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Nucleotide Sequence of the Primer RNA for DNA Replication of Filamentous Bacteriophages

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We determined the nucleotide sequence of RNA synthesized in vitro by *Escherichia coli* RNA polymerase at the complementary-strand replication origin on the single-stranded viral DNA of bacteriophages f1 and IKe (ori-RNA) by using chain-terminating ribonucleoside triphosphate analogs. The results indicated that the start site of f1 ori-RNA synthesis is 20 nucleotides downstream from the site previously reported (K. Geider, E. Beck, and H. Schaller, Proc. Natl. Acad. Sci. USA 75:645–649, 1978) and that the RNA sequence [(5′)pppAGGGC GAUGGCCCACUACGU-OH(3′)] is complementary to the f1 DNA sequence from nucleotides 5736 to 5717, with minor heterogeneity at the 3′ end. IKe ori-RNA had a sequence identical to that of f1 ori-RNA, except for a single base substitution, and IKe RNA was complementary to a region of IKe DNA (from nucleotides 6441 to 6422) that was homologous to the f1 sequence. Phenotypes and ori-RNA sequences in the relevant region of the genome of f1 deletion mutants were consistent with the presently determined sequence of ori-RNA. A possibility that ori-RNA synthesis is initiated by a mechanism similar to that for general transcription is suggested as a result of the new assignment of the ori-RNA start site. The double-origin plasmid assay of minus-strand origin activity, a sensitive in vivo method for detecting *cis*-acting elements for the initiation of DNA replication on a single-stranded DNA template, is described.

The F plasmid-specific filamentous Ff bacteriophages (f1, M13, and fd) have a circular, single-stranded DNA genome, which replicates via two mechanisms: (i) synthesis of the minus strand on a single-stranded template to yield the double-stranded replicative form and (ii) synthesis of the plus strand on the replicative form by rolling-circle-type replication that is initiated by the phage-encoded gene II protein. The origins for both minus-strand and plus-strand syntheses are located close to each other within an intergenic region on the phage genome (for a review, see references 20 and 26).

Synthesis of the minus strand is initiated by an RNA primer (ori-RNA) that is synthesized at a unique site on the viral DNA by the host RNA polymerase (10). The 5′-terminal nucleotide of ori-RNA was reported by Geider et al. (9) to correspond to nucleotide 5756 of phage f1 or M13 DNA (see Fig. 3). Near this site are two inverted repeats that can form two hairpin structures, named B and C (see Fig. 6). These inverted repeats are protected by the RNA polymerase from nuclease digestion when the enzyme forms a complex with the viral DNA in the presence of the *Escherichia coli* single-strand binding protein (SSB) (24). Large portions of the inverted repeats are required for a normal level of minus-strand synthesis (18; unpublished data). Deletion mutant phages that lack the entire region of the minus-strand origin can grow, although very poorly, producing less than 1% the normal phage yield (12, 18).

The nucleotide sequence of an N plasmid-specific filamentous bacteriophage, IKe, has been determined and compared with those of Ff bacteriophages (22). A high degree of sequence homology between IKe and Ff in the region including hairpins B and C suggests that the initiation of complementary-strand synthesis is accomplished in both phages by the same RNA polymerase-dependent mechanism. However, the IKe and Ff sequences differ from each other in a short region near nucleotide 5756 of phage f1. Furthermore, the results of deletion studies of the relevant region of phage f1 (unpublished data; see also below) suggested that the nucleotides near 5756 can be altered without marked effects on the origin function. These observations prompted us to reexamine the nucleotide sequence of ori-RNA. The results described in this paper indicate that the start site of ori-RNA in both f1 and IKe is located 20 nucleotides downstream from the previously reported site.

MATERIALS AND METHODS

Bacteria, phages, and plasmids. *E. coli* K38 (Hfr) (15) and JE257/N3 (2) were used for the growth of phages f1 and IKe, respectively. K902 (Hfr *supE recA*) (8) was used for the double-origin plasmid assay. R227, a bacteriophage f1 mutant, was obtained from the laboratory collection of N. D. Zinder, Rockefeller University. R368 (12) is a derivative of f1 that carries an *Eco*RI site at position 5615 (for nucleotide numbering of f1, see reference 13) and a *Pst*I site at position 5769. R377 (12) carries a ca. 150-bp deletion between positions 5615 and 5769, thus lacking the entire origin region for minus-strand synthesis.

pAR2 is a derivative of pBR322 (1) that carries an *Hpa*II-H fragment (from positions 5615 to 5996) of wild-type f1 inserted at the *Bam*HI site in the counterclockwise orientation. pDOWT was constructed by inserting the *Hpa*II-H fragment of R368 at the *Eco*RI site of pAR2. pDO377 was constructed by inserting an *Hae*II (position 5568 of f1)-*Hpa*II (position 5996 of f1) fragment of R377 at the *Hin*dIII site of pAR2. The orientation of these inserts was the same as that of the first insert at the *Bam*HI site in pAR2.

