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Biochemistry

Filamentous coliphage M13 as a cloning vehicle: Insertion of a *Hin*dII fragment of the *lac* regulatory region in M13 replicative form *in vitro*

(single-stranded DNA phage/blunt end ligation/lactose operon/α complementation)

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ABSTRACT **A *Hin*dII restriction fragment comprising the *Escherichia coli lac* regulatory region and the genetic information for the α peptide of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) has been inserted into 1 of the 10 *Bsu* I cleavage sites of M13 by blunt end ligation. A stable hybrid phage was isolated and identified by its ability to complement the *lac* α function. Further characterization of the hybrid phage includes retransformation studies, agarose gel electrophoresis, DNA·DNA hybridization, and heteroduplex mapping. The insertion point has been localized at 0.083 map unit on the wild-type circular map—i.e., within the intergenic region. The results prove that part of the intergenic region is nonessential and that the phage can be used as a cloning vehicle.**

Two vector systems, plasmids of the ColE1 family or pSC 101 and the bacteriophage λ genome (1–8), have been developed to clone prokaryotic and eukaryotic DNA in *Escherichia coli*. Vectors generated from filamentous phages like M13 containing single-stranded circular DNA may offer some special advantages. Upon infection, phage DNA is converted into a double-stranded supercoiled replicative form (RF I) and amplified to about 300 copies per cell, and the phage-producing cells do not lyse but continue to replicate (9). In addition to the double-stranded DNA from the infected bacteria, single-stranded DNA can easily be obtained from the progeny phages, thus exerting a pronounced advantage over the isolation procedure of plasmid DNA from transformed bacteria. Because bacterial growth is slightly retarded by M13 infection, infected bacteria can be detected by "turbid-plaque" formation (9) and no selective pressure, as for instance for drug resistance factors, would be necessary to detect bacteria transfected by M13 DNA. Finally, due to the filamentous shape of the phage, it can be expected that additional DNA could be packed over a broad range of size.

The existence of a nonessential region in a phage genome can be considered as a useful precondition in constructing a viable hybrid phage (5). However, for M13, it is known that amber mutations in almost any gene lead to host killing under non-permissive conditions (9). Therefore, it was an open question if insertions could be made at any site of the M13 wild-type genome without loss of viability. In order to search for a potential nonessential region, a population of RF molecules with single cuts per molecule but at different sites might be used for *in vitro* insertions. After insertion of foreign DNA into one of those cleavage sites, a stable hybrid phage should be identifiable by an appropriate screening procedure. Furthermore, it would be desirable that the inserted DNA exhibit properties allowing eventual expression of additional DNA.

Following this concept, a nonessential region was detected within the intergenic region (10), and a hybrid phage containing the *Hin*dII fragment from the *lac* regulatory region (11) in a functional state (12) has been constructed. This hybrid phage is considered as an intermediate form in developing a safe vector from filamentous phages.

MATERIALS AND METHODS

E. coli K-12 71-18 (Δ[*lac,pro*], F′ *lac* I^qZ Δ M15 *pro*$^+$) was used as a host for M13 (13). Plasmid pMG 1106/71-18 is described in the *text;* RSF 2124/C 600 was provided by J. Collins. M13 (wild type) was from our own stock.

Preparation of supercoiled DNA was essentially as described (2) except for the use of 1% Triton X-100 as detergent in the lysis procedure. The cleared lysate was obtained by low-speed centrifugation of the lysed cells. After cesium chloride equilibrium centrifugation in the presence of ethidium bromide, the dye was extracted three times with 1-butanol before dialysis of the DNA solution. DNA was further purified over a neutral 5–20% sucrose gradient as described (14). Propagation and purification of M13 phage have been described (10). Isolation of viral single-stranded DNA and purification of λ $cI_{857}S_7$ was carried out as described (10, 15). DNA was isolated from the λ-transducing phage λ imm^{434}ts $susS_7b_{221}$ carrying the *lac* λ *c*I fusion (no. 22-2) and in the following termed 21_{II} DNA (13). λ $cI_{857}S_7$plac 5 DNA was kindly provided by J. Kania.

Restriction endonucleases *Eco*RI, *Bsu* I and *Hin*dII were purified and assayed according to Greene *et al.* (16), Bron *et al.* (17), and Phillipsen *et al.* (18), respectively.

