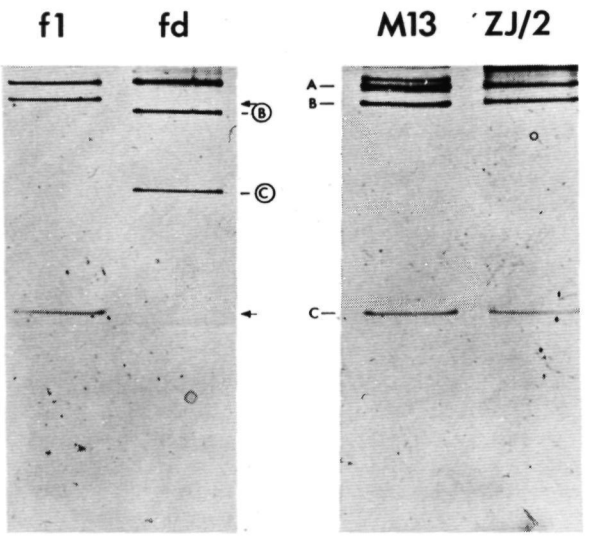

Fig. 3.: *Electrophoresis of fragments produced by the digestion of various phage RF-I with endonuclease R.*Hae *II*. The conditions for endoR.*Hae* II digestion of various phage RF-I were as before (legend to Fig. 1). Before analysis, each sample was enriched with 0.01 volume of 10% Sarcosyl and subsequently layered on a 2.5% to 7.5% polyacrylamide gradient gel, which was prepared as described by Jeppesen (21). Electrophoresis was for 24 h at 20 mA. The capital letters A through C refer to the positions of the endoR.*Hae* II fragments of M13 RF. The function of the symbols is as before (legend to Fig. 1).

fragment Hae III-A and Hae III-F is absent, which in turn, gives rise to the appearance of a slightly larger fragment Hae III-A.

EndoR.*Hae* II, a second restriction endonuclease from *H.aegyptius*, cleaves M13 RF at only three specific sites yielding the fragments Hae II-A, Hae II-B and Hae II-C, respectively. The sizes of these fragments are given in Table 1.

The physical order of endoR.*Hae* II fragments has been determined for phage M13 (17) and for f1 (20) and both appear to be identical (Figure 8). Upon electrophoresis on a linear 2.5-7.5% polyacrylamide gradient gel, the endoR.*Hae* II digest mixture of ZJ/2 RF gives a cleavage pattern which is identical to the pattern of M13 and f1 (Figure 3). Hence, it is very probable that the order of fragments given for M13 and f1 is valid also for ZJ/2.

The pattern of fd RF, however, deviates in such a way that the fd fragment Hae II-B (1900 basepairs) is smaller and the Hae II-C fragment (760 basepairs) is larger than the respective M13 counterparts. Digestion of the individual endoR.*Hae* II fragments of fd with endoR.*Hap* II, endoR.*Hae* III and endoR.*Alu* I and subsequent analyses of the double-digest mixtures have shown that only the

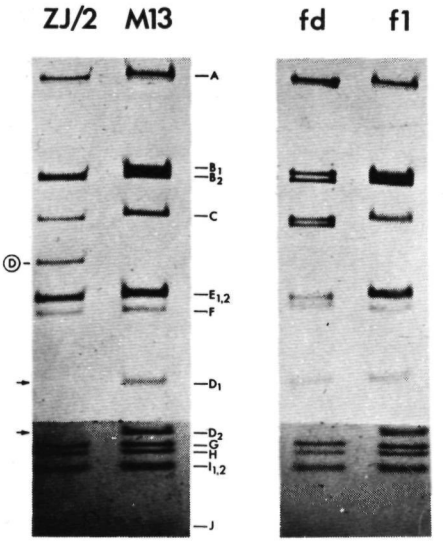

Fig. 4.: *Electrophoresis of double-digest fragments of various phage RF-I, produced with endonuclease R.*Hind *II and endonuclease R.*Hap *II.* Double digestion of phage RF with endoR.*Hind* II and endoR.*Hap* II was performed as described previously (14). The conditions for electrophoresis of double-digest fragments were identical to those described in the legend of Fig. 1. The capital letters A through J refer to the positions of the double-digest fragments of M13 RF. The function of the symbols is as before (legend to Fig. 1).

cleavage site between Hae II-B and Hae II-C in fd is located in a position which is not identical to the position of the corresponding cleavage site in M13 (van den Hondel, unpublished data).

Cleavage pattern of M13, fd, f1 and ZJ/2 with endonuclease R.*Hind* II

We have demonstrated previously (13, 14) that endoR.*Hind* II has a single cleavage site on the M13 RF molecule. From electrophoretic analysis of a double digest of M13 RF with both endoR.*Hap* II and endoR.*Hind* II, we established that this cleavage occurs in fragment Hap II-D (14). The latter fragment, being absent in the double-digest pattern, is split up into two new M13 fragments, designated D_1 and D_2 with respective sizes of 300 and 260 basepairs (Figure 4).

