Deletion Analysis of the Cloned Replication Origin Region from Bacteriophage M13

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A cloned 270-nucleotide fragment from the origin region of the M13 duplex replicative form DNA confers an M13-dependent replication mechanism upon the plasmid vector pBR322. This M13 insert permits M13 helper-dependent replication of the hybrid plasmid in *polA* cells which are unable to replicate the pBR322 replicon alone. Using in vitro techniques, we have constructed several plasmids containing deletions in the M13 DNA insert. The endpoints of these deletions have been determined by DNA sequence analysis and correlated with the transformation and replication properties of each plasmid. Characterization of these deletion plasmids allows the following conclusions. (i) The initiation site for M13 viral strand replication is required for helper-dependent propagation of the chimeric plasmid. (ii) A DNA sequence in the M13 insert, localized between 89 and 129 nucleotides from the viral strand initiation site, is necessary for efficient transformation of *polA* cells. A chimeric plasmid containing the viral strand initiation site, but lacking this additional 40 nucleotide M13 sequence, transforms helper-infected cells at a frequency approximately 10^4 -fold less than that of plasmids containing this additional DNA segment. (iii) The entire M13 complementary strand origin can be deleted without affecting M13-dependent transformation by the hybrid plasmids. We propose a model in which replication of one strand of duplex chimera initiates by nicking at the gene II protein nicking site in the viral strand of the M13 insert, followed by asymmetric single-strand synthesis. Initiation of the complementary strand possibly occurs within plasmid sequences.

The origins of replication of both the viral and the complementary strands of bacteriophage M13 are contained within a 508-base-pair intergenic space (8, 23). This region of the single-stranded phage genome also contains DNA sequence information required for the expression of the adjacent gene II (24). Still other biological functions may be associated with specific domains of this intergenic space.

To carry out a genetic analysis of this noncoding region of the M13 genome, we have inserted DNA fragments containing all or part of the M13 intergenic space into a plasmid cloning vector, pBR322 (2). In *polA*⁺ strains of *Escherichia coli*, the cloned DNA sequences are not required for plasmid replication and are therefore available for mutagenesis. However, in *polA* mutant cells, the replication of pBR322 is inhibited by the *polA* mutation (2) and consequently depends on a functional M13 origin (*ori*M13) under conditions where phage-specific proteins are provided by a helper phage.

Initial characterization of the replication properties of the *ori*M13 plasmids (3) revealed the

following: (i) a hybrid plasmid containing the DNA sequence complementary to the RNA primer for the complementary strand of the replicative form (RF) but lacking two major hairpin structures considered to be a component of the origin (6) is capable of M13-dependent replication, and (ii) DNA sequences on the gene II-proximal side of the viral strand initiation site (17, 18) are required for high-frequency transformation of M13-infected *polA* cells.

Plasmids containing the complementary strand RNA primer sequence and the viral strand initiation site on an M13 *Hae*III fragment of 142 nucleotides transformed helper-infected *polA* cells extremely inefficiently (approximately 10 transformants per μ g of input DNA), whereas plasmids containing an additional 128-nucleotide region adjacent to gene II transformed helper-infected *polA* cells at a high frequency ($\geq 10^5$ transformants per μ g of DNA). The high frequency of transformation is comparable to the frequency of transformation of *polA*⁺ cells by the chimeric plasmids.

To determine which, if any, of the DNA se-

quences implicated in the phage complementary strand origin is essential for *oriM13* plasmid replication and to map more precisely the region affecting transformation frequency by the chimeric plasmid DNA, we have constructed deletions from both ends of the M13 insert. Our results indicate that there is no requirement for the M13 complementary strand primer sequence for *oriM13* plasmid replication *in vivo*. Also, the region essential for high-frequency transformation has been limited to within a 40-nucleotide, adenine-plus-thymine-rich sequence 89 nucleotides from the initiation site of viral strand replication.

MATERIALS AND METHODS

Bacterial strains. *E. coli* strains used were K37 (Hfr SupD) and H560 (F^+ *polA endA thy rpsL*).

