DNA Requirements at the Bacteriophage G4 Origin of Complementary-Strand DNA Synthesis

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An in vivo assay was used to define the DNA requirements at the bacteriophage G4 origin of complementary-strand DNA synthesis (G4 origin). This assay made use of an origin-cloning vector, mRZ1000, a defective M13 recombinant phage deleted for its natural origin of complementary-strand DNA synthesis. The minimal DNA sequence of the G4 genome sufficient for the restoration of normal M13 growth parameters was determined to be 139 bases long, located between positions 3868 and 4007. This G4-M13 construct was also found to give rise to proper initiation of complementary-strand synthesis in vitro. The cloned DNA sequence contains all the regions of potential secondary structure which have been implicated in primase-dependent replication initiation as well as additional sequence information. To address the role of one region which potentially forms a DNA secondary structure, the DNA sequence internal to the G4 origin was altered by site-directed mutagenesis. A 3-base insertion at the AvaII site as well as a 17-base deletion between the AvaI and AvaII sites both resulted in loss of origin function. The 17-base deletion was also generated within the G4 genome and found to dramatically reduce the infectious growth rate of the resulting phage. These results are discussed with respect to the role of the G4 origin as the recognition site for primase-dependent replication initiation and its possible role in stage II replication.

Bacteriophage G4 is a single-stranded DNA phage which infects Escherichia coli C (11). Upon infection, the single-stranded G4 genome is converted to a double-stranded replicative intermediate. This replication event is dependent upon the synthesis of an RNA primer by the host dnaG gene product, primase, at a unique position on the G4 genome, the origin of complementary-strand DNA synthesis (3). The mechanism by which primase interacts on the G4 genome is different from its mode of interaction on other genomes, such as that of bacteriophage φX174, in which a multiprotein complex termed the primosome is required for primase activity (1). The primosome is also thought to be necessary for the synthesis of RNA primers by primase during E. coli chromosomal replication as well as lambda DNA replication (9, 10). On the other hand, both in vivo and in vitro studies indicate that primase activity on the bacteriophage G4 genome does not require the primosome (2, 4, 27) but is dependent solely upon the addition of stoichiometric amounts of single-stranded DNA-binding protein (SSB). The features of the G4 origin that cause primase to interact independently of the primosome are not understood.

The DNA sequence of the G4 genome was determined, and the position of primase-dependent RNA synthesis was mapped to a location within the gene F-G intercistronic region (8, 13). Sequence comparison with the primase-dependent (primosome-independent) origins of three closely related, single-stranded DNA bacteriophages, φK, α3, and st-1 (25), revealed several interesting features. First, there are two regions of strong sequence conservation within the intercistronic regions of these phages. Second, these regions of conserved sequence can potentially form secondary structures (stemloops I and II; see Fig. 2 and 8). Whether these sequence homologies are significant with respect to origin function or are simply a consequence of the relatedness between the phages has not been determined; however, the high degree of sequence conservation within this region compared with other regions of these genomes implicates a functional role (25).

In this study, the DNA sequence requirements at the G4 origin are defined. We used an in vivo assay for origin activity that measured the ability of a DNA fragment to restore normal infectious growth character to a defective M13 phage. Normal infectious growth was found to correlate with proficient rifampin-resistant DNA replication assayed in vitro. The boundaries of the G4 origin as well as the requirements for regions within this DNA sequence were determined. In addition, the effect of destroying G4 origin activity within phage G4 was addressed.

MATERIALS AND METHODS

Bacterial strains and phage stocks. E. coli K12 strains JM101 [F' traD-36 lacI2 lacZM15 proA’B’/Δ(lac-pro)supE thy] and CSH26/Pox38-gen (CSH26 is described below; Pox38-gen is an F' plasmid encoding gentamycin resistance which was constructed as described by Johnson and Reznikoff [15]) were used for infectious growth curves and efficiency of infection experiments. Strain CSH26 [ara Δ(lac-pro) thi] was used for transfection experiments. E. coli C was used for infectious growth of bacteriophage G4 and derivatives constructed in this study. Bacteriophage M13mp8 was purchased from Bethesda Research Laboratories. M13A.E101 was a gift from D. Ray, University of California at Los Angeles, Los Angeles. Construction of all other phages is described below.