Deletions Δ410 and Δ411 were obtained by treating a *Pst*I-cleaved R368 DNA fragment with exonuclease Bal-31 under stringent conditions (4) and then ligating the fragment to a *Pst*I linker. The R368 fragment in pDOWT was replaced

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with fragments carrying deletions Δ410 and Δ411, yielding plasmids pDO410 and pDO411, respectively. Phages carrying the deletions were constructed by ligating the smaller *Eco*RI-*Pst*I fragments of pDO410 and pDO411 with the larger *Eco*RI-*Pst*I fragment of R368 and were named R410 and R411, respectively. The nucleotide sequence of the deletions was determined by the method of Sanger et al. (23).

Enzymes and chemicals. *E. coli* RNA polymerase holoenzyme and SSB were generously provided by A. Ishihama and N. Shimamoto, respectively, National Institute of Genetics. *Saccharomyces cerevisiae* capping enzyme was a generous gift from K. Mizumoto, Kitasato University. Exonuclease Bal-31 was from Bethesda Research Laboratories. 3′-deoxyribonucleoside triphosphates (3′-dNTPs) and nonradioactive nucleoside triphosphates were from Boehringer Mannheim. [α-^{32}P]GTP (800 Ci/mmol) was from DuPont/NEN Research Products. Oligoribonucleotides with the sequences (5′)AGGGCGAUGGC(3′) (11-mer), (5′)AGGGCGAUGGCCCA(3′) (14-mer), (5′)AGGGCGAUGGCCCACUACGU(3′) (20-mer), and (5′)AGGGCGAUGGCCCACUACGUG(3′) (21-mer) were from Bex Co. Ltd., Tokyo, Japan. G(5′)ppp(5′)G and G(5′)ppp(5′)A were from Pharmacia.

In vitro capping of primer RNA and cap analysis. RNA synthesis was carried out with a 100-μl reaction mixture containing 0.5 pmol of single-stranded phage DNA, 600 pmol (12 μg) of SSB, 40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–KOH (pH 7.5), 130 mM KCl, 0.02 mg of bovine serum albumin per ml, 5 mM $MgCl_2$, 500 μM each ATP, CTP, and UTP, 50 μM GTP, 25 μCi of [α-^{32}P]GTP (800 Ci/mmol), and 5 pmol of RNA polymerase holoenzyme at 30°C for 20 min. The reaction was stopped by the addition of 200 μl of stop solution (7 M urea, 0.5% sodium dodecyl sulfate, 0.3 M NaCl, 0.5 mM EDTA, 20 mM Tris-HCl [pH 7.5]), and nucleic acids were extracted with phenol-chloroform and precipitated with 2.5 volumes of ethanol. The precipitate was dissolved in 40 μl of 2 M ammonium acetate, precipitated twice with ethanol, washed with 70% ethanol, and dried. Cap formation was carried out as described previously (16) with a 20-μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mg of bovine serum albumin per ml, 20% glycerol, 10 μM GTP, 60 μCi of [α-^{32}P]GTP (800 Ci/mmol), and 1 to 2 U of yeast capping enzyme. After incubation at 30°C for 60 min, 60 μl of stop solution was added, and nucleic acids were extracted with phenol-chloroform, precipitated with ethanol, dissolved in blue mix (80% formamide containing bromophenol blue and xylene cyanol), and subjected to electrophoresis through 18% polyacrylamide–8 M urea gels at 1,800 V. ori-RNA was isolated from the gels and extracted with phenol. After treatment with P1 nuclease and alkaline phosphatase, the resulting products were subjected to paper electrophoresis on Whatman DE81 paper (19), and autoradiography was carried out with a Fuji image analyzer.

