Mutational Mechanisms by Which An Inactive Replication Origin of Bacteriophage M13 is Turned On Are Similar to Mechanisms of Activation of ras Proto-Oncogenes

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M13 viral strand synthesis is initiated by nicking of the viral strand of the duplex replicative form by the M13 gene II initiator protein at a specific site within a sequence of about 40 base pairs having dyad symmetry. Efficient replication of the M13 viral strand also requires the presence of an adjacent sequence of ca. 100 base pairs. Together these sequences constitute the minimal origin for M13 viral strand synthesis. A pBR322 derivative having a 182-base-pair insert of M13 DNA contains a functional M13 viral strand origin and, when provided with M13 gene functions in trans, replicates under conditions nonpermissive for the parent plasmid. Chimeric plasmids containing deletions within the sequence flanking the viral strand origin are unable to replicate under these conditions. We isolated spontaneous mutants of M13 based on their ability to activate replication of such plasmids. The mutations found in these strains, as well as several produced by oligonucleotide-directed mutagenesis, all result in the substitution of any of at least four different amino acids for a specific glycine residue near the amino-terminal end of the initiator protein. Other studies have shown that overproduction of the wild-type initiator protein also restores replication. These alternate mechanisms are discussed in terms of their striking similarity to the mechanisms of activation of the ras proto-oncogenes which can be activated either by increased expression of the wild-type protein or by substitution of any of several amino acids for a glycine residue near the amino terminus.

Bacteriophage M13 is a filamentous coliphage closely related to fl and fd (7, 28). It consists of a 6,407-nucleotide single-stranded circular DNA molecule encoding 10 genes. The only phase-specific proteins required for viral DNA replication are the gene II and gene V proteins. Gene II protein is essential for the replication of both double-stranded replicative form (RF) and single-stranded viral DNA. Upon infection, the filamentous phage M13 viral strand is converted into a supercoiled double-stranded circular molecule by host enzymes (17, 37, 39). Progeny viral strand synthesis is then initiated by gene II protein which introduces a specific nick at the viral strand origin (15, 18, 31). Gene V protein, a single-stranded DNA-binding protein (29, 37), is required for viral single-strand DNA synthesis (35). The shift from RF replication to the asymmetric synthesis of viral single strand is determined by the availability of gene V protein later in infection. The other M13 proteins are involved in phage morphogenesis and packaging (20). A 574-nucleotide intergenic space (IS), between gene II and gene IV, contains both the viral and complementary strand replication origins and a sequence required for phage morphogenesis.

The functional role of the intergenic space has been studied by using chimeric plasmids that contain different portions of the intergenic space as well as neighboring regions. Three functional domains have thus been identified in the intergenic space of fl (9). The proximal part of gene II is responsible for the prevention of male-specific phage adsorption and penetration. The middle region of the IS contains both the viral and complementary strand origin of replication. Sequences at the gene IV and IS junction are important for phage morphogenesis. All three domains can be inserted into separate sites of a plasmid and function independently of one another (4, 9, 51).

The functional origin of viral strand synthesis has been defined by deletion analysis of the intergenic fragment of M13 cloned into the plasmid vector pBR322. When gene II protein is provided in trans, a functional M13 viral strand origin can direct replication of the pBR322 chimeric plasmid in a polA host and thereby confer ampicillin resistance (4).

The viral strand replication origin has been divided into two domains (4, 5, 9–13, 26). A core region that is absolutely required for viral strand synthesis contains three distinct but partially overlapping signals: (i) the gene II protein recognition sequence, which is necessary for viral strand initiation and termination, (ii) the termination signal, and (iii) the initiation signal (10). The second domain, termed the replication enhancer sequence (26), is about 100 nucleotides long and is required exclusively for viral strand initiation (12). An oriM13 chimera containing 182 nucleotides of the M13 intergenic region, the M13 HaeIII G fragment, plus the neighboring 40 nucleotides (pORI182; see Fig. 1) contains the functional origin of M13 viral strand replication. However, deletion of either this terminal 40-base-pair sequence (4, 5) or sequences around the AvaI site in the replication enhancer (12, 26) strongly inhibits plasmid replication.