An identical cleavage pattern to M13 is observed when f1 RF is double digested with both endoR.*Hind* II and endoR.*Hap* II. Since the positions of the f1 analogues of the M13 double-digest fragments D_1 and D_2 correspond exactly with the positions of their M13 counterparts, it is clear that the unique endoR.*Hind* II cleavage site,

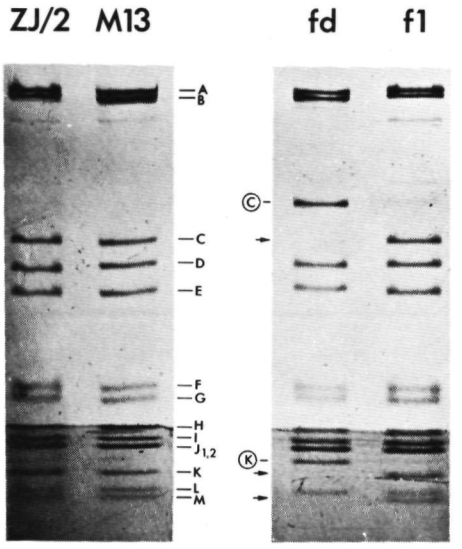

Fig. 5. *Electrophoresis of fragments produced by the digestion of various phage RF-I with endonuclease R.*Alu *I.* The conditions for endoR.*Alu* I digestion and electrophoresis of fragments were exactly identical to those described in the legend of Fig. 1. The capital letters A through M refer to the positions of the endoR.*Alu* I fragments of M13 RF (The fragments N are not visible on this gel). The function of the symbols is as before (legend to Fig. 1).

present in f1 and M13, is located in exactly the same position on the genome.

Phage fd RF is cleaved also at a single site by endoR.*Hind* II (34, 39). This site is located in the fd analogue of fragment Hap II-D, designated Hap II-F (*cf.* Fig. 1). Since this fragment is the only one which is missing in the double-digest mixture (Fig. 4) and gives rise to two new fragments, of which one comigrates with its M13 counterpart D_1, we conclude that the position of the endoR.*Hind* II cleavage site in fd is identical to M13. The difference existing between the two cleavage patterns can readily be explained on the basis of differences already detected in the endoR.*Hap* II digests of M13 and fd RF.

In contrast to M13, f1 and fd, the RF of phage ZJ/2 contains no cleavage site for the endoR.*Hind* II. This conclusion is based on the fact that covalently closed RF I of ZJ/2 is not split into full-length linear RF III molecules upon digestion with endoR.*Hind* II (van den Hondel, unpublished data). Consequently, the cleavage pattern of the endoR.*Hap* II digest of ZJ/2 RF does not change after prolonged incubation with the endoR.*Hind* II (see Fig. 1 and 4).

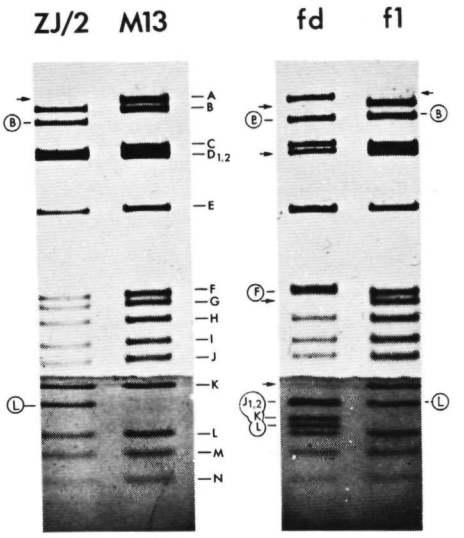

Fig. 6.: *Electrophoresis of fragments produced by the digestion of various phage RF-I with endonuclease R.*Hha. The conditions for endoR.*Hha* digestion and electrophoresis of fragments have been described in the legend to Fig. 1. The capital letters A through N refer to the positions of the endoR.*Hha* fragments of M13 RF. The function of the symbols is as before (legend to Fig. 1).

Cleavage pattern of M13, fd, f1 and ZJ/2 with endonuclease R.*Alu* I

As is shown in Figure 5, endoR.*Alu* I, a restriction endonuclease from *A. luteus*, generates 18 fragments of M13 RF which can readily be separated on the discontinuous polyacrylamide gel. All bands are singlets, except for band J which contains the doublet fragments Alu-J_1 and J_2, and band N (not shown) which appears to be a quartet of DNA fragments, the sizes of which are limited to about 20-30 basepairs (Table 1). The physical order of the endoR.*Alu* I fragments of M13 RF has recently been determined (17) and is given in Figure 8.

The cleavage patterns of f1 and ZJ/2 RF, obtained with endoR.*Alu* I are completely identical to the pattern of M13 RF (Fig. 5). This is in contrast to the case with fd RF. The fd analogues of the M13 fragments Alu I-C (600 basepairs), Alu I-K (160 basepairs) and Alu I-M (115 basepairs) are missing in the digest of fd RF. On the other hand, two new fd fragments can be distinguished, namely Alu I-C (705 basepairs) and Alu I-K (180 basepairs). It has to be emphasized, that the length of the former new fd fragment is about equal to the sum of the length of the M13 fragments Alu I-C (600 basepairs) and Alu I-M (115 basepairs). The size of

the other new fd fragment Alu I-K equals the sum of M13 fragments Alu I-K (160 basepairs) and Alu I-N (20 basepairs). This, together with the fact that the physical order of the missing M13 fragments is - C - M - N - K - (Figure 8) makes it very probable that two endoR.*Alu* I cleavage sites are missing in the fd RF molecule. One site is located between the fd analogues of M13 fragment Alu I-C and Alu I-M and the other between the fd analogues of Alu I-N and Alu I-K.

Cleavage pattern of M13, fd, f1 and ZJ/2 with endoR.*Hha* restriction endonuclease

EndoR.*Hha*, a restriction endonuclease from *H.haemolyticus*, cleaves M13 RF at 15 specific sites to yield 15 fragments, which have been named Hha-A through N (Fig. 6). From analysis of the ^{32}P-label content of each band *versus* the electrophoretic mobility of each fragment, it could be deduced that all the bands are singlets except for band D which actually is composed of two fragments D_1 and D_2 of nearly equal size. The lengths of the individual endoR.*Hha* fragments of M13 RF are given in Table 1. The physical order of these fragments is not known.

Comparing the fd and M13 digests, at least four fd analogues of M13 fragments are missing in the fd digest while six new fragments of fd can be detected (Fig. 6).