T_4 DNA ligase was purchased from Miles Co. and assayed as described (2). Ligation of blunt ends was performed essentially as described (2) except for the amount of enzyme, which was increased from 0.1 to 8 nmol.

Purified *lac* repressor was a gift of J. Kania.

Slab gels (0.7 or 1.5%) were run in a vertical electrophoresis apparatus with Tris·borate buffer (16) containing ethidium bromide (0.5 μg/ml). The bands were visualized under a shortwave ultraviolet S 68 lamp (UV Products, San Gabriel, CA) and photographed with a Polaroid Land camera (CU 5).

The assay of the *lac* α function of the *Hin*dII fragment from the *lac* regulatory region was performed in *E. coli* 71-18. The F episome of this strain, in which the entire *lac* operon is deleted, carries the M15 deletion in the operator proximal region of the β-galactosidase (β-D-galactoside galactohydrolase, EC

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Abbreviations: RF, replicative form; IPTG, isopropyl-thiogalactoside; Mdt, megadaltons; bp, base pairs.

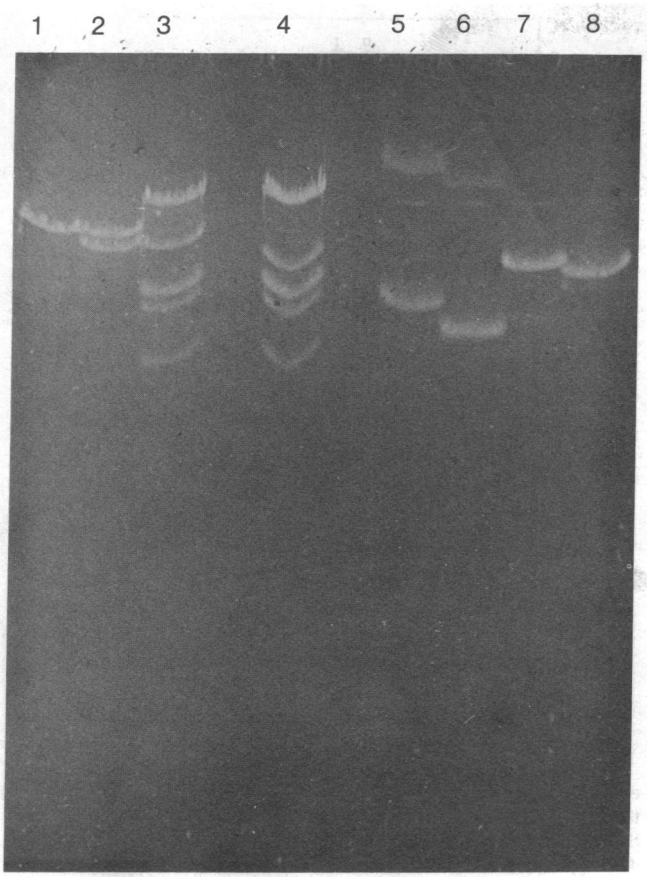

FIG. 1. Analysis of pMG 1106 and M13 mp1 RF by agarose gel electrophoresis. Lanes: 1–4: cleavage pattern of *Eco*RI digests—1, RSF 2124; 2, pMG 1106; 3, 21_{II}DNA; and 4, λ wild type. 5–6: uncleaved DNAs—5, M13 mp1 RF I; and 6, M13 wild-type RF I. 7–8: *Hin*dII-cleaved DNAs—7, M13 mp1 RF III; and 8, M13 wild-type RF III. Concentration of agarose in the slab gel was 0.7%.

3.2.1.23) gene. Therefore, in this strain, a defective β-galactosidase protein (lacking amino acid residues 11–41) is produced (12). In addition, *lac* repressor is overproduced by a factor of about 10 due to the I^q promoter mutation on the episome (13). The *Hin*dII fragment comprises the *lac* regulatory region and the base sequence for the first 145 amino acids of β-galactosidase (W. Gilbert, personal communication). It has been shown that the peptide produced by this fragment has α donor activity in intracistronic complementation of the M15 protein *in vitro* (19).

Hybrid phages were plated with *E. coli* 71-18 in soft agar containing isopropyl-thiogalactoside (IPTG), an inducer of the lactose operon, and 5-bromo-4-chloro-indolyl-β-D-galactoside, a colorless compound which, when hydrolyzed by β-galactosidase, releases deep blue 5-bromo-4-chloro-indigo (15). Thus, infection of cells by hybrid phages can be traced by the appearance of blue plaques.