Mutations resulting in increased expression of the gene II protein have also been shown to overcome deletions in the replication enhancer sequence (13). In this paper, we report the discovery of M13 mutants named M13UK, which can compensate for deletions in the replication enhancer sequence by providing altered trans-acting gene II proteins having single-amino acid substitutions at glycine73 (Gly73). The substitution of cysteine, alanine, arginine, or serine for Gly73 was found to rescue replication enhancer mutants. These initiator mutants represent a novel and distinct class

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FIG. 1. Physical map of the M13 intergenic region and the M13 fragments present in oriM13 plasmids. Dotted lines indicate sequences deleted around the A\textit{val} restriction site within the M13 insert in the pSJ plasmids (26). Deletion endpoints are marked with the relative distance from the gene II protein-nicking site; e.g., pSJ101 contains a deletion of nucleotides 45 to 54. The replication enhancer sequence is indicated by a double arrow. Cleavage sites for various restriction enzymes are shown above the M13 sequence. Also included on the map are the start of M13 gene II mRNA and its promoter region and the site of viral strand cleavage (ori\textsubscript{I}) by the gene II protein (gIIp). Possible hairpin structures in the single-stranded viral DNA are indicated by boxes. The hatched boxes represent hairpins protected by RNA polymerase at the origin of complementary strand synthesis. The RNA primer for complementary strand synthesis is indicated by a squiggled arrow. The lower line is a scale of the M13 sequence in base pairs by using the nucleotide coordinates of van Wezenbeek et al. (48).

of M13 mutants that allow the use of a greatly reduced minimal origin. A similar strategy has been used (44) to isolate simian virus 40 initiator mutants that overcome ori\textsubscript{I} mutations.

MATERIALS AND METHODS

Strains, media, and buffers. Escherichia coli strains K37 Hfr SupD, RL108 F\textsuperscript{T} Tet\textsuperscript{r} recA56 leu met r\textsubscript{x} m\textsubscript{xy} V, and H560 F\textsuperscript{+} pola enda thy rps L were used. SOB, TBE, and M9 media have been described (21, 40). TE buffer is 10 mM Tris-hydrochloride (pH 8.0)-5 mM disodium EDTA (pH 8.3). TFB contains 100 mM KCl, 45 mM NaCl\textsubscript{2}, 10 mM CaCl\textsubscript{2}, 3 mM hexamethylenetetraamine (HTTA), and 10 mM potassium-2-(N-morpholino)-ethanesulfonic acid (pH 6.0).

Marker rescue and in vitro recombination experiments. M13UK3 RF was prepared (24) and digested to completion with HaeIII, HphII, or HinfI restriction enzyme (prepared from Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Restriction fragments were purified from agarose gels after electrophoresis by transferring each fragment onto NA-45 DEAE membranes (Schleicher & Schuell, Inc., Keene, N.H.) and then eluting them in high-salt buffer (50). Fragments were hybridized to the wild-type single-stranded viral DNA and converted to RF molecules with pol (Klenow fragment) and deoxyribonucleoside triphosphates (22) in vitro before transfection by the procedure of Hanahan (21). Individual plaques or pooled plaques were tested for the UK phenotype as helper viruses for the rescue of pORI142 transformation of pola cells.

For in vitro recombination experiments, two restriction enzymes that recognize unique sites in M13 RF were used to generate two fragments from both wild-type and M13UK RF. The large and small fragments from each source were isolated as above and ligated back together in the four possible combinations before transfections.

DNA sequencing analysis. Single-stranded DNAs were sequenced by the dideoxynucleotide-substitution technique (42). Two different oligonucleotides were synthesized by the phosphotriester method (25, 32) and used to prime the DNA sequencing reactions. One oligonucleotide (5\textsuperscript{'-TCTGGTGCGTGTAG 3\textsuperscript{'}; 13-mer) hybridizes in the middle of the gene II protein structural region 198 nucleotides from the HincII restriction enzyme recognition site, and the other (5\textsuperscript{'-TGGCGGGAGAACCTTT 3\textsuperscript{'}; 15-mer) hybridizes near the HincII site and 187 nucleotides from the AccI recognition site (see Fig. 3).