RNA sequencing. RNA was synthesized in a 20-μl reaction mixture containing 0.1 pmol of single-stranded phage DNA, 120 pmol (2.4 μg) of SSB, 40 mM HEPES–KOH (pH 7.5), 130 mM KCl, 0.02 mg of bovine serum albumin per ml, 5 mM $MgCl_2$, 10 μCi of [α-^{32}P]GTP (800 Ci/mmol), 1 pmol of RNA polymerase holoenzyme, and the following concentrations of ribonucleotides in each reaction: reaction 1 (standard), 500 μM each ATP, CTP, and UTP and 50 μM GTP; reaction 2 (A+), 1 mM 3′-dATP, 50 μM ATP, 500 μM each CTP and UTP, and 50 μM GTP; reaction 3 (A−), 50 μM ATP, 500 μM each CTP and UTP, and 50 μM GTP; reaction 4 (G+), 1 mM 3′-dGTP, 25 μM GTP, and 500 μM each ATP, CTP, and UTP; reaction 5 (G−), 25 μM GTP and 500 μM each ATP, CTP, and UTP; reaction 6 (C+), 500 μM 3′-dCTP, 50 μM CTP, 500 μM each ATP and UTP, and 50 μM GTP; reaction 7 (C−), 50 μM CTP, 500 μM each ATP and UTP, and 50 μM GTP; reaction 8 (U+), 1 mM 3′-dUTP, 25 μM UTP, 500 μM each ATP and CTP, and 50 μM GTP; and reaction 9 (U−), 25 μM UTP, 500 μM each ATP and CTP, and 50 μM GTP. After incubation at 30°C for 20 min, the reactions were stopped by the addition of 80 μl of stop solution, yielding final concentrations of 80 mM NaCl and 10 mM EDTA. The nucleic acids were precipitated with 250 μl of ethanol, dried, and suspended in 5 μl of blue mix. The samples were boiled for 2 min, chilled on ice, and subjected to electrophoresis through 18% polyacrylamide–8 M urea gels at 1,800 V. The gels were dried, and autoradiography was carried out with a Fuji image analyzer.

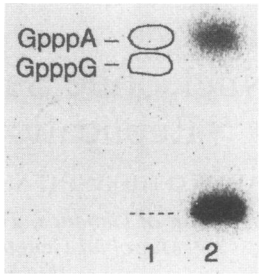

FIG. 1. 5′-Terminal nucleotide of f1 ori-RNA. RNA synthesized in vitro on f1 single-stranded DNA by *E. coli* RNA polymerase was subjected to a capping reaction with yeast capping enzyme and [^{32}P]GTP and to subsequent digestion with P1 nuclease and analyzed by paper electrophoresis as described in Materials and Methods (lane 2). The positions of two markers are indicated in lane 1. The dashed line represents the origin. GpppC and GpppU (not shown) move faster than GpppA (7).

Double-origin plasmid assay. K902 cells carrying a double-origin plasmid were grown at 37°C in TY medium (25) to an optical density at 660 nm of 0.3 and divided into two parts. One was infected with f1 phage at a multiplicity of infection of 50, while the other remained uninfected. After further incubation at 37°C for 30 min, cells were collected. Intracellular DNA was extracted as described previously (6) and electrophoresed through 0.7% agarose gels containing 0.5 μg of ethidium bromide per ml.

RESULTS

Nucleotide sequence of ori-RNA of wild-type f1. We determined the 5′-terminal nucleotide of in vitro-synthesized f1 ori-RNA by labeling it with [α-^{32}P]GTP by use of the yeast capping enzyme. This enzyme can efficiently cap the 5′ di- or triphosphate end of RNA but not the monophosphate end of RNA or DNA by adding a guanine nucleotide in a 5′-5′ condensation (16). The capped RNA was digested with P1 nuclease and electrophoresed as described in Materials and Methods. The results shown in Fig. 1 indicate that the 5′-terminal residue of ori-RNA is adenine, in agreement with the previous report (9).

The sequence of f1 ori-RNA was determined by synthesizing it on the single-stranded DNA template with *E. coli* RNA polymerase in the presence of chain-terminating ribonucleoside triphosphate analogs (3′-dNTPs) (21). An excess amount of *E. coli* SSB was added to the reaction to prevent nonspecific initiation (see Materials and Methods). The gel