Biochemicals. Restriction enzymes, E. coli DNA polymerase I, and DNA nuclease were purchased from New England BioLabs, Bethesda Research Laboratories, Inc., or Promega Biotec and used as specified by the supplier. T4

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DNA ligase was a gift from R. Simoni, Stanford University, Stanford, Calif. SSB was a gift from M. Cox. Deoxy- and dideoxy-nucleoside triphosphates were purchased from P-L Pharmacia Biochemicals, and radioisotopes were purchased from Amersham Corp.

Phage constructions. The origin vector mRZ1000 was constructed by ligating the 761-base-pair (bp) fragment of M13mp8 containing the lac region, generated by cleavage at the AvaII and Hgal sites and purified by polyacrylamide gel electrophoresis, into the filled-in EcoRI site of M13ΔE101. The resulting DNA was transformed into competent JM101 cells, and minute lac+ plaques were screened for on TYE agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside and isopropyl-β-D-thiogalactopyranoside (19). DNAs from these phage were further analyzed by extensive restriction enzyme cleavage analysis. The resulting phage genome organization is illustrated in Fig. 1. The 274-bp Alal restriction fragment from duplex G4 DNA was blunt-end ligated into the filled-in EcoRI site of mRZ1000 to generate mRZ1200 (Fig. 2). This phage gave rise to normal-sized plaques which were still lac+, owing to the generation of a fusion protein which starts at a G4 translational start codon and continues in frame into the α-complementing region of lacZ. Minute lac− plaques were also found which have the opposite orientation of the G4 fragment inserted into the origin-cloning vector (mRZ1205). The constructs mRZ1211- mRZ1213 were generated by inserting the respective restrict-
primase-dependent RNA synthesis. In the far right column of Table 1 is indicated the number of picomoles of $[^{3}H]$dTMP incorporated into a trichloroacetic acid-insoluble precipitate as measured by the fraction II replication assay. These assays were done in triplicate in the presence of rifampin (see Materials and Methods).

Transfection assay. Competent F$^-$ cells (CSH26) were transfected for 30 min on ice with 50 ng of phage DNA prepared by the method of Sanger et al. (23) and quantitated on agarose gels by using appropriate standards. After heat shock at 42°C for 2 min, a small portion was plated on top agar containing 0.3 ml of a saturated culture of F$^+$ cells [CSH26/Pox38-gen] and underlaid with gentamycin 1 h after plating. This allowed the determination of the number of transfectants for each type of phage DNA. The rest of the transfection stock was outgrown in 4 ml of LB medium for 6 h, during which time portions were removed and the phage produced were titrated as described for the infectious growth assay. The maximal rate of progeny phage production (Table 1) was determined by maximizing the value for the quotient $(y_n - y_{n-1})(x_n - x_{n-1})$, where $y_n$ is the number of phage per transfectant at time point $n$, $y_{n-1}$ is the number of phage per transfectant at the previous time point, $n - 1$, $x_n$ is the time (in minutes) at time point $n$, and $x_{n-1}$ is the time at the previous time point, $n - 1$.

Bacteriophage G4 infectious growth assays. The burst size for wild-type phage G4 was determined by infecting a 5-ml culture of E. coli C with 1 PFU at early log phase. Because the rate of progeny phage production for the G4oriI mutant was extremely low, cells were infected with 10 PFU in this case. Portions were taken every 10 minutes, and the phage

TABLE 1. In vivo growth characteristics of origin-cloning vector mRZ1000

<table>
<thead>
<tr>
<th>Construct</th>
<th>Efficiency of infection of:</th>
<th>Transfection</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>JM101</td>
<td>Lag (min)</td>
</tr>
<tr>
<td></td>
<td>CSH26(Pox38-gen)</td>
<td></td>
</tr>
<tr>
<td>M13</td>
<td>2.2 x 10$^{12}$</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Single-stranded DNA</td>
<td>24 x 10$^{12}$</td>
</tr>
<tr>
<td>mRZ1000</td>
<td>3.1 x 10$^{11}$</td>
<td>45 x 10$^{11}$</td>
</tr>
<tr>
<td></td>
<td>Single-stranded DNA</td>
<td>38 x 10$^{11}$</td>
</tr>
<tr>
<td></td>
<td>Double-stranded DNA</td>
<td></td>
</tr>
<tr>
<td>mRZ1200</td>
<td>2.0 x 10$^{12}$</td>
<td>24 x 10$^{12}$</td>
</tr>
<tr>
<td></td>
<td>Single-stranded DNA</td>
<td>24 x 10$^{12}$</td>
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<tr>
<td></td>
<td>Double-stranded DNA</td>
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</table>