According to the National Institutes of Health guidelines, these experiments were carried out under P1 conditions.

RESULTS

Preparation of the *lac Hin*dII Fragment. As a source of DNA for the *lac Hin*dII fragment, we used the chimeric plasmid pMG 1106. This plasmid is a derivative of RSF 2124 (8) containing an *Eco*RI fragment of a λ-transducing phage, in which the *lac* regulatory region is fused to the λ immunity region by *in vivo* recombination (13). It is able to exhibit α complementation of the β-galactosidase gene in an appropriate host and to overproduce λ repressor 70 times more than a normal lysogen.

The DNA could be cleaved by *Eco*RI in the two parental molecules, the vector and the inserted fragment with molecular masses of 7 megadaltons (Mdt) [11,200 base pairs (bp)] and about 6 Mdt (9600 bp), respectively (Fig. 1, lanes 1–3). The hybrid plasmid could be amplified by chloramphenicol treatment, and the high yield of plasmid DNA per cell facilitated the preparation of a sufficient amount of the appropriate *Hin*dII fragment. After digestion of pMG 1106 with *Hin*dII restriction endonuclease, the fragment carrying the *lac* operator was separated from the mixture of other DNA fragments by the *lac* repressor binding assay on nitrocellulose filters (19). The DNA fragment was eluted from the filter by IPTg, the allosteric effector of the *lac* repressor.

After this procedure, one fragment, of 0.5 Mdt, was retained on nitrocellulose filters as shown in Fig. 2. The eluted DNA fraction represented in lane 2 was treated with phenol, dialyzed extensively, and used for insertion into the RF of the filamentous phage M13.

Preparation of M13 RF *Bsu* I DNA of Unit Length. The RF of M13 was cleaved by *Bsu* I restriction endonuclease into 10 fragments which were ordered on a circular map (10). Cleavage was performed at room temperature with a limited amount of enzyme to create mainly RF molecules which were cut only once and still had full genome length. This class of molecules was separated from partial digests by agarose gel electrophoresis and potassium iodide equilibrium centrifugation (20).

Construction of the M13 Hybrid Phage by Blunt End Ligation of the Purified Restricted DNAs. The restriction endonucleases *Bsu* I and *Hin*dII recognize the sequences G-G-C-C and G-T-Py-Pu-A-C, respectively, and cleave these sites in their symmetry axes, generating blunt ends. After purification of the linearized RF molecules, the *Bsu* I ends of the DNA were joined to the *Hin*dII ends of the *lac* fragment by T_4 DNA ligase as described in *Materials and Methods*. The complete ligation mixture was used to transform *E. coli* strain 71-18 by using the calcium chloride technique (21). The cells were plated in soft agar containing IPTG and 5-bromo-4-chloro-indolyl-β-D-galactoside. After an incubation period of 48 hr, two blue plaques were found among several hundred colorless ones. From these, infected cells were seeded for single colonies and one clone, which was able to produce blue plaques again, was chosen for further analysis. This hybrid phage has been denoted "M13 mp1"; the first number after "mp" is reserved for modifications of the M13 mp as a cloning vehicle, whereas a second number separated from the first by / represents the individual DNA inserted in M13 mp *in vitro*.

Characterization of the Hybrid Phage M13 mp1. Phages from a purified bacterial clone were propagated and analyzed by centrifugation through a sucrose gradient. Because the inserted DNA should influence the particle size of the phage DNA only to a small extent, phage M13 mp1 sedimented nearly at the same position as wild-type M13 (30 S) (data not shown). Electron micrographs of negatively stained phages allowed precise measurement of the particle size. The length of the filaments of hybrid phages increased by about 12.5 ± 0.3% compared to the wild type. This increase corresponded to the molecular mass of the additional *Hin*dII DNA fragment (0.5 Mdt), which corresponds to 12% wild-type DNA.