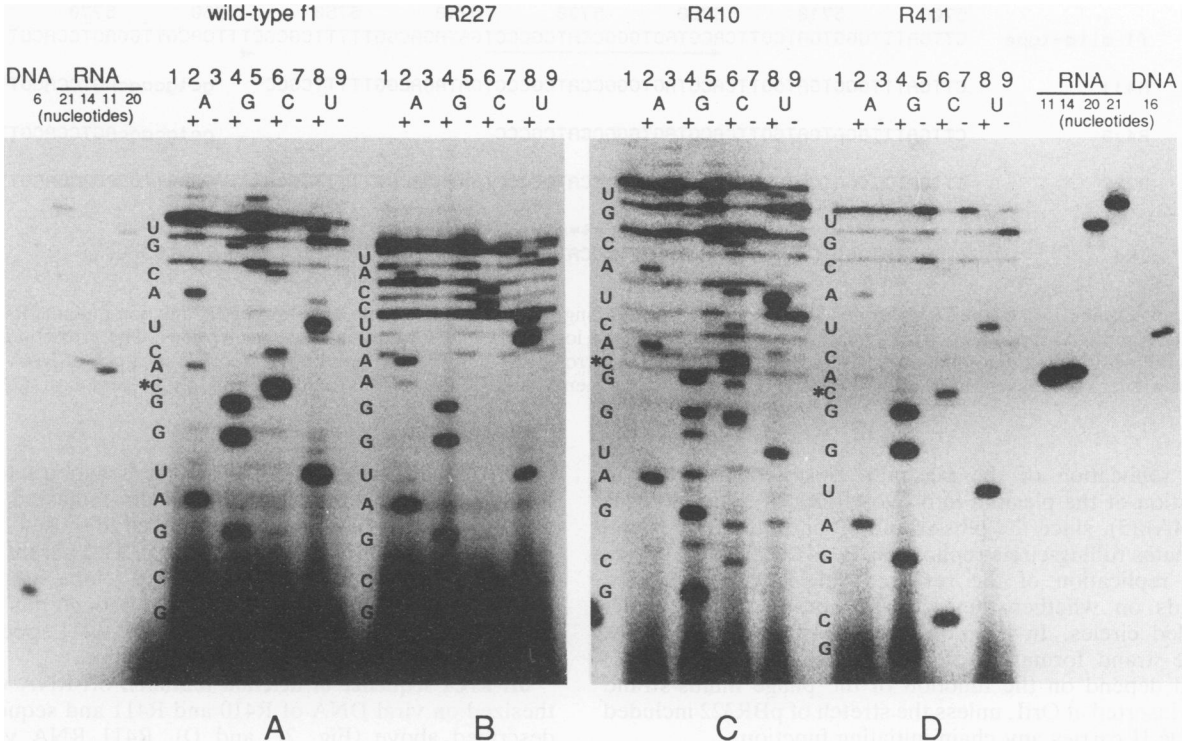

FIG. 2. Sequencing gel electrophoresis of ori-RNAs. RNAs labeled with [α-^{32}P]GTP were synthesized in vitro on single-stranded DNA templates in the presence or absence of chain-terminating ribonucleoside triphosphate analogs (3′-dNTPs) and electrophoresed as described in Materials and Methods. The template DNA used was as follows: A, wild-type f1; B, R227; C, R410; D, R411. Lanes: 1, RNA synthesized under standard conditions; 2, 4, 6, and 8, RNAs synthesized in the presence of 3′-dATP, 3′-dGTP, 3′-dCTP, and 3′-dUTP, respectively; 3, 5, 7, and 9, controls synthesized in the absence of 3′-dNTPs. 5′-Terminally labeled oligoribonucleotides (11-mer, 14-mer, 20-mer, and 21-mer; see Materials and Methods) and oligodeoxyribonucleotides (6-mer and 16-mer) were electrophoresed in parallel and are shown on both sides of the figure. The bottom portion of the figure is overexposed so that a few bands at the bottom of panels A and B are difficult to see. However, these bands were clearly observed on less-exposed films.

pattern obtained (Fig. 2A) showed the sequence (5′). . . GCGAUGGC*ACUACGU(3′), where . . . represents a few nucleotides at the 5′ end that were not resolved in the gel and C* represents a somewhat broad band in lane C. This sequence is complementary to the f1 DNA sequence of nucleotides 5733 to 5717, except that the three consecutive G's from 5724 to 5726 in the DNA sequence are represented by a single C band (C*) rather than three C bands.

Since this result was probably due to the compression of RNA bands in this particular region, in which the expected sequence was (5′)GGCCC(3′), we used two synthetic oligoribonucleotides with the following sequences as electrophoretic markers: (5′)AGGGCGAUGGC(3′) (11-mer) and (5′)AGGGCGAUGGCCCA(3′) (14-mer). These sequences should have been identical to the ori-RNA sequence if it started at nucleotide 5736 and terminated at nucleotides 5726 and 5723, respectively. As shown in Fig. 2, the mobility of these oligoribonucleotides clearly indicated the presence of compression of the three C bands. Moreover, a comparison of the sequence ladder and the mobility of the oligoribonucleotides indicated that ori-RNA must start at nucleotide 5736. Thus, the entire sequence of ori-RNA is complementary to that of f1 DNA from nucleotides 5736 to 5717, 20 nucleotides in length, although there is minor heterogeneity at the 3′ terminus (Fig. 2).