Oligonucleotide-directed mutagenesis. A mixed 20-mer oligonucleotide, 5\textsuperscript{'-TAAATGCATGTNGTGANAA 3\textsuperscript{'}, was synthesized by solid phase phosphoramidite methods with the model 380A DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.). All four nucleotides were incorporated in equimolar ratios at positions 12 and 13, corresponding to the first two positions of the Gly73 codon (underlined). The resulting mixed oligonucleotide was hybridized to wild-type single-stranded M13 viral DNA at molar ratios (oligonucleotide/DNA template) of 0.1, 1, and 10. Small portions of these template primer mixtures were used for dideoxy sequencing to confirm their specificity. The remainder of each sample was converted to a double-stranded form by extending the oligonucleotide primer with Klenow fragment and all four deoxynucleotides (23, 49) and was then cotransformed into H560 pola cells along with pORI142 or, as a control, pORI182. The ratio of ampicillin-resistant colonies produced in cotransformations with pORI142 to that produced with pORI182 was used as a measure of the frequency of introduction of the UK phenotype into the M13 helper. pORI142 transformants were colony purified and used to produce small phage preparations for DNA sequence analysis.

RESULTS

Isolation of M13UK phages and their ability to function in trans to rescue deletion mutations in the M13 replication origin. The chimeric plasmids pORI142 and pORI182, containing the M13 HaeIII G fragment (142 base pairs) and the HaeIII G fragment plus the adjacent 40-base-pair AT-rich sequence on the 3\textsuperscript{'}-site side of the viral strand origin (182 base pairs), have been described elsewhere as pMC122A90-11 and pMC122Δ60-43, respectively (5; Fig. 1). The parental plasmid pBR322 requires primers I for its replication and as a consequence cannot replicate in a pola mutant host. However, the insertion of a replication origin that does not require polymerase I allows the chimeric plasmid to replicate in a pola host and to confer ampicillin resistance. The pORI182 plasmid contains a functional M13 origin which replicates the chimeric plasmid when the M13 gene II protein is supplied in trans by a helper phage. Thus, transformation of an M13-infected pola host by this plasmid confers ampicillin resistance on the host cell. In contrast, pORI142 only transforms M13-infected pola strains of E. coli to ampicillin resistance at extremely low frequencies (4), we explore here the possibility that transformation by pORI142 might depend on mutant helper phage in the wild-type population.

We picked 11 ampicillin-resistant colonies transformed by pORI142 after M13 infection and found that all isolates retained the pola mutation as observed previously. Each
infected clone was then used to produce phage which were plaque purified and tested for their ability to serve as helper phage for transformation by pORI142. In all cases, phage isolates were obtained that allowed transformation of polA strains by pORI142 at the same frequency as that of pORI1182. Results for two such isolates, M13UK3 and M13UK4, are shown in Table 1. M13UK mutants are indistinguishable from wild-type M13 with regard to plaque morphology and restriction digest patterns of the RF DNAs with 10 different enzymes (data not shown). Mutants M13UK3 and M13UK4 both serve as efficient helper phage for transformation of a polA host by pORI142 as well as by pORI1182 (Table 1).

Because the 40-base-pair sequence deleted in pORI142 has been proposed to be a part of a larger sequence termed a replication enhancer which extends to within 20 to 30 base pairs of the gene II protein-nicking site (oriI), we also examined the transformation properties of oriM13 plasmids containing deletions closer to the nicking site. A series of deletion mutants described elsewhere (26) contains deletions at the Aval site in the oriM13 plasmids (Fig. 1). These plasmid mutants were able to be helped by wild-type M13 to various degrees depending on the extent of deletion. The plasmid pSJ101, in which only 10 base pairs were deleted, transformed the polA strain in the presence of a wild-type M13 helper phage at an efficiency approaching that of the pORI1182 plasmid. However, the plasmid pSJ103, lacking 42 base pairs around the Aval site, transformed under these conditions at more than a 102-fold-lower efficiency. No transformants were detected in the presence of a wild-type helper phage with pSJ105, which lacks 59 base pairs around the Aval site.