Upon induction of the *lac* operon by IPTG, the hybrid phages caused formation of blue plaques in strain 71-18. In the absence of inducer (IPTG), the plaques were colorless like M13 wild-type plaques under the same condition. The stability of the

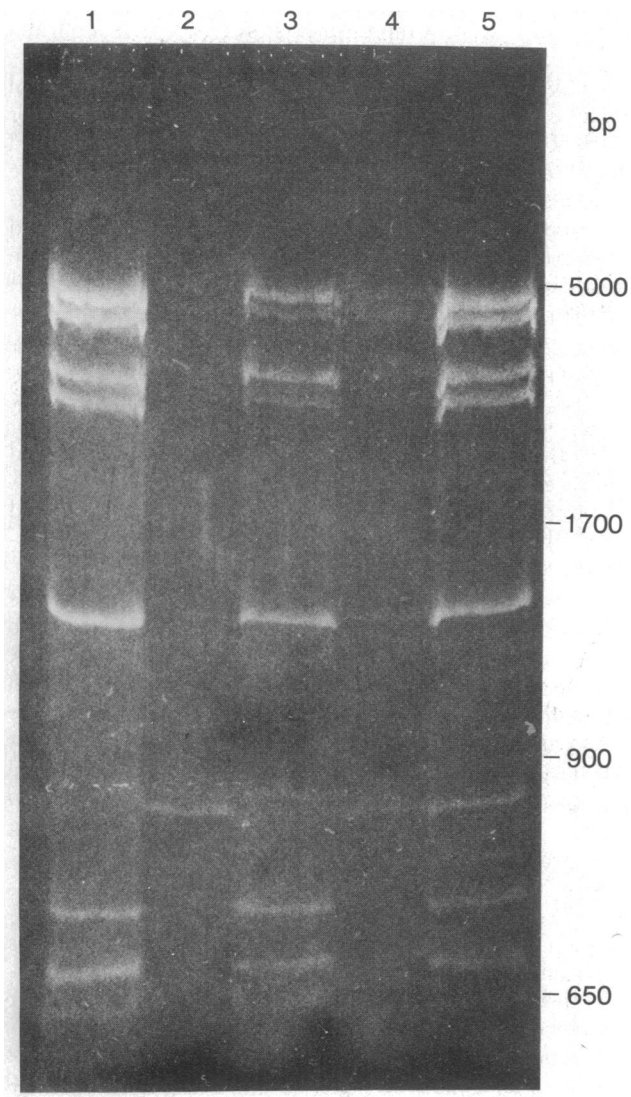

FIG. 2. *lac* repressor binding fragment from the plasmid pMG 1106 cleaved by *Hin*dII. About 100 μg of pMG 1106 was digested with *Hin*dII to completion. The reaction was stopped by heating the mixture to 65° and diluting into DNA binding buffer (19). Twelve micrograms of purified *lac* repressor was added to half of the DNA solution and 10 times more repressor to the other half. The mixtures were passed through nitrocellulose filters. Filter-bound DNA was eluted with binding buffer containing 1 mM IPTG. Aliquots of IPTG-eluted and nonretained DNAs were electrophoresed through a 1.5% agarose slab gel (lanes 1–4). Molecular weights of DNA fragments of pMG 1106 were calibrated with *Hpa* II-cleaved M13 RF DNA (not shown). Lanes: 1 and 2, nonretained and IPTG-eluted DNA, respectively, with 12 μg of *lac* repressor; 3 and 4, nonretained and eluted DNA, respectively, with 10-fold amount of *lac* repressor; and 5, unfiltered DNA.

inserted fragment in M13 mp1 was tested and confirmed by multiple passages. No differences in the growth rate of M13 mp1 and wild-type phage have been observed.

Characterization of Hybrid DNA. To characterize the hybrid DNA, RF I molecules were purified from infected cells. They sedimented in neutral sucrose gradients with approximately the same sedimentation constant as wild-type RF molecules (Fig. 3). After isolation from sucrose gradients, these molecules were able to transform strain 71-18 with an efficiency of 6×10^4 plaque-forming units/μg of DNA. A total of approximately 10^5 plaques were observed, and all of them were blue due to the α complementation in the host cells.