To verify the RNA sequence, we determined the sequence of ori-RNA of R227, an f1 mutant in which 13 nucleotides from 5714 to 5726 are replaced by 4 nucleotides with the sequence (5′)AATT(3′) (Fig. 3). Thus, R227 lacks the region which showed the compression on the sequencing gel. The results shown in Fig. 2B indicated that R227 ori-RNA started at the same nucleotide as wild-type ori-RNA and that its sequence was (5′). . .GCGAUGGAAUUCCAU(3′), complementary to the mutant DNA sequence. The termination site of R227 ori-RNA was more variable than that of the wild type, and the major species of mutant RNA had a total length of 18 nucleotides.

In vivo origin function of deletion mutants. Two f1 mutants, R410 and R411, in which parts of the relevant region were deleted, were obtained as described in Materials and Methods. R411 lacks nucleotides 5755 to 5766 (Δ411), while R410 lacks nucleotides 5736 to 5766 (Δ410). Because a *Pst*I linker was used to construct the mutants, the deleted nucleotides were replaced by an octanucleotide with the sequence (5′)GCTGCAGC(3′) (Fig. 3). R411 showed normal plaques, while R410 showed tiny, turbid plaques.

We assayed the chain-initiating function of the mutant origins in vivo by using a double-origin plasmid system (Fig. 4A). We inserted two copies of an f1 restriction fragment, each of which carried functional origins for both plus- and minus-strand replication, in the same orientation into two sites of pBR322. In the absence of phage, this plasmid replicates by use of the authentic plasmid origin. When a cell harboring the plasmid is infected with f1, the phage-encoded initiator protein (gene II protein) acts on the plus-strand origins on the plasmid and leads to the phage-type rolling-

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                5700      5710      5720      5730      5740      5750      5760      5770
f1 wild-type    CTTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTT

R411            CTTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCC    gctgcagcAGTCCACGTT

R410            CTTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCC                        gctgcagcAGTCCACGTT

R227            CTTGATTTGGGTGATGGaa          ttCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTT

                **** * ********************************** ** ******  ******  *** **
IKe             CTTGTTATGGGTGATGGTTCACGTAGTGGGCCATCGCCTTGTTAGACGTTTTTTTCTTTTGGTGATCG
                     6410      6420      6430      6440      6450      6460
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FIG. 3. Comparison of the DNA sequence in the region surrounding the ori-RNA start site of wild-type f1, deletion mutants R411, R410, and R227, and wild-type IKe. Blank spaces indicate deletions, and lowercase letters show inserted nucleotides. The arrow indicates the position of ori-RNA as determined in the present study, while the arrowhead indicates the previously reported start site of RNA synthesis (9). The numerals represent nucleotide numbers. *, homology between f1 and IKe sequences, as determined by Peeters et al. (22).

circle replication of the plasmid. This action results in resolution of the plasmid into two circles of different sizes (Fig. 4A) (5), since the plus-strand origin both initiates and terminates rolling-circle replication (3, 14). However, subsequent replication of the resolved single-stranded circles depends on whether they can be converted to double-stranded circles. In the configuration shown in Fig. 4A, double-strand formation of the smaller circle (named II) should depend on the function of the phage minus-strand origin inserted at OriI, unless the stretch of pBR322 included in circle II carries any chain-initiating function.

When the minus-strand origin inserted at OriI was the wild type (pDOWT), circle II replicated upon f1 infection (Fig. 4B). On the contrary, when the insert at OriI had a deletion of the entire minus-strand origin (pDO377), little replication of circle II was observed (Fig. 4B). Thus, the replication of circle II does depend on the function of the minus-strand origin in the insert at OriI.

We constructed double-origin plasmids pDO410 and pDO411, which contain fragments carrying Δ410 and Δ411, respectively, inserted at the OriI site, and tested them for origin function. The results shown in Fig. 4B indicated that circle II carrying Δ411 replicated effectively, while circle II carrying Δ410 replicated less efficiently. The intensity of the bands on the gel was quantitated by densitometric tracing of Polaroid 665 negative film. The results indicated that the relative amounts of circle II, normalized to those of circle I, for pDOWT, pDO377, pDO410, and pDO411 were 100, 10, 50, and 90%, respectively. Thus, Δ410 retains a significant level of minus-strand origin function, although this function is somewhat reduced. On the other hand, Δ411 appears fully active.