Enzymes and biochemicals. Restriction endonucleases *Bam*HI, *Ava*I, and *Hpa*II and T4 ligase were purchased from New England Biolabs. *Eco*RI was from Boehringer Mannheim Corp. *Hae*III was prepared by S. Suggs (23). BAL-31 nuclease and *Eco*RI methylase were purchased from Bethesda Research Laboratories Inc. *Eco*RI dodecanucleotide linkers were the generous gift of K. Itakura. *S*-Adenosyl-L-methionine and ethidium bromide were from Sigma Chemical Co. Sea Plaque agarose was from Marine Colloids.

Preparation of plasmid DNAs. Cells containing plasmids were grown and amplified according to Clewell (4) and lysed with Triton X-100 as described by Katz et al. (10). DNA from cleared lysates was concentrated by precipitation with polyethylene glycol 6000 according to Humphreys et al. (9) before banding in dye-cesium gradients.

In vitro recombinant DNA methods. Plasmid DNA at 200 μ g/ml was partially digested with *Bam*HI at 1 U/ μ g of DNA in the presence of 120 μ g of ethidium bromide per ml for 12 h at 25°C. Linear DNA molecules were purified from 0.7% Sea Plaque agarose according to Langridge et al. (12). DNA was methylated by *Eco*RI methylase as described previously (13). BAL-31 nuclease digestions (1 U/ μ g of DNA) were for 30, 60, or 90 s according to Gray et al. (7). Ligation of *Eco*RI linkers was performed essentially as described by Bahl et al. (1), except that the *Eco*RI linkers were labeled with polynucleotide kinase and [γ - 32 P]ATP to monitor ligation to plasmid DNA. Transformations were as described previously.

Clone analysis. Amp^r cells were screened directly for plasmid DNA containing deletions as previously described (3). Restriction digests were performed on DNA prepared from minilysates according to Klein et al. (11).

Nucleotide sequence determination. Restriction fragments were labeled with polynucleotide kinase and [γ - 32 P]ATP (>5 Ci/mol) from International Chemical and Nuclear Corp., using T4 polynucleotide kinase from P-L Biochemicals, Inc. 5'-Labeled DNA was sequenced according to Maxam and Gilbert (15) after secondary restriction cleavage of labeled fragments.

Helper phage growth inhibition assay. K37 (*polA*⁺) cells, grown in tryptone-yeast extract broth

to an absorbancy at 595 nm (A_{595}) = 0.1, were infected with M13 at a multiplicity of infection of 1 to 2. After 6 h, culture supernatants were heated at 70°C for 30 min, and titers were determined for phage production.

RESULTS

Construction and characterization of deletions in an *oriM13* plasmid. The starting *oriM13* plasmid used for construction of deletions was pJMC122 (Fig. 1), which contains a 270-nucleotide fragment from the right-hand end of the M13 intergenic region (3). The procedure used for isolating deletions from either end of the M13 insert is outlined in Fig. 2. Plasmid DNA was partially cleaved with *Bam*HI in the presence of ethidium bromide, and purified linear DNA was subsequently incubated with *Eco*RI methylase blocking the single *Eco*RI site in the vector DNA. Samples were then digested with BAL-31 nuclease, and *Eco*RI dodecanucleotide linkers (C-A-T-G-A-A-T-T-C-A-T-G) were ligated to the samples. Finally, the linkers were cleaved by *Eco*RI, the DNA was circularized by T4 DNA ligase, and the deletion products were transformed into K37 cells. From 148 Amp^r transformants sized on 0.7% agarose gels for plasmid DNA containing small deletions, isolates found to be smaller than or equal to pJMC122 but larger than pBR322 were characterized by further restriction analysis (data not shown). Preparations of minilysate DNAs were cleaved by *Eco*RI to detect insertion of the *Eco*RI linker (6% of those tested possessed a second *Eco*RI site), and the approximate size of the deletions were mapped further by restriction with *Hae*III and *Hpa*II. From 78 isolates initially characterized in this manner, five plasmid DNAs (Fig. 1) were examined for their transformation properties and sequenced to determine end-points of the deletions. Autoradiograms of several sequencing gels are shown in Fig. 3 and illustrate the junction region of the deletions.