In contrast to sucrose gradient centrifugation, agarose gel

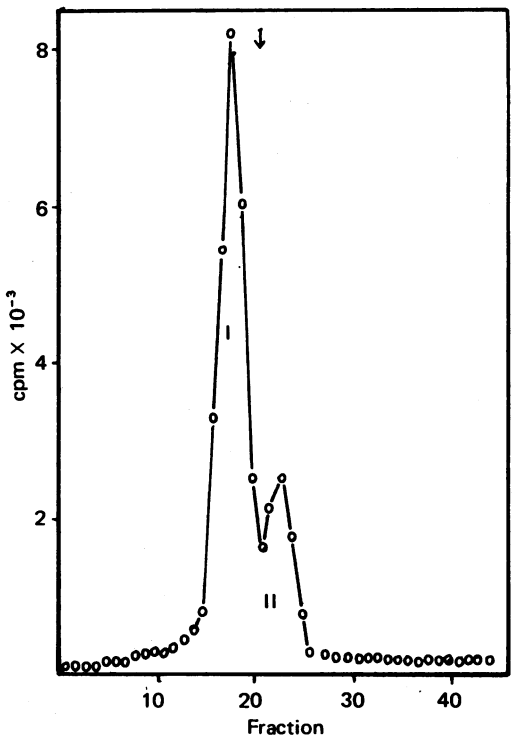

FIG. 3. Sedimentation of M13 mp1 RF DNA in a sucrose gradient. M13 mp1 RF DNA was labeled with ^{32}P (10) and then centrifuged through 5–20% sucrose gradients in TES buffer (0.01 M Tris·HCl, pH 8.0/1 mM EDTA/1.0 M NaCl) in a Spinco SW 27 rotor for 14.5 hr at 4°. ^{3}H-Labeled ΦX 174 RF DNA was included as a marker. Fractionation of the gradients and assay procedures have been described (14). Density increases from right to left. Position of the marker is indicated by an arrow. I and II correspond to RF I and RF II DNA, respectively.

electrophoresis resolved the hybrid and wild-type RF molecules by size. *Hin*dII endonuclease cleaved the hybrid molecules in one site like wild-type molecules. The hybrid molecules increased in size to about 12% resulting in a molecular mass of about 4.5 Mdt (Fig. 1, lanes 5–8). Hybridization to different DNA species showed that the additional DNA incorporated into M13 was indeed *lac* DNA. Single-stranded DNA of M13 mp1 hybridized to wild-type RF molecules and to λ-plac DNA but not to λ DNA used as an internal control; single-stranded DNA of wild-type M13 hybridized only to M13 mp1 RF molecules but to neither λ-plac nor λ DNA (Table 1).

Table 1. Hybridization of ^{32}P-labeled single-stranded DNA to RF DNA of wild-type and hybrid M13 phages, to λ, and to λ-plac DNA on filters

DNA, μg per filter	cpm hybridized to filter-bound DNA from ss M13 wt	ss M13 mp1
λ-plac, 1.5	9	123
λ, 1.5	8	11
RF wt, 0.25	5501	6415
RF mp1, 0.25	5529	6638

DNA·DNA hybridization was carried out on individual filters, loaded with the amounts of DNA indicated, for 12 hr at 65° in 1 ml of PM buffer as described (22). Input of single-stranded M13 and M13 mp1 DNA was 30 ng (both DNAs had a specific radioactivity of 0.9×10^6 cpm/μg DNA). See *Materials and Methods* and Fig. 3 for isolation of plasmid, phage, and RF DNA. ss, Single stranded; wt, wild type.