Like pORI142, the plasmids pSJ101 and pSJ103 were efficiently rescued by the M13UK3 and M13UK4 phages. This similarity lends further support to the hypothesis that the replication enhancer includes sequences both within the 40 base pair fragment and surrounding the Aval site. The inability of pSJ105 to be efficiently rescued by M13UK3 and M13UK4 suggests that the deletion in this plasmid has extended into the core sequence of the origin.

Inhibition of M13UK phage growth in the presence of oriM13 plasmids. oriM13 plasmids containing the replication enhancer sequence have been shown previously (4, 5, 26) to interfere with M13 phage growth in polA+ cells. Deletions in the oriM13 plasmid replication enhancer sequence reduce or eliminate this interference. However, since the UK plasmids were selected on the basis of their ability to rescue the replication defect in oriM13 deletion plasmids, such plasmids might have a greater ability to interfere with M13UK phage growth than with that of wild-type M13. To examine the interference phenotype of these mutant plasmids with oriM13 plasmids, we infected polA+ cells harboring the plasmids pBR322, pORI142, pORI182, pSJ101, pSJ103, and pSJ105 with M13UK3 or M13UK4 at a low multiplicity of infection for 6 h. Culture supernatants were then titrated for phage production.

In cells containing pBR322, M13 infection produced a normal titer of 1012 PFU/ml (Table 1). However, in polA+ cells containing pORI182, the M13 titer was reduced to 4.0 × 109 PFU/ml, demonstrating the interference phenotype previously observed with oriM13 plasmids. Plasmids containing

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**TABLE 1. Assay for transformation and phage growth interference phenotypes of M13UK phages in the presence of oriM13 plasmids**

<table>
<thead>
<tr>
<th>Phage, plasmid</th>
<th>Plasmid DNA transformation efficiency of infected polA+ cells (CFU/µg)a</th>
<th>Phage production in polA+ cells harboring plasmid (PFU/ml)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13</td>
<td>pBR322</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td></td>
<td>pORI142</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>pORI182</td>
<td>5.2 × 106</td>
</tr>
<tr>
<td></td>
<td>pSJ101</td>
<td>1.5 × 106</td>
</tr>
<tr>
<td></td>
<td>pSJ103</td>
<td>1.7 × 105</td>
</tr>
<tr>
<td></td>
<td>pSJ105</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>M13UK3</td>
<td>pBR322</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td></td>
<td>pORI142</td>
<td>3.6 × 106</td>
</tr>
<tr>
<td></td>
<td>pORI182</td>
<td>4.0 × 106</td>
</tr>
<tr>
<td></td>
<td>pSJ101</td>
<td>3.7 × 106</td>
</tr>
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<td>pSJ103</td>
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<td>pSJ105</td>
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</tr>
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<td>M13UK4</td>
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</tr>
<tr>
<td></td>
<td>pORI142</td>
<td>1.8 × 106</td>
</tr>
<tr>
<td></td>
<td>pORI182</td>
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<tr>
<td></td>
<td>pSJ101</td>
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<td></td>
<td>pSJ103</td>
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</tr>
<tr>
<td></td>
<td>pSJ105</td>
<td>40.0</td>
</tr>
</tbody>
</table>

a Transformation efficiency is defined as the number of Amp' CFU per microgram of plasmid DNA on TYE plates containing ampicillin (80 µg/ml).

b Exponentially growing cells harboring plasmids were infected with M13 or M13UK phages at a multiplicity of 10-3 and grown for 6 h in M9 media with aeration. Culture supernatants were heated at 68°C for 30 min and phage titers were determined (27).
deletions in the replication enhancer sequence showed little, if any, ability to interfere with wild-type phage growth. However, when the growth of either M13UK3 or M13UK4 was examined in plasmid-containing cells, the intact pORI182 plasmid showed a 100-fold-greater interference toward the mutant phages than toward wild-type M13. The plasmids pORI142, pSJ101, and pSJ103, which had lost the ability to interfere with wild-type phage replication, still retained significant ability to interfere with M13UK replication. This result is consistent with the production by the UK phages of an initiator protein (gene II protein) with an increased ability to use an M13 origin containing a deletion in the replication enhancer sequence. The greatly increased interference of the M13 origin towards M13UK3 phage growth as compared with that expressed towards wild-type M13 suggests that the M13UK initiator proteins also interact strongly with the intact M13 origin.