Only the M13 viral strand origin is necessary for *oriM13*-dependent transformation. Three of the isolates examined contained significant deletions of the left portion of the *oriM13* insert (Fig. 1). In one isolate, Δ 60-2, 90 nucleotides of M13 DNA sequence are deleted from the hybrid plasmid (Fig. 4). Both the complementary and the viral strand initiation sites have been removed, leaving only the adenine-plus-thymine-rich portion of the insert proximal to gene II. The deletion extends 34 nucleotides beyond the cleavage site of the viral strand endonuclease, gene II protein (17), and, as expected, the plasmid is unable to transform infected *polA* cells (Table 1). Preliminary restriction mapping of two other isolates, Δ 30-16 and Δ 30-36 (both of which lack a new *Eco*RI site),

FIG. 1. Physical map of the M13 intergenic region and the M13 fragments present in plasmid pJMC122 and its deletion derivatives. Restriction enzyme cleavage sites are indicated on the top line. A scale of the region in base pairs, according to van Wezenbeek et al. (25), is shown beneath. Regions capable of forming secondary structures are indicated by boxed arrows. The boundaries for genes IV and II are included on the left and right, respectively. The gene II protein (gIIp) nicking site, the site of viral strand initiation, and the complementary strand primer RNA, oriRNA, are also indicated. Hatched boxes indicate RNA polymerase-protected viral strand hairpins, postulated to represent the complementary strand oriDNA (6, 22). M13 fragments contained in pJMC122 and the deletion plasmids constructed *in vitro* are indicated by horizontal lines.

indicated that they contained deletions of 40 to 70 nucleotides (data not shown). When tested for *oriM13* function, both were found to transform M13-infected *polA* cells at levels comparable to that of the parent plasmid pJMC122 (Table 1). Most importantly, sequence analysis of Δ30-16 and Δ30-36 (Fig. 3) showed that the isolates possess deletions extending 9 and 10 nucleotides, respectively, beyond the start of the complementary strand RNA primer (Fig. 4). Thus, the entire phage complementary strand primer sequence can be deleted without affecting *oriM13*-dependent transformation.

Localization of the DNA segment affecting transformation frequency by *oriM13* plasmids. As discussed above, the right-hand portion of the cloned M13 intergenic region was found to increase transformation by *oriM13* plasmids over 10^4 -fold. Our previous results indicated that this activity resides in the adenine-plus-thymine-rich region (24) bound by the *HaeIII* site to the right of the viral strand origin and the *HpaII* site at the start of gene II (Fig. 1). Two isolates, Δ60-63 and Δ90-11, with large deletions in this region were sequenced (Fig. 3) and tested for *oriM13* function (Table 1). In

isolate Δ60-63, an 88-nucleotide deletion removed 69% of the adenine-plus-thymine-rich segment, and yet the plasmid still transformed at a high frequency in the *oriM13* assay. In contrast, a larger deletion of 128 nucleotides in isolate Δ90-11 eliminated high-frequency, *oriM13*-dependent transformation. However, this plasmid still transformed at a very low frequency, identical to our previous result with the plasmid pJMC67 (Table 1), which contains the 142-nucleotide M13 *HaeIII* fragment comprising the left half of the insert in pJMC122 (Fig. 4; reference 3). Thus, the M13 DNA segment required for high-frequency transformation by *oriM13* plasmids extends a maximum of 41 nucleotides beyond this *HaeIII* site but does not include most of the adenine-plus-thymine-rich DNA sequence at the right-hand end of the intergenic space (24).

In *polA*⁺ cells that contain only pBR322, M13 infection produces a normal titer of $>10^{12}$ PFU/ml (14). However, in *polA*⁺ cells containing *oriM13* plasmids capable of transforming infected *polA* cells at a high frequency, the resulting M13 titer drops to less than 3% of the control. Listed in Table 1 is the amount of M13 phage

FIG. 2. Schematic diagram showing the strategy used to isolate deletions in the cloned M13 DNA fragment of pJMC122. Details of the procedure are discussed in the text.

produced by infection of *polA*⁺ cells harboring various *oriM13* plasmids. Isolate Δ60-63 also retains the interference phenotype previously observed with *oriM13* plasmids containing this region (3), whereas isolate Δ90-11 does not. The M13 phage yield from cells containing the Δ60-2 plasmid demonstrates that a functional viral strand origin is required for interference. Thus, the same DNA element that permits efficient *oriM13*-dependent transformation of M13-infected *polA* mutants is also required for the inhibition of normal phage production in *polA*⁺ cells.