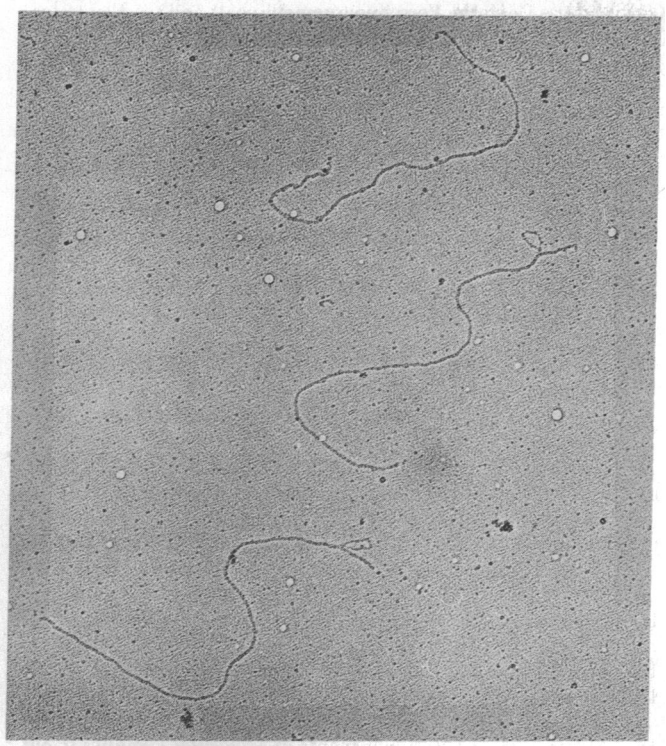

FIG. 4. Heteroduplex molecules of *Hin*dII-cleaved M13 RF and M13 mp1 RF. The replicative forms of M13 and M13 mp1 were cleaved by *Hin*dII endonuclease. After phenol treatment and extensive dialysis, DNAs were mixed, denatured, and reannealed. DNA was mounted for electron microscopy by the formamide technique of Davis *et al.* (23) and examined in a Siemens EM Elmiskop 102. The lengths of about 40 molecules of double-stranded DNA with and without the insertion loop, respectively, were measured. Standard deviation was less than 2% of unit length. (× 26,000.)

Heteroduplex Mapping of the Inserted DNA on the M13 Genome. The hybridization data can be confirmed by heteroduplex mapping between wild-type and hybrid RF molecules. The inserted DNA should form a single-stranded loop whose position can be mapped relative to the common unique *Hin*dII cleavage site. For this purpose, the RFs of M13 and M13 mp1 were linearized by *Hin*dII endonuclease, and heteroduplexes were formed by denaturing and reannealing a mixture of the two cleaved DNAs. An electron micrograph (Fig. 4) showed linearized double-stranded DNA molecules, two of them with single-stranded insertion loops close to one end. Double-stranded DNA represented wild-type DNA and the small single-stranded insertion loop corresponded to one strand of the inserted *lac* fragment. The length of individual molecules was measured and the location of the insertion was mapped. Assuming a total of 6400 bp as 100% for the wild-type genome (10), the insertion was located about 530 bp away from the *Hin*dII cleavage site. Because the *lac* fragment was inserted into a *Bsu* I site, the only possible position is thus the *Bsu* I site at 0.083 map unit, taking the *Hin*dII cut as the zero reference point (10). The physical mapping of the *Bsu* I site by gel electrophoresis of DNA fragments was in good agreement with the distance of the insertion loop from the *Hin*dII end of the molecule corresponding to 8.3% of the wild-type genome length. The increased length of M13 mp1 RF *Hin*dII as measured from electron micrographs corresponded to 12.38% or 792 bp. This value fits well with the sequenced length of 789 bp of the *lac* *Hin*dII fragment (W. Gilbert, personal communication). From heteroduplex mapping and the known physical and genetic map, it could be deduced that the *lac* fragment had been inserted into the intergenic region close to the NH_2-terminal end of gene *II* at map position 0.083. The mapping data are summarized on a circular cleavage map of wild-type M13 (Fig. 5).