An identical cleavage pattern is observed between the endoR.*Hha* digestion products of f1 and ZJ/2 RF. Both patterns, however, deviate from the cleavage pattern of M13 RF (Fig. 6). In ZJ/2 and f1 the analogue of the M13 fragment Hha-A (990 basepairs) is missing, while two new fragments, Hha-B (820 basepairs) and Hha-L (160 basepairs), are present. The sum of the lengths of the latter fragments is equal to the length of M13 fragment Hha-A. From this we infer that the positions of Hha-B and Hha-L are adjacent in f1 and ZJ/2 RF and that this cleavage site between both fragments is the only one which is missing in M13 RF.

Cleavage pattern of M13, fd, f1 and ZJ/2 with endonuclease R.*Hinf*

EndoR.*Hinf*, a restriction endonuclease from *H.influenzae* strain Rf, cleaves M13 RF into at least 21 fragments, the electrophoretic separation of which is illustrated in Figure 7. Based upon analysis of the ^{32}P-content of each band, we have concluded that most bands are singlets except for band E, H, I, J and P which contain doublet fragments of almost equal size. The lengths of the endoR.*Hinf*

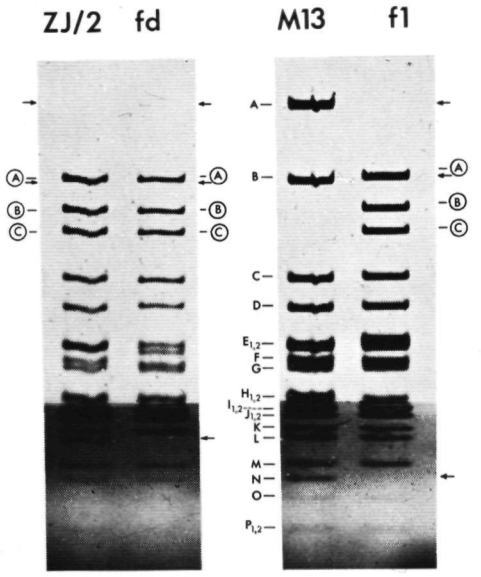

Fig. 7.: *Electrophoresis of fragments produced by digestion of various phage RF-I with endonuclease R.*Hinf. The conditions for endoR.*Hinf* digestion and electrophoresis of fragments have been described in the legend to Fig. 1. The capital letters A through P refer to the positions of the endoR.*Hinf* fragments of M13 RF. The function of the symbols is as before (legend to Fig. 1).

fragments of M13 RF, as calculated from their ^{32}P-content, are given in Table 1.

In contrast to the restriction endonucleases applied in the previous sections, endoR.*Hinf* generates a cleavage pattern of RF which appears to be different for each of the four phages. The most predominant differences are within the pattern of M13 RF, *i.e.* the presence of a large fragment, designated Hinf-A (1320 basepairs), which appears to be unique for M13 and the absence of three additional large fragments, namely Hinf-A, B and C, which are characteristic for fd, f1 and ZJ/2.

Characteristic differences are also noted among the patterns of f1, fd and ZJ/2. The most striking difference is the presence of fragments Hinf-L and Hinf-N in the ZJ/2 digest, whereas the former is absent in fd while the latter is missing in f1. Differences are also noted in the region containing band E, F and G. The M13 doublet fragments constituting band E are clearly separated in fd, but not in ZJ/2. In f1, band E is a doublet which runs ahead of a new fragment, the size of which is slightly larger than the doublet E fragments. Due to the relatively large number of doublet fragments present in the endoR.*Hinf*-digest mixture of each phage, it can not be ascertained yet whether additional differences exist among the

cleavage patterns of these phages.

Cleavage of M13, fd, f1 and ZJ/2 with other restriction endonucleases

Several other restriction endonucleases have been studied for their capability to cleave the various phage RF's so as to produce cleavage patterns in which characteristic differences for each of the phage molecules might be distinguished.

Digestion of phage RF with endoR.*Eco* RI does not cause fragmentation of either one of the RF molecules. Similar results were observed with endoR.*Hsu* from *H.suis* and endoR.*Sma* from *S.marcescens*. Apparently, these enzymes have no cleavage site on RF molecules of either one of the phages studied.

DISCUSSION

In this study, the restriction enzyme cleavage patterns were studied which were produced upon digestion of RF derived from the closely related filamentous coliphages M13, fd, f1 and ZJ/2. The enzymes applied were endoR.*Hap* II, endoR.*Hae* II, endoR.*Hae* III, endoR.*Hind* II, endoR.*Alu* I, endoR.*Hha*, endoR.*Hinf*, endoR.*Eco* RI, endoR.*Sma* and endoR.*Hsu*. Except for the latter three enzymes, all restriction endonucleases were able to cleave the various phage RF molecules into specific but different sets of fragments. In order to correlate these differences, we considered it useful to compare the respective patterns in more detail, and hence, to construct additional cleavage maps of f1, fd and ZJ/2 as well. Moreover, constructing such maps and tracing their differences might be of value for similar studies on other groups of filamentous bacterial viruses, like the *Pseudomonas* phage Pf1 and I-pili specific phages, the genetic content of which has been suggested to be partially related to the DNA of the F-specific coliphages (3, 48).

In previous studies we ordered the endoR.*Hind* II, endoR.*Hae* III, endoR.*Hap* II and the endoR.*Alu* I fragments of M13 RF into circular enzyme cleavage maps (14, 17). These maps have now been used here as a guide for constructing similar maps of fd, f1 and ZJ/2.