To directly observe oriM13 plasmid-mediated interference towards wild-type and UK phage DNA replication, we analyzed cell lysates by gel electrophoresis. polA+ cells harboring various plasmids (pORI142, pORI182, pSJ101, pSJ103, and pBR322) were grown to a density of 5 × 10^6 per ml in the presence of ampicillin (100 µg/ml) and infected with either wild-type M13 or M13UK4 at a multiplicity of infection of 2 to introduce different initiator proteins under the same conditions. Samples of each culture were removed 90 min after infection, the cells were lysed, and intracellular DNAs were analyzed directly by agarose gel electrophoresis. After the gels were photographed, densitometry was performed to quantitate the relative amounts of DNA present (Fig. 2; Table 2). Phage DNA synthesis by both wild-type M13 and M13UK4 (Fig. 2e) was strongly inhibited by the pORI182 plasmid relative to that of the pBR322 control (Fig. 2c). This result indicates that the UK4 gene II protein and the wild-type protein are both efficiently functionally seques-
tered by the intact M13 origin in pORI182. In contrast, pORI142, which is capable of M13UK4-dependent replication but not wild-type M13-dependent replication, shows strong inhibition of M13UK4 phage DNA synthesis but very little inhibition of wild-type M13 DNA synthesis (Fig. 2d; Table 2). Plasmids pSJ101 and pSJ103, carrying deletions at the Avai site in the M13 replication enhancer sequence, appeared to be more defective in sequestering the M13UK4 gene II protein than did pORI142, as shown by the increased UK4 phage DNA synthesis in the presence of these plasmids relative to that which occurred in the presence of pORI142 (Fig. 2a, b, and d; Table 2).

**Fragment mapping of UK mutations.** The UK mutations have been mapped within gene II by marker rescue experiments. Purified restriction enzyme fragments from M13UK RF were hybridized to M13 wild-type single-stranded viral DNA. The resulting partial heteroduplex molecules were transfected into bacteria after conversion to a fully double-stranded RF with Klenow fragment and subsequent ligation with T4 ligase to make closed double-stranded RF molecules (see above). The resulting plaques were pooled in each case and assayed for their ability to serve as a helper phage for transformation of polA cells by pORI142.

Only HaeIII B, HpaII D, and HindIII EI fragments of M13UK3 gave positive signals for the UK phenotype (Fig. 3). These results were confirmed by in vitro recombination experiments. Taking advantage of the unique restriction enzyme sites for AccI and HincII, which are conveniently located around HindIII EI fragment, we generated two fragments from both M13 RF and M13UK3 RF. The fragments were purified by agarose gel electrophoresis and binding to and elution from a DEAE membrane (50) and then were ligated in all four possible combinations. The 315-base-pair AccI-HincII fragment from M13UK RF was found to be required for production of the UK3 phenotype in ligations involving the large fragment from either source. This result localizes the UK mutation to a small region near the amino terminus of gene II and distinguishes it from the gene V mutants of similar phenotype discovered by Dotto and Zinder (13). The M13UK4 mutation has also been localized to the 315-base-pair AccI-HincII fragment in similar experiments.