ori-RNA sequence of deletion mutants. ori-RNA was synthesized on viral DNA of R410 and R411 and sequenced as described above (Fig. 2C and D). R411 RNA yielded a sequence identical to that yielded by wild-type f1 RNA. The sequencing gel pattern of R410 RNA showed some heterogeneity, but the major species appeared to be synthesized starting from 1 nucleotide upstream of and terminating at the same nucleotide as the wild type. Thus, the major species of R410 ori-RNA was 21 nucleotides long.

ori-RNA sequence of IKe. ori-RNA of the N-plasmid-specific filamentous phage IKe was sequenced in the same way. The results shown in Fig. 5 indicate that IKe ori-RNA has the same sequence and start site as f1 ori-RNA. Thus, ori-RNA of IKe starts at nucleotide 6441 of the IKe DNA sequence, which corresponds to nucleotide 5736 of the f1 DNA sequence. Some of the gel bands shown in Fig. 5 are

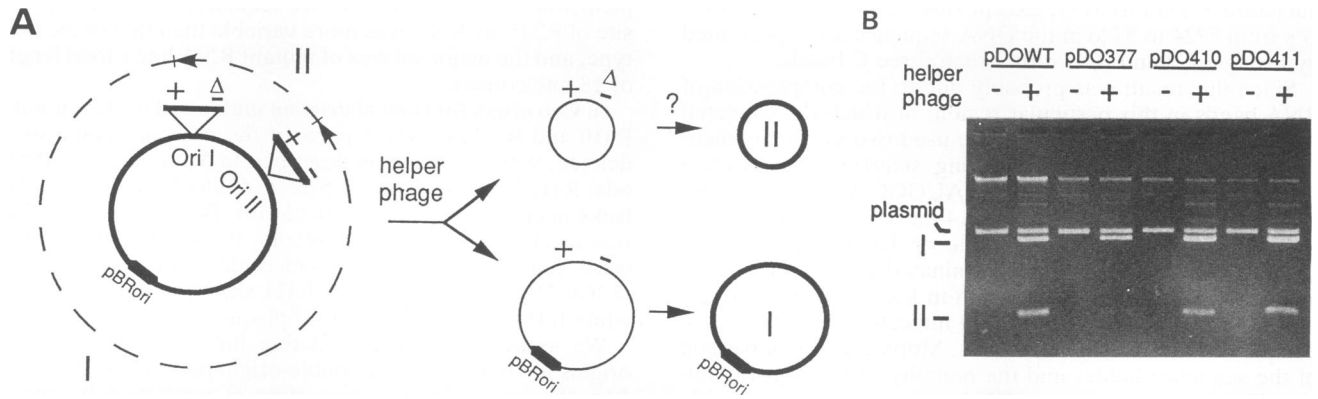

FIG. 4. Double-origin plasmid assay of minus-strand origin function. Plasmid pDOWT contains two wild-type f1 origins (the inserts indicated as OriI and OriII) in direct repeats. In pDO377, the entire minus-strand origin at OriI is deleted (indicated by Δ). Upon infection with helper phage, rolling-circle replication of the plasmid results in the production of single-stranded circles of two different sizes. Further replication of the smaller circle (labeled II) depends on the function of the minus-strand origin inserted at OriI. (A) Schematic presentation of the strategy (for details, see the text). pBRori indicates the replication origin of pBR322. (B) K902 cells harboring pDOWT, pDO377, pDO410 or pDO411, were grown at 37°C for 30 min in the presence (+) or absence (−) of phage f1 (multiplicity of infection, 50). The intracellular DNA was extracted and analyzed on a 0.7% agarose gel containing ethidium bromide. The positions of negatively supercoiled DNA of the parental plasmid, circle I, and circle II are indicated on the left.