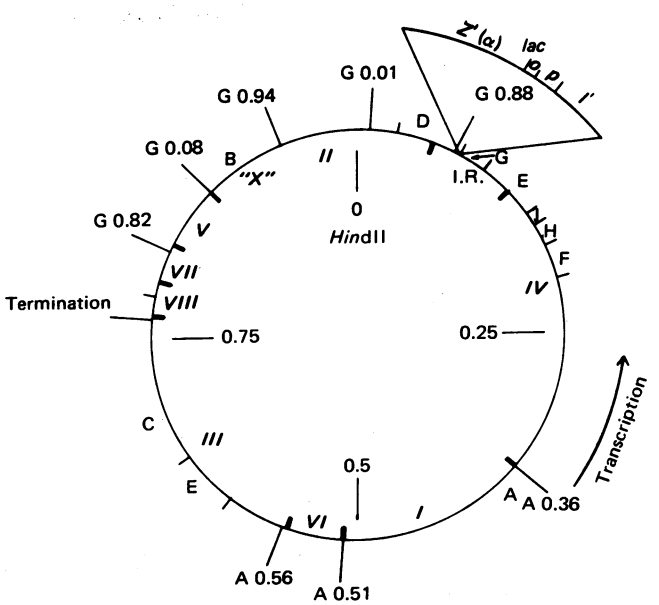

FIG. 5. Physical and genetic map of M13 mp1. The physical order of the *Bsu* I fragments of M13 wild-type (10) is shown outside of the circle. Fragments are indicated by capital letters, and the *Hin*dII cleavage site was taken as zero point of the map. Inside of the circle, the order of genes is shown by Roman numerals. "*X*" represents the "X protein" within gene *II* and I.R. represents the intergenic region with the initiation site for replication. Positions of the five G and the three A promoters are indicated by long bars. One map unit corresponds to 6400 bp and map units are given as distance from the *Hin*dII cleavage site per length of M13 wild-type RF. The direction of transcription is counterclockwise on the genetic map (26). The inserted *lac* fragment is represented on an outer circle segment on the same scale of map units as the inner one. Its orientation has been analyzed by the *Bsu* I and *Hpa* II cleavage patterns of M13 mp1 and wild-type RF (J. Messing and B. Gronenborn, unpublished data). I', Part of the *lac* repressor gene; *p*, *lac* promoter; *o*, *lac* operator; *Z'* (α), α region of β-galactosidase.

DISCUSSION

It has been shown that phage M13 DNA contains a nonessential sequence that can be utilized for *in vitro* recombination. Foreign DNA can be inserted by blunt end ligation. The same ligation technique has been used for fusing "linker" molecules to synthetic *lac* DNA (24) and for the ring closure of recombinant plasmid DNA (25).

The stable integration of a DNA fragment carrying the regulatory region of the *lac* operon and the part of the β-galactosidase gene that codes for the α peptide in M13 RF has been proven on the basis of several criteria. Hybrid phages are able to express the inserted genetic marker. As shown by electron micrographs, the filaments of the hybrid phages increased in size proportionally to the amount of additional packed DNA. Supercoiled RF DNA which has the expected size of hybrid molecules can be hybridized to M13 wild-type and λ-plac DNA. Retransformation of sensitive cells with M13 mp1 RF DNA leads to the expression of the same genetic properties as purified hybrid phage particles—i.e., α complementation and formation of blue plaques upon induction of the *lac* operon.

As shown by heteroduplex mapping, the insertion point for foreign DNA can be localized at the *Bsu* I site at 0.083 map unit. According to the genetic map of M13 (10), this point is within

the intergenic region that is located between genes *II* and *IV* (Fig. 5). Although no codogenic information has been found within this region, it carries the indispensible recognition site for the initiation of replication and, in addition, the promoter controlling gene *II* (26). Our results prove that at least a stretch of sequences neighboring the *Bsu* I site at 0.083 map unit comprises a nonessential region. Further experiments will be required to show if other parts of the intergenic region are nonessential, too.

The analysis of the cleavage pattern of M13 mp1 and wild-type RF with *Hpa* II and *Bsu* I indicates that the orientation of the inserted fragment is as depicted in Fig. 5. Because the initiation of replication is assumed to require RNA synthesis (14), transcription from the *lac* promoter would possibly interfere here if the inserted fragment would have the alternative orientation. Thus, the resulting order of promoters is supposed to be a prerequisite for replication of the hybrid phage.

The hybrid phage can be maintained without any disadvantage as compared to the wild type. No difference in the growth rates of the phages has been observed. Even after several passages, no parent revertant could be observed. This is in clear contrast to hybrid phages that are obtained by insertion of foreign DNA within an essential region. For instance, the insertion of DNA carrying the information for kanamycin resistance into the unique *Hin*dII site by the poly(dA·dT) "linker" method (27, 28) has revealed kanamycin-resistant phages. They can be replicated in the presence of a helper phage but are unstable even under selection pressure (J. Messing, unpublished data).

The hybrid phage M13 mp1 might serve as a better vehicle than phage M13 itself. *In vitro* insertion of foreign DNA in the *lac* region offers two screening possibilities. Loss of α complementation leads again to the appearance of white plaques. This can be used to discriminate between rejoined parental molecules and the new class of hybrid phages. If, on the other hand, further insertion of foreign DNA can be identified by other criteria, a site of insertion between the *lac* ribosomal binding site and the functional part of α would offer an assay system for transcription of the inserted DNA. Whether *lac* controlled transcripts of new inserted genetic information can be translated into a functional protein remains to be proved.

The upper limit of foreign DNA that can be inserted into M13 is not yet known. Recently, it has been shown that a kanamycin transposon can be inserted *in vivo* into the DNA of the related filamentous phage fd (H. Schaller, personal communication). Therefore, at least DNA up to 10 Mdt can be packed as intact phage particles.

Finally, it should be mentioned that according to the National Institutes of Health guidelines on recombinant DNA research, M13 as a cloning vehicle should be used for EK 1-type experiments only. However, experiments to improve the safety of this vector system are under discussion.

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