**DNA sequence analysis.** Since both M13UK3 and M13UK4 have been mapped within the 315-base-pair AccI-HincII fragment, we used two different oligonucleotide primers to allow unambiguous sequence analysis of the entire fragment. One oligonucleotide (5' TCTGGTGCAGTAG 3') hybridizes in the middle of the gene II protein structural region 198

<table>
<thead>
<tr>
<th>Phage</th>
<th>pBR322</th>
<th>pORI142</th>
<th>pORI182</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13</td>
<td>0.92</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>M13UK4</td>
<td>0.46</td>
<td>0.53</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Phage DNA was quantitated by measuring the total area under peaks for RF and single-stranded DNA from each panel in Fig. 2. The level of total phage DNA present in cells containing pBR322 was set equal to 1.0.
nucleotides from the HincII restriction enzyme recognition site on the 3′ side of the viral strand, and the other (5′ TGGGAGGGCTT 3′) hybridizes within the Accl-HincII fragment 187 nucleotides from the Accl recognition sequence. The entire Accl-HincII region was sequenced by the dideoxy-substitution technique (42).

The resulting DNA sequences showed that M13UK3 and M13UK4 differ from wild-type M13 at only a single nucleotide position, and in both cases, the mutation is within the Gly73 codon (Fig. 4). The M13UK3 mutation changed Gly73 to cysteine (GGC to TGC), whereas the M13UK4 mutation changed Gly73 to alanine (GGC to GCC). Of 11 M13UK phages isolated from a single phage stock, 7 had the same point mutation as M13UK3, and four isolates had the M13UK4-type point mutation. So far, no other UK-type mutations have been found in our laboratory M13 phage stocks, even though other mutations are known that give a similar phenotype (13).

**Oligonucleotide-directed mutagenesis.** To confirm that these point mutations are solely responsible for the UK phenotype and also to learn whether cysteine and alanine are the only amino acid substitutions at Gly73 that suppress deletion mutations in the M13 replication origin, we prepared a mixed 20-mer oligonucleotide (5′ TAAATGCAATGNNTGAGTAA 3′) complementary to the region surrounding the glycine codon and containing all four possible nucleotides in the first two positions of the glycine codon. This mixture of 16 oligonucleotides was then used for directed mutagenesis (49).

Oligonucleotide-dependent mutant phages were directly selected by cotransformation of polA cells with pORI142 and heteroduplex M13 molecules prepared by hybridization of the mixed oligonucleotide with the wild-type single-stranded viral DNA followed by enzymatic conversion to a circular duplex form by using the Klenow fragment of polymerase I (19, 23, 49; see above). Since M13UK phage mutants can direct the replication of pORI142 as well as pORI182, whereas wild-type M13 can only direct pORI182 replication, the relative frequency of recovery of M13UK mutants can be estimated by comparing the number of ampicillin-resistant polA transformants produced by cotransformation of pORI142 and pORI182 with the oligonucleotide-mutagenized M13 molecules as helpers. The relative frequency of pORI142-dependent transformation was increased ca. 100-fold with the oligonucleotide-mutagenized molecules as helper as compared with that observed with the wild-type M13RF as helper (Table 3).

Five ampicillin-resistant isolates were colony purified and used to produce phage which were then plaque purified and tested for the UK phenotype. All of the isolates yielded new UK phage mutants. Sequence analysis of the region between the Accl and HincII sites revealed that the Gly73 codon was mutated in each case (Fig. 5). Mutants UK11 and UK12 proved to be identical in sequence to UK3 and UK4, respectively, whereas mutation UK13 led to the substitution

| TABLE 3. Production of M13UK mutants by oligonucleotide-directed mutagenesis |
|---------------------------------|------------------|-----------------|
| Cointransforming helper molecules | Transformation efficiency (Amp<sup>+</sup> CFU/µg)<sup>a</sup> | Relative frequency of UK phenotype (10<sup>3</sup>)<sup>b</sup> |
| pORI142 | pORI182 | M13UK3RF |
| I (0) | <5.0×10<sup>5</sup> | 3.5×10<sup>4</sup> | <0.14 |
| II (0.1) | 2.0×10<sup>5</sup> | 3.8×10<sup>3</sup> | 0.53 |
| III (1) | 9.8×10<sup>4</sup> | 7.7×10<sup>4</sup> | 1.6 |
| IV (10) | 2.3×10<sup>4</sup> | 1.5×10<sup>4</sup> | 1.6 |
| M13RF | 5.0×10<sup>4</sup> | 5.7×10<sup>4</sup> | <0.0087 |
| M13UK3RF | 1.8×10<sup>5</sup> | 1.8×10<sup>4</sup> | 100 |