$^a$ Number of PFU for an equivalent amount of phage, as indicated in Materials and Methods.

$^b$ Time in minutes at which 0.01 PFU per transfectant occurs.

$^c$ Rate of progeny phage production (maximal rate is given in phage per minute per transfectant).
titers were determined. Phage G4 was found to produce a burst of approximately 200 to 400 phage at a time between 20 and 30 min after infection, while the G4Δori mutant gave rise to only 15 progeny phage after approximately 60 min.

In vitro DNA replication assay. DNA synthesis was measured by using the fraction II protein extract described by Fuller et al. (9). The fraction II protein extract was prepared from E. coli E177 (CGSC strain no. 5029; thr-1 leuB6 thi-1 thyA6 deoC1 dnaA177 lacY1 strA67 tonA21 supE44) exactly as described, with a 28% ammonium sulfate cut. The concentration of fraction II protein used in the DNA synthesis reactions described below was optimized by using single-stranded M13 DNA as template. The DNA synthesis reactions were carried out in a final volume of 20 μl and contained the following components: 40 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.6), 10 mM magnesium acetate, 40 mM creatine phosphate, 0.5 mM CTP, GTP, and UTP, 2 mM ATP, 0.1 mM dATP, dCTP, and dGTP, 0.1 mM [methyl-3H]dTTP (specific activity, 2,000, dpm/μmol of dTTP), 0.1 mg of creatine kinase per ml, 1 μg of SSB, 7% (wt/vol) polyvinyl alcohol 24,000 (Sigma), and 200 ng of single-stranded DNA template. Rifampin was added, when indicated, at a final concentration of 300 μg/ml. After the components were combined at 0°C, 325 μg of fraction II protein was added, and the reaction mixtures were incubated at 30°C for 20 min. Incorporation of nucleotide into trichloroacetic acid-precipitable material was measured by liquid scintillation quantitation.

RESULTS

Construction of the origin-cloning vector mRZ1000. To study G4 origin function in vivo, we constructed an origin-cloning vector, mRZ1000, the growth of which strongly relies upon the introduction of a functioning origin of complementary-strand DNA synthesis. The design of this vector and its use in screening DNA inserts for origin function is based on studies of M13 mutants deleted for their origin of complementary-strand DNA synthesis (16). The phage grow very poorly, giving rise to minute, turbid plaques; however, normal growth can be restored by the introduction of a DNA fragment carrying the G4 origin (21). This observation has led to the use of these M13 mutants as vectors for the isolation of single-stranded DNA replication determinants from bacterial episomes (20). For our study, we modified one of these mutant phages, M13ΔE101, by introducing the region of lacZ encoding the α chain, as present in M13mp8 (28). The resulting construct, mRZ1000 (Fig. 1), has several advantages over the parent phage M13ΔE101 as a cloning vector. The presence of unique restriction enzyme sites within the coding region for lacZ permits the insertion of DNA fragments and the screening of resulting clones owing to the insertional inactivation of lacZ. In addition, complementarity to the universal primer permits rapid DNA sequence determination by using the dideoxy sequencing protocol (23).

Characterization of mRZ1000 and cloned G4 origin activity. The viability of the phage was measured by infectious growth assays, in which early-log-phase cultures of host bacteria (F+ strain, JM101) were infected with phage at a low multiplicity of infection and the production of phage was monitored over the remaining period of log-phase growth (Fig. 3). The origin-cloning vector mRZ1000 grew very poorly in comparison with wild-type M13; however, introduction of the 274-bp AflI restriction fragment from phage G4, containing the G4 origin of complementary-strand DNA synthesis, resulted in high turbidity, indicating that the origin contained in this fragment was functional.
synthesis (see mRZ1200, Fig. 2), restored normal growth parameters (Fig. 3A). In addition, the efficiency of infection for mRZ1000, as measured in two different strains, was only 11 to 14% that of wild-type M13, while the efficiency of infection for the G4 origin clone mRZ1200 was between 75 and 90% that of wild-type M13 (Table 1).