From the foregoing results and the results reported by others (20, 34, 41) we know that M13, fd and f1 all have a single cleavage site for endoR.*Hind* II. Furthermore, the location of this site is within fragment Hap II-D, which gives rise to fragments of which the largest fragment, namely D_1, is shared by all three phages (Fig. 4). This supports the previous conclusion of Seeburg and Schaller (34) that the location of this unique endoR.*Hind* II site is identical in

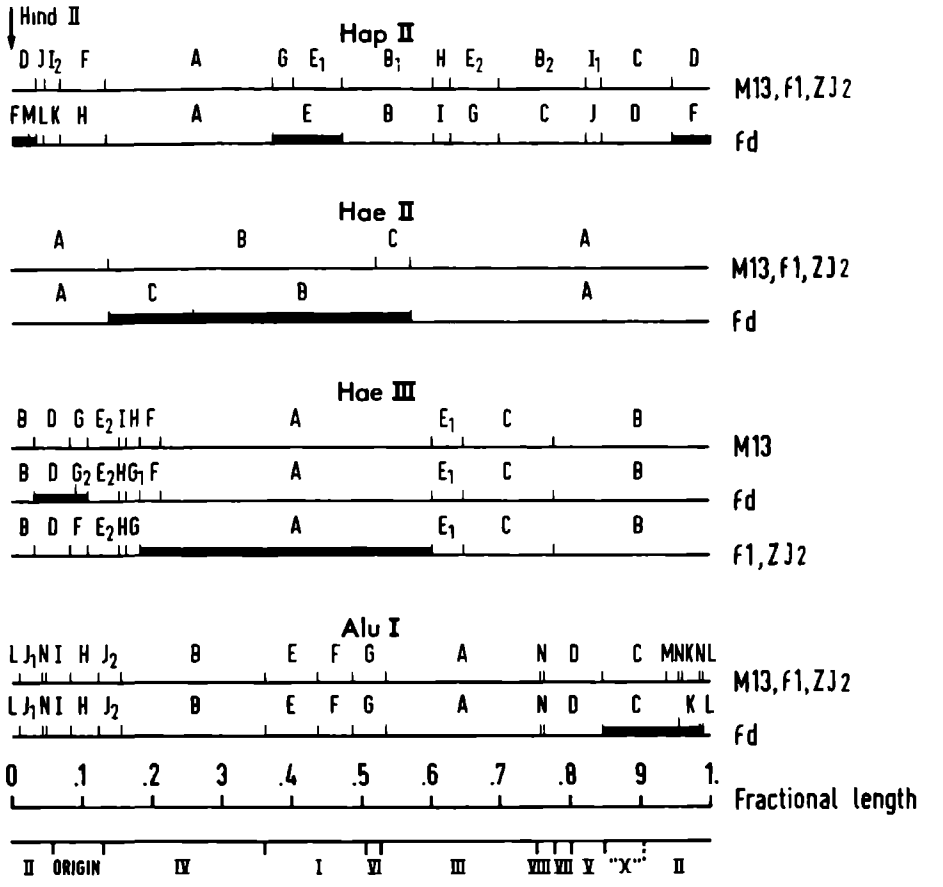

Fig. 8.: *Endonuclease R.*Hap *II, endoR.*Hae *II, endoR.*Hae *III and endoR.*Alu *I cleavage map of bacteriophage M13, fd, f1 and ZJ/2.* The phage RF fragments produced by each enzyme are denoted by capital letters and have been numbered for each phage individually in order of decreasing fragment size. In addition, arabic numerals are used as a suffix in order to designate fragments of (nearly) equal size, *i.e.* E_1, E_2 etc. The horizontal lines represent the physical order of fragments, the sizes of which are denoted by the vertical lines. The length of each genome is divided into ten equal map units with the endoR.*Hind* II cleavage site as the reference (zero) point (14, 20, 34, 41). The presented physical order of endoR.*Hap* II, endor.*Hae* II, endoR.*Hae* III and endoR.*Alu* I fragments of M13 RF have been determined previously (14, 17). The order of endoR.*Hap* II fragments of fd (34, 41) and of f1 (34), as well as the order of endoR.*Hae* II and endoR.*Hae* III fragments of f1 (20) have been taken from the results already reported by other groups.

Black bars denote fragments, present in phage RF, which are missing in M13 RF. Roman numerals refer to the filamentous phage genes, the order and size of which are represented by the bottom line. "Origin" means an intergenic space containing the origin of phage RF replication (17, 44).

each of the phage genomes. Therefore, the selection of the unique *Hind* II site as a reference point (zeropoint) in the physical maps of M13, f1 and fd, as indicated in Figure 8, has real physical sense. It should be noted that ZJ/2 RF has no cleavage site for endoR.*Hind* II. The zeropoint of the ZJ/2 map, therefore, is arbitrarily chosen to coincide with the zeropoints of the other maps.

From the digest patterns, presented in Fig. 1, it is clear that the endoR. *Hap* II cleavage maps of f1 and ZJ/2 are identical to the M13 map (Fig. 8). The differences between M13 and fd are due to an additional scission in the fd analogue of fragment Hap II-D at a distance of about 30 nucleotides from the cleavage site between Hap-D and Hap-J (map position 0.025) and a missing scission at map position 0.40, between fragment Hap II-E_1 and Hap II-G. The location of the new fd fragments Hap II-E, Hap II-F and Hap II-M are presented in the physical map of fd (Fig. 8). The same differences in the M13, f1 and fd cleavage maps have been found by Seeburg and Schaller (34) using endoR.*Hpa* II instead. It can not be excluded that besides the major differences already given, some minor differences exist between the cleavage maps of M13 and fd. For instance, it has repeatedly been observed that the fd analogue of fragment Hap II-F is slightly larger than the M13 (and f1, ZJ/2) counterpart (*cf.* Fig. 1 and 4). Since the position of fragment Hap II-I_2, which is contiguous to Hap II-F, is identical in both the fd and M13 cleavage pattern, it is assumed that the fd analogue of Hap II-A is smaller than Hap II-A itself. A difference in size of about 20-30 basepairs between both large fragments, however, can not be observed reliably on the polyacrylamide gels.