DISCUSSION

The initial step in M13 DNA replication is the conversion of the infecting single-stranded viral DNA to a duplex RF. Initiation of the complementary strand involves RNA priming by the *E. coli* RNA polymerase at a unique site within a 508-nucleotide intergenic space between genes II and IV (5). After covalent closure of the newly synthesized complementary strand and the introduction of supercoils, through the action of DNA gyrase, the duplex supercoil is nicked at a unique site located 24 nucleotides upstream from the complementary strand initiation site on the viral strand. The 3'-hydroxyl terminus generated as a consequence of this site-specific nicking by the M13 gene II protein serves as a primer for the initiation of viral strand synthesis by a roll-

FIG. 3. Autoradiographs of sequencing gels showing the deletion end regions of (a) Δ60-63, (b) Δ90-11, (c) Δ30-16, and (d) Δ30-36. Δ60-63 and Δ90-11 were digested with *EcoRI*, labeled with [γ -³²P]ATP, and cleaved with *BamHI*. Labeled fragments containing the deletion were sequenced by the technique of Maxam and Gilbert (15) and analyzed on polyacrylamide gels. Δ30-16 and Δ30-36 DNAs (which do not contain a new *EcoRI* site at the deletion junction) were digested with *AvaI*, labeled with [γ -³²P]ATP, and then cleaved with *EcoRI*. Labeled fragments containing the deletion junction were isolated and used for sequence analysis. Arrows indicate the junction between M13 DNA and the *EcoRI* linker from the top down (a and b) or M13 DNA and pBR322 DNA from the bottom up (c and d).

FIG. 4. Viral strand nucleotide sequence of deletion mutants constructed from pJMC122. That portion of the M13 intergenic region between the *HaeIII* site at the end of the complementary strand RNA primer and the *HpaII* site adjacent to gene II is shown. The DNA sequence deleted in each isolate is indicated by horizontal lines above the sequence. The *HaeIII* site which marks the right end of *oriM13* plasmid pJMC67 (3) is also shown. *gIIp*, Gene II protein.

TABLE 1. Assay for transformation and phage growth interference properties of *oriM13* deletion plasmids

Plasmid	DNA transformation frequency ^a		Phage production in <i>polA</i> ⁺ cells (PFU/ml)
	K37(<i>polA</i> ⁺)	M13-infected H560(<i>polA</i>)	
pJMC122 Deletion mutant			
Δ30-16	6.1×10^{-3}	3.2×10^{-3}	4.6×10^{10}
Δ30-36	6.5×10^{-3}	8.0×10^{-3}	4.3×10^{10}
Δ60-2	4.3×10^{-3}	$<1 \times 10^{-7}$	3.3×10^{12}
Δ60-63	7.3×10^{-3}	3.0×10^{-3}	8.7×10^{10}
Δ90-11	5.5×10^{-3}	2.1×10^{-7}	3.0×10^{12}
Controls			
pBR322	5.4×10^{-3}	$<5.0 \times 10^{-8}$	2.8×10^{12}
pJMC122	4.7×10^{-3}	6.7×10^{-3}	6.9×10^{10}
pJMC67	4.4×10^{-3}	4.9×10^{-7}	9.4×10^{11}

^a Transformation frequency is defined as Ap^r colony-forming units per microgram of DNA divided by total cells per incubation. The value $<1 \times 10^{-7}$ indicates that no transformants were detected.

ing circle mechanism. Early in the infectious process, duplex replication (RF $\rightarrow$ RF) appears to consist of two steps: (i) asymmetric viral strand synthesis (RF $\rightarrow$ SS), in which viral single strands (SS) are produced, and (ii) conversion of the viral single-strand circles to RF by synthesis of a complementary strand (SS $\rightarrow$ RF) (8).