The production of progeny phage was also measured by a single-cycle transfection assay. This assay, in which competent F− cells are transfected with viral DNA and the production of progeny phage is monitored during outgrowth, provides several advantages over the standard infectious growth assay. First, by introducing the viral DNA directly into the cells, potential differences in the accessibility of packaged DNA templates to the cellular replication apparatus are eliminated. This may be of importance, as it is known that the M13 genome is oriented specifically within the virion, such that the M13 origin of complementary-strand DNA synthesis is located at the end of the filament which is attached to the cell surface upon adsorption (29). Also, the viral DNA does not finish entering the cell until after complementary-strand DNA synthesis has begun (5). Therefore, the successful establishment of an infection could be affected by these packaging constraints. Second, introduction of the DNAs into competent cells is synchronized by the heat shock step followed by the addition of media at the start of outgrowth. This permits the determination of the lag period before which progeny phage appear. Third, because the competent cells are F−, they cannot be infected by progeny phage (filamentous phage require the F plasmid-encoded pili for adsorption to the cell surface). Consequently, by determination of the frequency of transfection, the rate of progeny phage production per transfectant may be determined.

The results of such an experiment are presented in Fig. 4 (the lag times and rates of progeny phage production derived from this experiment are presented in Table 1). The rate of mRZ1000 progeny phage production was less than 1% that of wild-type M13, and the lag time for the detection of mRZ1000 progeny phage was 45 min, in comparison with 24 min for wild-type M13. In contrast, the G4 origin construct mRZ1200 behaved very similarly to wild-type M13, having a rate of progeny phage production 90% that of wild-type M13 and a lag time equivalent to that of wild-type M13. In a parallel experiment, double-stranded replicative form DNAs were transfected and the production of phage was monitored (Fig. 4). There was little difference in the behavior of single-stranded versus double-stranded DNAs for M13 and mRZ1200, whereas double-stranded mRZ1000 DNA had a shorter lag time, 38 versus 45 min, and a rate of progeny phage synthesis 6 times higher than that of single-stranded mRZ1000 DNA (Table 1). The values obtained for double-stranded mRZ1000 were, however, much lower than the values for the origin-competent phages M13 and mRZ1200. This observation suggests a role for the origin of complementary-strand replication in stage II replication (see Discussion).

To measure DNA replication on the mRZ1000 template in comparison with wild-type M13 and the G4 origin clone mRZ1200, in vitro replication assays were performed by using the Kornberg fraction II system (9). DNA synthesis programmed by single-stranded mRZ1200 DNA, as measured by the incorporation of [methyl-3H]dTTP into acid-precipitable DNA (220 and 186 pmol of dTTP incorporated in the absence and presence of rifampin, respectively) was more than 5 times greater than synthesis programmed by mRZ1000 (39 and 21 pmol of dTTP incorporated in the absence and presence of rifampin, respectively). DNA synthesis programmed by mRZ1200 was not due to RNA polymerase-mediated primer formation, since rifampin had little effect on the extent of DNA synthesis, in contrast to a 6-fold reduction in DNA synthesis seen with the wild-type M13 template (114 and 18 pmol of dTTP incorporated in the absence and presence of rifampin, respectively). The data indicate that the origin-cloning vector is indeed deficient in DNA replication initiation and that the efficient rate of DNA synthesis measured on mRZ1200 is due to rifampin-resistant replication initiation, as expected from the primase-dependent G4 origin.