The differences in the endoR.*Hae* III cleavage maps of M13 and fd originate from a missing enzyme cleavage site in fd, which is located between the M13 fragments Hae III-D and Hae III-G (map position 0.08), and an additional cleavage site in the fd analogue of M13 fragment Hae III-G, most probably located at about 60 basepairs from the missing site (map position 0.09). These data account for the existence of the fd fragments Hae III-D and Hae III-G_2 (Fig. 8). Combination of these data with the M13 cleavage map gives the endoR.*Hae* III cleavage map of fd

The observed differences in the cleavage pattern of M13 on the one hand and ZJ/2 on the other, are caused by a missing endoR.*Hae* III cleavage site between the f1 and ZJ/2 analogues of fragment Hae III-A and Hae III-F. This results in the appearance of a f1 fragment Hae III-A which is slightly larger than the M13 analogue (Fig. 2). Consequently, the endoR.*Hae* III cleavage map of f1 and ZJ/2 deviates from the M13 map by a missing scission at map position 0.22 (Fig. 8). A similar difference in the fragmentation of f1 and M13 by endoR.*Hae* III was recently noted by Horiuchi *et al* (20).

From the results already reported by Zinder and coworkers (20) together with our results (17) we have concluded that the physical order of endoR.*Hae* II fragments of M13 RF is identical to f1. We conclude now that the physical order of ZJ/2 is also identical to M13. This is based upon the observations (van den Hondel, unpublished results), that digestion of the individual endoR.*Hae* II fragments of ZJ/2 with a second endonuclease generates a set of double-digest fragments which can not be distinguished from a similarly preparated set of M13 or f1. This, however, appears not to be the case for fd. We infer from the results of such analyses, that the observed difference in the endoR.*Hae* II cleavage pattern of fd is caused by a missing cleavage site at map position 0.53 and an additional scission at position 0.26, which gives rise to the appearance of a smaller Hae II-B fragment and a larger Hae II-C fragment (Fig. 8).

It is worth mentioning that the positions of the endoR.*Hae* II cleavage sites in fd, as deduced here, coincide, with the endoR.*Hin*H-1 cleavage sites in fd, as reported by Takanami *et al.* (41). This is in agreement with a recent report of Roberts *et al.* (33) which showed that endoR.*Hin*H-1 is an isoschizomer of endoR.*Hae* II.

As judged from the positions of the respective band on the polyacrylamide gel, we have concluded that the endoR.*Alu* I cleavage patterns of M13, f1 and ZJ/2 are identical. The pattern of fd RF, however, deviates in that two endoR. *Alu* I cleavage sites are missing.One site is absent between the fd analogues of fragment Alu-C and Alu-M, which results in the appearance of the fd fragment Alu-C. The other is missing between the analogues of Alu-K and Alu-N, which leads to the presence of a new fragment Alu-K. The endoR.*Alu* I cleavage map of fd, therefore, is identical to the M13 map except for the cleavage site at map position 0.94 and a site which is located either at map position 0.96 or 0.99 (Fig. 8).

As expected, the constructed cleavage maps of these phages are indeed very similar but not identical. The vast majority of cleavage sites coincide exactly within each particular map, although some differences in cuts were noted. These differences were found to occur at random over the phage DNA molecule. Interestingly, as far as differences in cleavage pattern is concerned, phage f1 and ZJ/2 revealed the highest degree of homology. The number and distribution of target sequences, recognized by each enzyme, were found to be identical for seven different restriction enzymes tested. It is apparent also that the patterns of f1 are more similar to M13 than the patterns of fd. These findings are somewhat different from the results reported by Tate and Peterson (42). Their data on pyrimidine-tract analysis suggest a more close relationship between fd and M13

than f1. Whether a simple comparison of either pyrimidine tracts or patterns of restriction enzyme fragments may be misleading as a measure of similarity between several DNA molecules, cannot be ascertained yet. In this respect the results have to be mentioned (7, 9) of similar comparative studies within the group of single-stranded DNA-containing icosahedral coliphages like ϕX-174, S13 and the G phages. The cleavage patterns of ϕX-174 and G4, a phage most distantly related to ϕX-174, have an overall dissimilarity of fragment sizes. Nevertheless, Godson's finding (7), that the cleavage maps of both phages can be superimposed and rotated in such a way that a considerable similarity of restriction cuts is observed, already indicates that phages can diverge considerably, but still remain a very similar pattern of cleavage sites.

From the data presented in this report, it is clear that endonuclease R.*Hinf* can discriminate between these phages since this enzyme gives rise to a set of fragments which is different for each of the four phage RF molecules (Fig. 7). The same arguments are valid for a combination of endoR.*Hae* III and endoR.*Hind* II or endoR.*Hha* and endoR.*Hind* II. Since the filamentous phages are very closely related and, hence, most of the biological properties tested so far are insufficient to distinguish these phages, a digestion with this restriction enzyme appears to be a new and powerful test of phage identification.

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In this thesis, the location of the eight structural genes and of some regulatory elements which contain information for replication and transcription on the genome of filamentous bacteriophage M13 is studied. This investigation was carried out to further an understanding of the mechanisms involved in the expression and multiplication of the M13 genome.

A short, general,introduction to the biology of the F-specific filamentous phages, in particular M13, is given in Chapter I.

In Chapter II the results of studies to determine the extent to which M13 DNA is sensitive to and can be fragmented by restriction endonucleases are described. Digestion with the restriction enzymes from *Haemophilus influenzae* (endoR.*Hind* II), *Haemophilus aegyptius* (endoR.*Hae* III) and *Haemophilus aphrophilus* (endoR.*Hap* II) produced 1, 10 and 13 fragments respectively. The 13 endoR.*Hap* II fragments were ordered into a physical map by analysis of partial digest products, while the 10 endoR.*Hae* III fragments were localized on this map by analysis of overlapping sets of fragments. In addition, the unique cleavage site of the restriction enzyme endoR.*Hind* II in double-stranded M13 DNA was localized on the map.