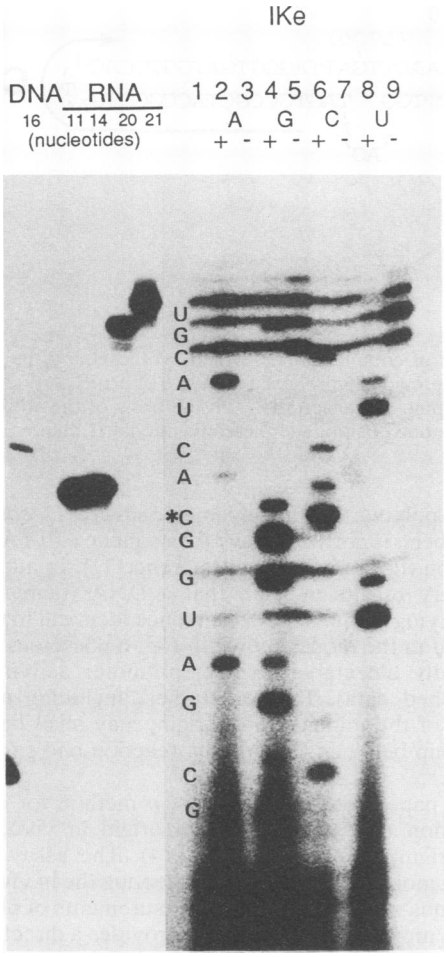

FIG. 5. Sequencing analysis of IKe ori-RNA. RNA synthesized on a single-stranded IKe DNA template in the presence or absence of chain-terminating ribonucleoside triphosphate analogs (3′-dNTPs) was electrophoresed as described in Materials and Methods. Lanes: 1, RNA synthesized under standard conditions; 2, 4, 6, and 8, RNA synthesized in the presence of 3′-dATP, 3′-dGTP, 3′-dCTP, and 3′-dUTP, respectively; 3, 5, 7, and 9, controls synthesized in the absence of 3′-dNTPs. Labeled oligoribonucleotides (11-mer, 14-mer, 20-mer, and 21-mer; see Materials and Methods) and an oligodeoxyribonucleotide (16-mer) electrophoresed in parallel are shown on the left.

accompanied by a more slowly moving, lighter band. This band may indicate the presence of a minor species of IKe ori-RNA that starts 1 nucleotide upstream of the major start site.

DISCUSSION

In this study, we determined the nucleotide sequence of RNA (ori-RNA) synthesized at the minus-strand origin on the viral DNA of f1 by using chain-terminating ribonucleoside triphosphate analogs (3′-dNTPs). Our data (Fig. 1 and 2) indicate that the total sequence of the major species is (5′)pppAGGGCGAUGGCCCACUACGU-OH(3′), which is complementary to the f1 DNA sequence from positions 5736 to 5717 (Fig. 3), with minor heterogeneity at the 3′ end. Although our sequencing gels did not resolve a few nucleotides at the 5′ end of the RNA, the mobility of the sequencing gel bands when compared with that of chemically synthesized oligoribonucleotides indicated that RNA synthesis started from position 5736. This result meant that the 5′ terminus of the RNA must be adenine, and our analysis by the capping reaction indicated that this was the case (Fig. 1).

Reading the RNA sequence was somewhat complicated by compression of the three consecutive C bands on the gels (Fig. 2A). However, the use of R227, an f1 mutant that lacks the region including these C residues, clearly showed that the RNA sequence is complementary to the DNA sequence of this region and that the synthesis of R227 RNA starts at the same nucleotide as that of wild-type f1 RNA, position 5736.

The genome of phage IKe, an N-plasmid-specific filamentous phage, is 476 nucleotides longer than that of phage f1. However, both genomes contain 10 homologous genes and one large intergenic space, the order of which is identical. Overall homology between their nucleotide sequences is approximately 55% (22). Extensive sequence homology was noted in the region including hairpins B and C in the intergenic region, suggesting that the initiation of minus-strand synthesis takes place at the same sequence. Our results (Fig. 5) indicate that the sequence of the RNA synthesized by the host RNA polymerase on IKe viral DNA is complementary to the IKe sequence DNA from nucleotides 6441 to 6422, which exactly corresponds to the f1 DNA sequence from nucleotides 5736 to 5717 (Fig. 3). Thus, the two RNAs are essentially identical, differing from each other by only 1 nucleotide, the second residue from the 5′ terminus being G in f1 and A in IKe.

Our results indicate that the start site of ori-RNA is located 20 nucleotides downstream from the site previously reported for phage fd by Geider et al. (9), which corresponded to nucleotide 5756 of f1 (Fig. 3). They analyzed ori-RNA by fingerprinting after digestion with nuclease T1 and studied the composition of each spot by nearest-neighbor analysis. The total sequence of ori-RNA was then deduced from the known DNA sequence in the relevant region. Their conclusion was that the RNA sequence was complementary to the DNA sequence from positions 5756 to 5727. It should be noted that seven consecutive nucleotides starting from 5756 are identical to those starting from 5736 [the RNA sequence (5′)AGGGCGA(3′)]. Thus, the 20- or 21-nucleotide-long RNA starting from position 5736 is expected to yield fingerprints and nearest-neighbor results similar to those reported by Geider et al. (9). They also mapped the 3′ terminus of ori-RNA by extension with DNA polymerase I, cleavage with restriction enzymes, and measurement of the lengths of the DNA fragments produced. The results showed a certain heterogeneity in the 3′ end of the ori-RNA, and even the longest DNA fragments observed were shorter by about 5 nucleotides than the lengths expected on the assumption that ori-RNA ended at position 5727. This result meant that the 3′ end of ori-RNA should have been in the region from 5722 to 5717, in agreement with our present results.