Defining the DNA requirements at the G4 origin. To define, at a gross level, the DNA requirements at the G4 origin, subfragments of the 274-bp AvaI restriction fragment from G4 were cloned into mRZ1000 and screened for origin function. Three subclones were chosen for study: mRZ1211, which contains the left half of the 274-bp region up to the AvaII sites; mRZ1212, which contains the central region between the two HindIII sites; and mRZ1213, which contains the right half of the 274-bp region starting from the AvaI site (Fig. 2). All three subclones were deficient in origin activity, as measured by infectious growth assays (Fig. 3A) and in vitro replication assays (Fig. 2). The second approach taken was to determine the boundaries of the G4 origin. The right boundary was defined by
deleting the G4 DNA sequence from the right end of the 274-bp AvaI restriction fragment by using S1 nuclease as a double-stranded DNA exonuclease (deletion constructs are shown in Fig. 2). The boundary was found to lie between positions 3990 and 4007 as defined by infectious growth characteristics (Fig. 3B) and in vitro DNA synthesis properties (Fig. 2) of the deletion constructs mRZ1216 and mRZ1217. Phage mRZ1216 was completely deficient in origin activity, whereas mRZ1217, and the deletion constructs mRZ1219 and mRZ1220 are functional in G4 origin activity, equivalent to that of mRZ1200. The left boundary, defined by a similar approach with mRZ1220 as the template for deletion formation and Bal 31 nuclease for removing the G4 DNA sequence from the left end, was found to lie between positions 3892 (deletion construct mRZ1221) and 3868 (deletion construct mRZ1222) (Fig. 2). The in vivo infectious growth assay was used to show that there was complete loss of origin activity in mRZ1221, whereas 100% origin activity was observed in the next deletion constructs, mRZ1222 and mRZ1223 (Fig. 3B). The in vitro DNA synthesis assays were not performed on these constructs. These results indicate that the G4 origin lies within the 139 bases defined by the deletion endpoints at positions 3892 and 4007.

Site-directed mutagenesis within the G4 origin. The DNA sequence within the G4 origin was altered by site-directed mutagenesis. A partial duplex was formed between the viral DNAs of two recombinant M13 phages, mRZ1200, containing the viral strand of the 274-bp AvaI restriction fragment, and mRZ1205, containing the complementary strand from the same fragment. The partial duplex was linearized by the restriction enzyme AvaI or AvaII. Both of these enzymes have more than one restriction site in the two M13 templates; however, only one enzyme cut within the double-stranded region of the partial duplex. The staggered ends generated by cleavage with either enzyme were made double stranded by being filled in with DNA polymerase I; the linear partial duplex with blunt ends was then ligated closed with T4 ligase. Before transformation, the partial duplexes were again subjected to cleavage by the restriction enzyme used originally. This step enriches for the desired product altered in its DNA sequence at the restriction enzyme site.

This technique was very efficient in generating small duplications at the AvaI (mRZ1206) and AvaII (mRZ1207) sites within the G4 DNA sequence. Likewise, a deletion between these restriction sites was generated (mRZ1208; Fig. 2). These constructs were analyzed for origin activity by the infectious growth assay (Fig. 3B) and in vitro replication assay (Fig. 2). The AvaI duplication mRZ1206 retained some origin activity, while the AvaII duplication mRZ1207 and the 17-base deletion construct mRZ1208 were completely deficient for origin activity.

To determine whether the 17-base deletion present in mRZ1208 results in the loss of G4 origin function in the parent phage, we repeated the site-specific mutagenesis procedure described above, with bacteriophage G4 viral DNA in place of mRZ1200 viral DNA. The resulting G4 phage DNA was transformed into E. coli C and found to give rise to small plaques. These small-plaque variants were grown up, and replicative-form DNA was isolated. The deletion construct of the AvaII site as a result of the deletion was confirmed by restriction analysis, indicating that the small-plaque variant is the result of the alteration of the DNA sequence within the G4 origin. In infectious growth assays, the small-plaque variant (G4Δori) grew poorly in comparison with wild-type bacteriophage G4 (Fig. 5), having a burst size of approximately 15 plaque in comparison with 200 to 400 plaque for wild-type G4.

FIG. 5. Bacteriophage G4 production in liquid culture. E. coli C was infected at early log phase of growth, and the production of G4 phage was monitored for wild-type G4 and G4Δori. See Materials and Methods for details.