* a, b The values <5.0×10<sup>5</sup> indicates that no transformants were detected with 0.02 µg of DNA. Transformation efficiency is expressed as the number of ampicillin-resistant transformants per microgram of plasmid DNA. The relative frequency of the UK phenotype is expressed as the ratio of the number of transformants produced by pORI142 to that produced by pORI182 in each cotransformation with various phage DNAs.

FIG. 4. Sequencing gel pattern showing the point mutations in M13UK phages. Sequence analysis was carried out by the dideoxy substitution technique as described in the text. The dideoxyribonucleotide used in each reaction is indicated at the top of each lane. Nucleotides corresponding to the glycine codon at amino acid position 73 in the M13 wild-type gene II protein are circled. Point mutations in M13UK3 and M13UK4 are indicated by dotted circles. Translated viral strand sequences are shown.
UK COMPLEMENTARY
OLIGOMER (20-mer) 3′ A T G A G T G G G T A A C G T A A A T G 5′

WILD-TYPE M13
VIRAL DNA 5′-C C T A C A C A T A C T C A G G C A T G C A T T A A A A T A-3′

geneII

-PrEgg -Thr35-G133 -Ser73 -Ser72 -Asp73 -Ile74 -Ala75 -Phe76 -Lys37 -Ile78-

IN VITRO MUTAGENIZED M13UK ISOLATES

UK11

5′-------------------------------c′ G C-----------------------------3′

-------------------------------Gly73-------

UK12

5′-------------------------------c′ G C----------------------------3′

-------------------------------Ala73-------

UK13

5′-------------------------------c′ G C----------------------------3′

-------------------------------Arg73-------

UK14

5′-------------------------------A G C----------------------------3′

-------------------------------Ser73-------

UK15

5′-------------------------------T C C----------------------------3′

-------------------------------Ser73-------

FIG. 5. M13UK mutants isolated from in vitro oligonucleotide-directed mutagenesis. Nucleotides 6207 to 6239 of wild-type M13 viral DNA and the synthetic oligodeoxyribonucleotide complementary to the region around the Gly73 codon are shown. The mutant positions are circled together with the corresponding nucleotides in the complementary oligodeoxyribonucleotide. N represents any of the four deoxyribonucleotides. Substituted nucleotides responsible for the UK phenotype are marked with dotted circles.

of an arginine residue at this position and mutations UK14 and UK15 led to the substitution of a serine residue. Thus, at least four different amino acid substitutions at this single site allowed the mutant gene II protein to recognize an M13 origin carrying deletions in the replication enhancer sequence.

DISCUSSION

Deletions and insertions of DNA sequences in the vicinity of the M13 viral strand origin of replication have established distinct functional domains within the 140 base pair minimal origin (4, 5, 10, 12). A core sequence of ca. 40 base pairs contains the nicking site for the gene II initiator protein plus an overlapping sequence required for termination of a round of asymmetric single-strand synthesis by a rolling circle mechanism. The remaining 100 base pairs, termed a replication enhancer sequence (26), are required for efficient viral strand synthesis and mediate interference between M13 and plasmids containing a functional M13 origin. Two functionally distinct regions have also been identified in the adenovirus 2 origin recently (38). A sequence adjacent to the essential origin sequence binds a cellular protein and thereby enhances the initiation reaction.

Plasmids lacking the distal 40 base pairs of the 100-base-pair M13 replication enhancer sequence are extremely defective in competing for available levels of gene II protein in M13-infected cells. This deficiency has provided a means for the direct selection of the phage mutants which we term M13UK mutants. Using a different genetic selection, Dotto and Zinder (13) have also obtained mutants having a similar phenotype. However, their mutants map within gene V, a multifunctional single-stranded DNA-binding protein. The gene V protein coats the single-stranded progeny DNA and thereby prevents synthesis of the complementary strand. In addition, gene V protein regulates expression of gene II at a translational level (33). The mutants isolated by Dotto and Zinder (13) appear to be defective in regulating gene II expression and lead to an overproduction of gene II protein by 5- to 10-fold. The increased level of gene II protein appears to compensate for mutations in the replication enhancer sequence.