The arrangement of the M13 genes into a genetic map has been determined in Chapter III. This arrangement could be achieved by analyzing the genetic content of the various Hap II and Hae III fragments. The genetic content of each fragment was determined in a genetic fragment assay in which wild type double-stranded DNA fragments are annealed to mutant single-stranded viral DNA. The hybrids are used to infect calcium-chloride-treated *Escherichia coli* cells. The production of wild type phages in the progeny indicates the presence of wild type allele on the tested fragment. From the results of this fragment assay and the cleavage maps of both types of restriction fragments a distribution of genetic markers along the physical map could be obtained. By combining these results and taking into account the individual gene sizes, the M13 genes could be arranged into a genetic map. This map is in agreement with a map previously published, except that the order of gene V and gene VII should be reversed.

In Chapter IV the possibility of expressing the information content of each Hap II and Hae III fragment *in vitro* has been explored. Hence, the template activity of these restriction fragments in a "coupled" transcription-translation system was determined. From the analyses of the *in vitro* products synthesized in this system, it was concluded that fragment Hap II-A directs the synthesis of gene IV protein, fragment Hap II-B_2 of gene VIII protein, fragment Hae III-B of gene V

protein and fragments Hap II-C and Hae III-B of the polypeptide, designated "X-protein". The genetic origin of this protein is unknown, but is undoubtedly a M13-specific product. Furthermore, it was concluded that the double-stranded M13 DNA is transcribed in a counterclockwise direction around the genetic map and that promoter sites are located on the genetic map before gene IV, the gene VII/gene VIII boundary and the "gene" coding for"X-protein".

In Chapter V we describe the determination of the physical order on the M13 genome of fragments which were produced by digestion of double-stranded M13 DNA with the restriction endonucleases from *Haemophilus aegyptius* (endoR.*Hae* II) and from *Arthrobacter luteus* (endoR.*Alu* I) respectively. The ordering of fragments into physical maps was achieved by (1) using each fragment as a primer for the *in vitro* synthesis of its respective neighbour and (2) by digestion of the isolated fragments with restriction endonucleases endoR.*Hae* III or endoR.*Hap* II and subsequently analyzing the overlapping sets of fragments. The cleavage maps were correlated with the M13 genetic map by the genetic fragment assay of the purified wild type fragments. From the results, obtained with the fragment assay, it was concluded that gene II and gene V are contiguous on the genetic map and that the C-terminal region of gene II codes for the "X-protein". Furthermore, we concluded that there is an intergenic space of considerable length (450-500 basepairs) on the M13 genome. This intergenic space was found to be located between gene II and gene IV.

In Chapter VI the localization of the origin of the complementary strand synthesis during the double-stranded M13 DNA replication on the M13 genome is described. It was shown, as a result of analysis of the label distribution in labeled replicative form (RF-I) M13 DNA, isolated shortly after the start of the replicative form DNA replication, that primarily the complementary strand of these RF-I molecules was labeled. This analysis also indicated that the complementary strand synthesis is unidirectional and clockwise around the genetic map. Furthermore, it was concluded that the origin of the complementary strand synthesis of the replicative form DNA replication is located in the intergenic region between gene IV and gene II.

In Chapter VII we studied the similarity between the DNA molecules of the bacteriophages M13, fd, f1 and ZJ/2 by analyzing their sensitivity to the restriction endonucleases endoR.*Hap* II, endoR.*Hae* II, endoR.*Hae* III, endoR.*Hind* II, endoR.*Alu* I, endoR.*Hha*, endoR.*Hinf*, endoR.*Hsu*, endoR.*Eco* RI and endoR.*Sma*. In M13 DNA the number of cleavage sites varied from 21 for endoR.*Hinf*, 18 for endoR.*Alu* I, 15 for endoR.*Hha*, 13 for endoR.*Hap* II, 10 for endoR.*Hae* III, 3 for endoR.*Hae* II to only one site for endoR.*Hind* II. In contrast to M13, fd and f1, the ZJ/2 DNA

molecule was not cleaved by endoR.*Hind* II. None of the phage DNAs was cleaved by the endonucleases endoR.*Hsu*, endoR.*Eco* RI and endoR.*Sma*. When compared to M13 DNA, several differences were noted in the number and sizes of cleavage products obtained with DNA of phage fd, f1 and ZJ/2. From the results of these analyses, using the M13 enzyme cleavage maps as a reference, the endoR.*Hap* II, endoR.*Hae* II, endoR.*Hae* III, endoR.*Hind* II and endoR.*Alu* I maps of the phages fd, f1 and ZJ/2 DNA could be obtained. Since characteristic differences exist within the endoR.*Hinf* enzyme cleavage pattern of all four phage DNAs, digestion with this enzyme provides a new and sensitive method of distinction of these closely related, filamentous coliphages.

In dit proefschrift wordt de ligging van de acht structurele genen en van enige "regulatory elements", die informatie bevatten voor de replicatie en transcriptie, op het genoom van de filamenteuze bacteriofaag M13 bestudeerd. Dit onderzoek werd uitgevoerd om een beter inzicht te verkrijgen in de mechanismen die betrokken zijn bij de vermenigvuldiging en het tot expressie komen van het M13 genoom.

In Hoofdstuk I wordt een korte, algemene, inleiding gegeven over de biologie van F-specifieke, filamenteuze bacteriofagen, en wel in het bijzonder van M13.