DISCUSSION

Use of mRZ1000 to assay origin function. We used the origin-cloning vector mRZ1000 to define the DNA requirements at the G4 origin. This vector was shown to be deficient in DNA replication in vitro and to grow poorly in vivo. Introduction of the 274-bp restriction fragment from bacteriophage G4, containing the G4 origin, resulted in efficient rifampin-resistant DNA replication in vitro and restored near-normal infectious growth in vivo. The G4 origin did not restore 100% of the wild-type M13 growth rate, as measured by both the infectious growth and transfection assays, which may simply indicate that the primase-dependent G4 origin is not perfectly equipped to replace the RNA polymerase-dependent M13 origin of complementary-strand DNA synthesis. The vector mRZ1000 has been used for the isolation of single-stranded DNA replication initiation determinants from a mini-F plasmid (referred to as M13ΔElac in this study) (14) as well as studies of the bacteriophage G4 origin of complementary-strand DNA synthesis (this study; P. F. Lambert, E. Kawashima, and W. S. Reznikoff, submitted for publication).

The characterization of this vector suggested an interesting insight concerning the mechanism of M13 replicative-form DNA synthesis. In the transfection experiment (Fig. 4), the rate of progeny phage production seen for double-stranded mRZ1000 DNA was extremely low, similar to that of the single-stranded form. This result strongly supports the hypothesis that the M13 RNA polymerase-dependent origin of complementary-strand DNA synthesis is necessary not only for the initial conversion of viral DNA to a double-stranded replicative intermediate (stage I replication), but also for the subsequent amplification of this replicative intermediate (stage II replication). This proposal is consistent with those made by Staudenbauer et al. (26), but is in
contrast with studies on the thermosensitivity of M13 replication in dnaG and dnaB strains. These results led to an alternative proposal for the mechanism of complementary-strand DNA synthesis during stage II replication, involving discontinuous synthesis (22). The proposed utilization of the M13 origin of complementary-strand synthesis in stage II replication is also consistent with the models for DNA replication for bacteriophage F1, øX174, and G4 (6, 7, 12).

One caveat to this interpretation of the results of the transfection assay (Fig. 3) is that, while it permits the determination of sequences that are essential for normal growth of mRZ1000, it cannot distinguish whether the function being rescued is merely a replicative function (our hypothesis), or whether there is some other additional function which may have been impaired by the original deletion in this M13 mutant. Although we have no evidence for the existence of an impaired function in addition to the origin of complementary-strand synthesis, we cannot preclude this possibility. Several facts, however, are consistent with the origin of complementary strand synthesis as the only defective function in this M13 mutant. First, the defective infectious-growth phenotype found with mRZ1000 and its parent deletion mutant M13ΔE101 is rescued by the introduction of DNA sequences from heterologous sources, including plasmids as well as bacteriophages (14, 20, 21). In each case, the DNA sequence was found to contain a single-stranded DNA replication initiation determinant. It seems highly implausible that in every case a second function, hypothetically impaired in the M13 mutant, has been fortuitously supplied in addition to the replication functions. Second, in our study, in all cases in which in vitro rifampin-resistant DNA replication assays were performed (Fig. 2), mRZ1200 derivatives defective in vivo were also found to be deficient in DNA replication in vitro. We did not find mutations which were proficient in DNA replication but defective in growth, nor, vice versa, a phenotype which could be expected were a second function encoded on the cloned G4 DNA sequence. These arguments are consistent with there being one function impaired in the M13 mutant, mRZ1000, and one function encoded for by the G4 DNA sequence; that is, the origin of complementary-strand synthesis.

DNA requirements at the G4 origin. In this study, the minimal region of the bacteriophage G4 genome sufficient for origin activity was determined to be 139 bp long. The DNA sequence includes all regions conserved among the origins of complementary-strand synthesis in the single-stranded DNA bacteriophages φX, α3, st-1, and G4.