In contrast, the M13UK mutants which we have isolated all contain base substitutions in the glycine73 codon of the 410-amino-acid gene II initiator protein. The resulting amino acid replacements leading to activation of a defective origin are surprising, ranging from the substitutions of the aliphatic amino acids alanine or serine to the substitutions of the sulfur-containing amino acid cysteine or the basic amino acid arginine. In view of the small number of mutants which we have characterized, still other substitutions at this site may be possible. This result indicates the existence of a second mechanism for compensating for deletions in the replication enhancer sequence in addition to the previously described mechanism (13) involving increased expression of the wild-type protein. Although we have not yet directly determined the level of expression of gene II protein in M13UK-infected cells, we consider it more likely that substitutions for Gly73 lead to an altered origin recognition specificity rather than an increased expression of the mutant protein. An in vitro analysis of origin recognition by gene II protein from wild-type and UK mutant-infected cells as well as a comparison of relative levels of gene II expression by these phages should provide a clearer understanding of the
biochemical mechanisms involved in the reduced specificity of the UK mutants.

Dotto and Zinder (13) have suggested that an increased concentration of the wild-type gene II protein might render the replication enhancer sequence unnecessary by altering the DNA-protein interactions involved in forming the initial replication fork. In addition to gene II protein, the in vitro replication of the M13 viral strand of the RF requires DNA polymerase III holoenzyme, rep helicase, and the single-strand-binding protein (16, 30). Elongation of the nicked M13 viral strand by DNA polymerase III requires concomitant unwinding of the duplex DNA ahead of the polymerase. This ATP-dependent unwinding of the RF by rep helicase (14, 43) requires the presence of gene II protein at least for initiation of unwinding. It is therefore reasonable to speculate that gene II protein may specifically interact with the replication enhancer sequence in the formation of a prerelaxation complex. A structural alteration of the active form of gene II protein may provide still another means of compensating for a deletion in the replication enhancer sequence. Our results provide support for a direct interaction between gene II protein and the replication enhancer sequence. An examination of the in vitro replication requirements of gene II protein purified from the M13UK mutants could provide a better understanding of the interactions of this initiator protein with the M13 replication origin and possibly with other components of the initiation complex.

The similarity between the mechanisms involved in activating the mutant M13 origin and those by which the ras proto-oncogenes are activated is remarkable. Increased expression of proto-oncogenes has been shown to result in oncogene activation. These experiments include the activation of the myc proto-oncogene occurring during leukemogenesis of avian retroviruses (34, 36) and the activation of clones of the rat and human c-Ha-ras proto-oncogenes by in vitro fusion of the genes to a retroviral long terminal repeat (3, 6). A second mechanism of activation of ras proto-oncogenes involves substitution of any of several amino acids for a glycine at position 12. Oncogene activation in the human EJ and T24 bladder carcinoma cell lines involves substitution of a valine for Gly12 (41, 46), and in human lung and colon carcinoma cell lines, valine and cysteine are substituted for Gly12 (2). In addition, the homologous transforming proteins encoded by Harvey, BALB/c, and Kirsten murine sarcoma viruses substitute arginine (8), lysine (41), and serine (47) at position 12, respectively. These examples provide a precedent for the potentiation or activation of a protein by the replacement of a glycine residue near the amino-terminal end of a protein with any of several amino acids. Because glycine lacks a side chain and can therefore participate in extreme bands in the polypeptide backbone, its replacement by other amino acids can have drastic structural consequences. An examination of the conformational changes involved in the activation of proteins by such substitutions will be of considerable interest. The finding here that this mechanism is also used by a bacteriophage initiator protein that is not otherwise related to the ras proteins indicates that this activational mechanism is not unique to oncogene activation but may be of more general significance.

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LITERATURE CITED