In Hoofdstuk II worden de resultaten beschreven van het onderzoek dat uitgevoerd werd om te bepalen in welke mate M13 DNA gevoelig is voor en gesplitst wordt door restrictie enzymen. Digestie met de restrictie enzymen uit *Haemophilus influenzae* (endoR.*Hind* II), *Haemophilus aegyptius* (endoR.*Hae* III) en *Haemophilus aphirophilus* (endoR.*Hap* II) leverde respectievelijk 1, 10 en 13 fragmenten op. De 13 endoR.*Hap* II-fragmenten werden in een fysische kaart gerangschikt door analyse van partiële digestie producten, terwijl de 10 endoR.*Hae* III-fragmenten op deze kaart werden gelocaliseerd door de overlappende gebieden van de Hap II- en Hae III-fragmenten te analyseren. Bovendien werd de unieke splitsingsplaats van het restrictie enzym endoR.*Hind* II op de kaart gelocaliseerd.

De rangschikking van de M13 genen in een genetische kaart is bepaald in Hoofdstuk III. Deze rangschikking kon worden bepaald door de genetische inhoud van de verschillende Hap II- en Hae III-fragmenten te analyseren. De genetische inhoud van ieder fragment werd bepaald met behulp van een genetische fragmenten test, waarin de Hap II- en Hae III-fragmenten afzonderlijk aan enkelstrengs DNA van M13 mutanten werden gehybridiseerd. Met deze hybriden werden *E.coli* cellen, die met calciumchloride behandeld waren, geïnfecteerd. De productie van wild type fagen was een aanwijzing voor de aanwezigheid van het wild type allel op het geteste fragment. Tesamen met de splitsingskaarten van de Hap II- en Hae III-fragmenten, leverden de resultaten van de fragmenten test een verdeling van genetische markers over de fysische kaart op. De combinatie van deze gen verdeling met de gen grootten maakte het mogelijk de genen van M13 in een genetische kaart te rangschikken. Deze kaart is in overeenstemming met een reeds eerder gepubliceerde kaart; de volgorde van gen V en gen VII bleek echter omgekeerd te zijn.

In Hoofdstuk IV is de mogelijkheid onderzocht om de informatie, die de verschillende Hap II- en Hae III-fragmenten bevatten, *in vitro* tot expressie te brengen. Daartoe werd de template activiteit van de endoR.*Hap* II-fragmenten en

de endoR.*Hae* III-fragmenten in een "gekoppeld" transcriptie-translatie systeem bestudeerd. Analyse van de *in vitro* gesynthetiseerde producten in dit systeem toonde aan, dat het fragment Hap II-A codeert voor het gen IV eiwit, het fragment Hap II-B_2 voor het gen VIII eiwit, het fragment Hae III-B voor het gen V eiwit en de fragmenten Hap II-C en Hae III-B voor een polypeptide, genaamd "X-eiwit". Hoewel dit eiwit ongetwijfeld M13 specifiek is, is de genetische oorsprong ervan onbekend. Bovendien kon worden geconcludeerd, dat de transcriptierichting van dubbelstrengs M13 DNA ten opzichte van de genetische kaart tegen de wijzers van de klok in verloopt en dat er voor het gen IV,voor het grensgebied gen VII/gen VIII en voor het "gen", dat codeert voor het "X-eiwit", promoters gelegen zijn.

In Hoofdstuk V wordt beschreven hoe twee additionele splitsingskaarten werden bepaald van de restrictie fragmenten die verkregen werden door dubbelstrengs M13 DNA te digesteren met de restrictie enzymen uit *Haemophilus aegyptius* (endoR.*Hae* II) en uit *Arthrobacter luteus* (endoR.*Alu* I). Deze splitsingskaarten werden bepaald door (1) ieder fragment als "primer" te gebruiken voor de *in vitro* synthese van zijn naaste buur en (2) de geïsoleerde fragmenten te digesteren met endoR.*Hap* II of endoR.*Hae* III en vervolgens de overlappende gebieden van deze fragmenten te analyseren. De splitsingskaarten werden gecorreleerd met de genetische kaart van M13 door de fragmenten te testen in de genetische fragmenten test. Uit de resultaten van deze test kon worden geconcludeerd, dat gen II en gen V naast elkaar gelegen zijn op de genetische kaart en dat het gebied aan het C-terminale uiteinde van gen II codeert voor het "X-eiwit". Bovendien kon worden geconcludeerd, dat er een gebied, "intergenic region" genaamd, van aanzienlijke lengte (450-500 baseparen) tussen gen IV en gen II gelegen is,dat geen informatie voor een M13 eiwit bevat.

In Hoofdstuk VI wordt beschreven waar de origin van de complementaire streng synthese gedurende de dubbelstrengs M13 DNA replicatie op het M13 genoom gelocaliseerd is. Analyse van de radioactiviteitsverdeling in gelabeld circulair dubbelstrengs gesloten (RF-I) M13 DNA, geïsoleerd kort na de start van de RF replicatie, liet zien dat voornamelijk de complementaire streng van deze RF-I moleculen gelabeld was. Deze analyse wees er tevens op, dat de richting van de complementaire streng synthese ten opzichte van de genetische kaart met de wijzers van de klok mee verloopt. Bovendien kon worden geconcludeerd, dat de "origin" van de complementaire streng synthese gelegen is in de "intergenic region" tussen gen IV en gen II.