Just to the right of stemloop I, there is a 42-base-long A+T rich region which shares 70% homology with the origin of replication for bacteriophage lambda (Fig. 6). Since the bacteriophage lambda origin is also primase dependent (16), it was postulated that this region of homology may be the site for primase binding. In addition, the location of this homologous sequence just upstream of the RNA primer site in the G4 origin also supports the concept of its being a binding site. The deletion endpoint in mRZ1217, however, contains less than half of this region of homology, and yet the mRZ1217 origin is fully functional in vivo. It is not clear whether the remaining portion of homology is important, as the next deletion endpoint, in mRZ1216, removes not only this region but also part of stemloop I. That at least part of this region homologous to the bacteriophage lambda origin is not necessary for origin function may not be surprising, since the mechanism of primase action at these origins is quite different. McMacken et al. (18) have suggested that primase binding to the lambda origin is primosome dependent at positions where the assembly of the primosome is directed by lambda O and P proteins. This is quite different from the primosome-independent binding by primase at the G4 origin. A significant aspect of this region of homology may be its A+T richness (21 of 26 bases conserved between these origins were A or T).
The minimal origin sequence includes regions of potential secondary structure, stemloops I and II, present in the gene F-G intercistronic region (Fig. 6). Neither region of secondary structure alone, however, was sufficient for origin function. Of particular interest are the subclones mRZ1212 and mRZ1213, which contain stemloop II and stemloop I, respectively. The infectious growth related of these constructs were no higher than that of the vector mRZ1000. DNA in addition to these regions of potential secondary structure must be necessary, based upon the origin-deficient character of deletion endpoint mRZ1221. This deletion contains G4 DNA sequence information up to and including the last base involved in stemloop II structure. The additional G4 DNA sequence present to the left of stemloop II in deletion endpoint mRZ1222 includes part of the direct repeat indicated in Fig. 6. This direct repeat is highly conserved in the origin sequences of the other primase-dependent single-stranded DNA bacteriophage (25). Furthermore, both copies of the direct repeat are located within the stemloop regions of the φK origin protected by primase from nuclease digestion (24). Another characteristic of this region is its high content of A residues, as is true of the region to the right of stemloop I.

Site-directed mutagenesis. The formation of partial duplexes in vitro between recombinant M13 viral DNAs allows for the alteration of DNA sequences at nonunique restriction enzyme sites. In this study, small insertions and deletions were generated within the G4 origin. The 3-base duplication at the AvaI II site (mRZ1207), as well as the 17-base deletion between the AvaI and AvaI II sites, resulted in the total loss of origin function. Both of these mutations destroy 1 bp at the base of stemloop II and affect the spacer region between stemloop II and stemloop I. In contrast, the 4-base duplication at the AvaI site (mRZ1206), which only changes the spacing between stemloop I and II, resulted in a slight decrease in origin activity as measured by the in vivo and in vitro assays. Therefore, the strong effect of the mutations in mRZ1207 and mRZ1208 may be due to the disruption of secondary structure.

Site-specific mutagenesis was also used to alter the G4 origin DNA sequence within the G4 genome. This resulted in a small-plaque phenotype, which may be similar to the phenotype of M13 mutants defective in their origin of complementary-strand DNA synthesis (16). That this 17-base deletion resulted in a defective phenotype in bacteriophage G4 is consistent with the effect it had on the G4 origin when assayed in the origin cloning vector mRZ1000. We also noticed that this G4 mutant reverted at a high frequency, giving rise to medium-sized plaques (data not shown). These revertants appear to be quite stable, although their rate of infectious growth is still quite poor in comparison with that of wild-type G4. It would be interesting to determine whether these revertants are due to a compensating change in the origin region or perhaps to the generation of a more functional secondary origin.

In conclusion, we have determined that the minimal sequence sufficient for G4 origin activity is no longer than 139 bases. Within this minimal origin are the two segments of DNA sequence highly conserved within the origins of the single-stranded DNA bacteriophages φX174, φ3, st-1, and G4. Not contained within the sequence is over 50% of the region of homology to the bacteriophage lambda origin of DNA replication. The role of secondary structure at primase-dependent origins of complementary-strand DNA synthesis has been suggested both by in vitro studies on primase interaction at these origins and by the sequence conservation

within the regions of potential secondary structure. More recently, single-base substitutions which disrupt intranuclear basepairing at stemloop I have been found to affect G4 origin function in vivo (Lambert et al., submitted). The results presented in this study confirm the requirement of these regions of potential secondary structure for G4 origin function in vivo and indicate the need for additional G4 DNA sequence outside of these regions.

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LITERATURE CITED