The precise boundaries of the DNA sequences specifying the viral and the complementary strand origins are unknown. It is also unknown what specific roles potential DNA secondary structures may play in initiation of either strand in vivo or what other functions may be associated with DNA sequences surrounding the replication origins.

To address these questions, we initially cloned a restriction fragment containing both replication origins (3) into the plasmid pBR322. In an M13-infected *polA* mutant, the cloned M13 origin region is required for plasmid replication. Our previous results with the derivative *oriM13* plasmid pJMC122, which lacks two major hair-

pins adjacent to the M13 complementary strand origin, suggested that the M13 complementary strand initiation mechanism might not be involved in duplex replication of the chimeric plasmids. As shown here, the transformation properties of two deletion mutants, Δ30-16 and Δ30-36, constructed from pJMC122 clearly indicate that the M13 complementary strand primer sequence is nonessential. We propose that synthesis of the complementary strand of these hybrid plasmids may be initiated at a site within pBR322 sequences. This conclusion is supported by recent results of Nomura and Ray (19), who have shown that a single-strand DNA fragment from the origin region of the plasmid ColE1 (of which pBR322 is a derivative [2]) will initiate parental complementary strand synthesis (SS $\rightarrow$ RF) in vivo when cloned into an M13 phage. Also, Zipursky et al. (26) have recently observed a similar result in vitro with a pBR322 recombinant plasmid containing the viral strand origin of phage ϕ X174.

The deletion in isolate $\Delta 30-36$ limits the left boundary of the M13 viral strand origin to 14 nucleotides from the *in vivo* origin of viral strand synthesis (17, 18). Thus, viral strand initiation has no requirement for DNA sequences in the adjacent complementary strand primer sequence. However, until the converse can be demonstrated, we cannot conclude that the origins are separable, as in the case of the icosahedral single-strand phage G4 (20). It may also be noted that the $\Delta 30-36$ deletion extends one nucleotide into the self-complementary sequence surrounding the cleavage site of gene II protein (17, 21). The gene II protein cleavage site is located at the top of the loop in this potential hairpin, but the significance of this secondary structure for recognition by gene II protein has not been established. Additional deletions and base substitutions in this region should lead to a clearer definition of the precise sequence requirement for the viral strand nicking site and the possible role of DNA secondary structure in this reaction.

The M13 DNA segment which strikingly affects the helper-dependent transformation properties of *oriM13* plasmids is located within a 40-nucleotide sequence beginning 89 nucleotides to the gene II side of the viral strand nicking site (Fig. 4). Whereas isolate $\Delta 60-63$ transforms efficiently, isolate $\Delta 90-11$ only rarely produces *Ap*^r transformants (Table 1). Isolate $\Delta 90-11$ contains the same M13 DNA fragment as does plasmid pJMC67, which we have also previously shown to be capable of M13-dependent replication but only feeble transformation (3). Most of the adenine-plus-thymine-rich DNA on the right side of the intergenic region has been eliminated in isolate $\Delta 60-63$, suggesting that these sequences are not essential for origin function. However, the 40-nucleotide additional sequence in $\Delta 60-63$ still contains a high adenine-plus-thymine content (80%). In addition to the effect on *oriM13*-dependent transformation, cells harboring *oriM13* plasmids containing both this sequence (Table 1) and the viral strand origin show a greatly reduced phage yield. Isolate $\Delta 60-2$ contains this region but lacks the gene II protein nicking site and does not interfere with phage production. Although the function of this domain remains unclear, these results suggest that the region, although not essential for replication per se, plays an important role in the establishment of replication by *oriM13* plasmids. This 40-base-pair sequence could possibly permit the *oriM13* plasmid DNA to compete effectively with M13 RF DNA for a factor or site required for an early stage of phage replication. The ability to insert foreign DNA into an M13 phage vector at the *Hae*III site shown in Fig. 4 (16)

implies that this *cis*-acting region is separable from the viral initiation site.

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