In Hoofdstuk VII is de homologie tussen de RF DNA's van de bacteriofagen M13, fd, f1 en ZJ/2 bestudeerd door de gevoeligheid voor de restrictie enzymen

endoR.*Hap* II, endoR.*Hae* II, endoR.*Hae* III, endoR.*Hind* II, endoR.*Alu* I, endoR.*Hha*, endoR.*Hinf*, endoR.*Hsu*, endoR.*Eco* RI en endoR.*Sma* te analyseren. Wat M13 DNA betreft, varieerde het aantal splitsingsplaatsen van 21 voor endoR.*Hinf*, 18 voor endoR.*Alu* I, 15 voor endoR.*Hha*, 13 voor endoR.*Hap* II, 10 voor endoR.*Hae* III, 3 voor endoR.*Hae* II tot slechts 1 voor endoR.*Hind* II. In tegenstelling tot M13, fd en f1 werd ZJ/2 DNA niet door endoR.*Hind* II gesplitst. Geen enkele splitsingsplaats van de restrictie enzymen endoR.*Hsu*, endoR.*Eco* RI en endoR.*Sma* werd op het DNA van de vier fagen aangetoond. Diverse verschillen werden waargenomen tussen het aantal en de grootte van de splitsingsproducten van fd, f1 en ZJ/2 enerzijds en die van M13 anderzijds. Door vergelijking van deze verschillen met de M13 splitsingskaarten konden de endoR.*Hap* II-, de endoR.*Hae* II-, de endoR.*Hae* III-, de endoR.*Hind* II- en de endoR.*Alu* I-kaarten van de fagen fd, f1 en ZJ/2 worden bepaald. Daar er karakteristieke verschillen bestaan in de endoR.*Hinf*-splitsingspatronen van de vier faag DNA's, werd geconcludeerd dat digestie met dit enzym een nieuwe en gevoelige methode is om deze nauwverwante filamenteuze colifagen van elkaar te onderscheiden.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 18 juli 1945 te Gouda. In 1963 werd het einddiploma H.B.S.-B aan het Aloysiuscollege te Den Haag behaald, waarna in hetzelfde jaar werd begonnen met de scheikunde-studie aan de Rijksuniversiteit te Utrecht. Het kandidaats examen, letter g, werd in maart 1968 afgelegd. In november 1971 werd het doctoraal examen afgelegd, met als hoofdvak Biochemie (Prof. Dr. J. Boldingh) en als bijvak Microbiologie (Prof. Dr. P. G. de Haan).

Per 1 december 1971 werd hij als wetenschappelijk medewerker aangesteld aan het Laboratorium voor Moleculaire Biologie van de Faculteit der Wis- en Natuurkunde van de Katholieke Universiteit te Nijmegen, waar het in dit proefschrift beschreven onderzoek werd uitgevoerd. Sinds 1 december 1975 is hij als wetenschappelijk medewerker werkzaam bij de Vakgroep Moleculaire Celbiologie van de Rijksuniversiteit te Utrecht om onderzoek te verrichten naar de gastheer-faag relatie bij Cyanophyceeën.

STELLINGEN

I

Het is waarschijnlijk dat de *in vivo* synthese van de complementaire streng tijdens de M13 dubbelstrengs DNA replicatie op dezelfde wijze verloopt als de complementaire streng synthese gedurende de *in vitro* omzetting van enkelstrengs M13 DNA in dubbelstrengs DNA.

K. Geider en A. Kornberg (1974) J. Biol. Chem. *249*, 3999-4005

Dit proefschrift

II

De conclusie van Martin en Godson, dat het A'-eiwit van bacteriofaag ϕX174 betrokken is bij het stopzetten van de gastheer DNA synthese, is aanvechtbaar.

D. F. Martin en G. N. Godson (1975) Biochem. Biophys. Res. Commun. *65*, 323-330

III

In hun model voor de buitenmembraan van *Salmonella typhimurium* RC-mutanten plaatsen Smit *et al.* ten onrechte alle fosfolipiden in een monolaag aan de binnenzijde van dit membraan.

J. Smit, Y. Kamio en H. Nikaido (1975) J. Bacteriol. *124*, 942-958

A. J. Verkley, E. J. J. Lugtenberg en P. H. J. Th. Ververgaert (1976) Biochim. Biophys. Acta *426*, 581-586

IV

De experimenten van Dunker en Anderson rechtvaardigen niet hun conclusie dat de stabiliteit van het gen 5 eiwit - DNA complex toeneemt bij lagere pH.

A. K. Dunker en E. A. Anderson (1975) Biochim. Biophys. Acta *402*, 31-34

V

De veronderstelling van Delaney en Carr, dat het genoom van de blauwgroenwier *Anacystis nidulans* zich bidirectioneel repliceert of unidirectioneel maar van meer dan één origin uit, wordt niet voldoende ondersteund door experimentele gegevens.

S. F. Delaney en N. G. Carr (1975) J. Gen. Microbiol. *88*, 259-268

VI

Uit de experimenten van Singh en Ray kan niet worden geconcludeerd dat de door hen geïsoleerde endonuclease een enkelstrengs endonuclease is.

S. Singh en D. S. Ray (1975) Biochem. Biophys. Res. Commun. *67*, 1429-1434

VII

Bij het door Liautard *et al.* verrichte onderzoek naar de binding van eiwitten aan heterogeen kern RNA van Hela cellen is geen rekening gehouden met de mogelijke binding van kern RNA aan nucleaire membraanstructuren.

J. P. Liautard, B. Setyona, E. Spindler en K. Köhler (1976) Biochim. Biophys. Acta *425*, 373-383

VIII

De twee door Dodgson *et al.* als nieuw aangemerkte genetische testen van niet-infectieuze DNA-fragmenten zijn niet nieuw.

J. B. Dodgson, I. F. Nes, B. W. Porter en R. D. Wells (1976) Virology *69*, 782-785

IX

Aangezien de gemiddelde lengte van de Nederlander gedurende de laatste dertig jaar is toegenomen, dient de hoogte van het volleybalnet hieraan aangepast te worden.

X

Het inhalen door vrachtwagens op de Nederlandse autosnelwegen dient ter vermijding van risico's voor medeweggebruikers aan strictere regels te worden onderworpen.

XI

De "groen-arme" verfraaiing die aangebracht wordt op het middenterrein van de Faculteit der Wiskunde en Natuurwetenschappen is in volledige harmonie met de architectonische betonconstructies die deze verfraaiing omhullen.

C. A. M. J. J. van den Hondel

Nijmegen, 10 juni 1976.