With position 5736 of f1 having been determined as the initiation site of ori-RNA, the nucleotide sequences surrounding the initiation sites in f1 and IKe would have been essentially identical. If the start site were position 5756 of f1, the surrounding sequences would have been different for the two phages (see Fig. 3), and it would have been necessary to explain how *E. coli* RNA polymerase could start synthesizing two ori-RNAs with different 5′-terminal sequences.

The phenotypes of f1 deletion mutants in the relevant

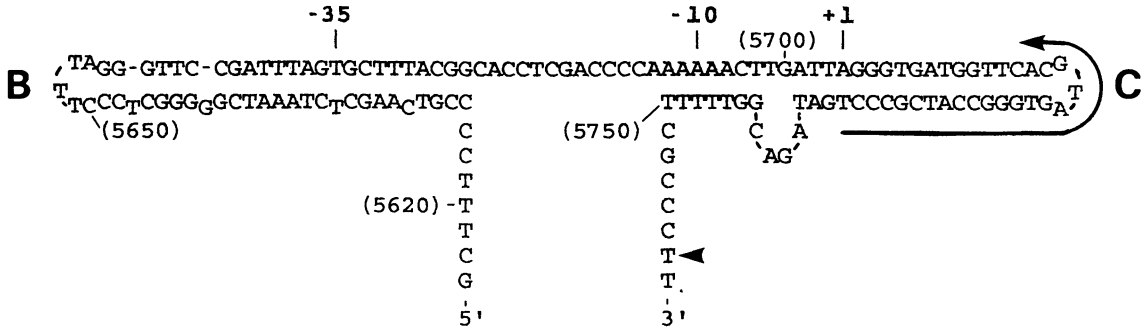

FIG. 6. Possible secondary structure of DNA (hairpins B and C) at the origin of complementary-strand synthesis and the location of ori-RNA of phage f1. The arrow represents ori-RNA. The arrowhead indicates the previously proposed start site of ori-RNA (9). Nucleotide numbers are shown with a + or − sign in a manner analogous to that used for general transcription, on the basis of the RNA start site determined in the present study. The numerals in parentheses represent the nucleotide positions in the conventional f1 map.

region were consistent with the assignment of the ori-RNA start site to position 5736. R411, in which the deletion ranges from 5755 to 5766, grew as efficiently as the wild-type phage and showed positive origin activity in the in vivo double-origin plasmid assay (Fig. 4). In vitro, its DNA produced ori-RNA that was indistinguishable from that of the wild type (Fig. 2). R410, in which the deletion ranges from 5736 to 5766 (Fig. 3), showed 50% reduced but definitely positive origin activity in the double-origin plasmid assay (Fig. 4). Its ori-RNA molecules showed a certain heterogeneity in the start site, but the major species had the same sequence as that in the wild type, except that it started 1 nucleotide upstream (Fig. 2). Such results would not be expected were wild-type ori-RNA to start at position 5756. We conclude that ori-RNA of f1 starts at position 5736.

In the minus-strand origin of R410, the entire DNA sequence upstream of the ori-RNA start site is missing, and the starting nucleotide of wild-type ori-RNA is changed (Fig. 3). Therefore, the fact that R410 showed an almost normal level (ca. 50%) of origin function indicates that the sequence elements important for origin function are located only downstream from the ori-RNA start site.

The mechanism by which ori-RNA is synthesized at a specific site is not well understood. It has generally been assumed that ori-RNA synthesis is initiated by some mechanism that is entirely different from that of the initiation of general transcription. One of the main reasons for this assumption was that, on the basis of the previous assignment of the ori-RNA start site (9), RNA synthesis should start and proceed in the direction toward hairpins C and B (Fig. 6), at which RNA polymerase binds. This situation is opposite that of RNA synthesis from promoters. On the other hand, Kaguni and Kornberg (17) reported that the specificity of the priming reaction strictly depends on the presence of the σ subunit of RNA polymerase. With the new assignment of the start site within the stem of hairpin C (Fig. 6), it is now possible to search for structures that are recognized by RNA polymerase in the priming reaction by taking the analogy of promoter recognition into account.

Examination of the nucleotide sequence of the top strand in the structure depicted in Fig. 6 reveals sequence elements such as TTTACG, GTGCTT, and TTTAGT in the region about 35 nucleotides upstream from the +1 position and sequence elements such as AAAAAA and AAAACT in the region about 10 nucleotides upstream from the +1 position. Although these sequence elements are not identical to those found in typical promoters, they might act as binding sites for RNA polymerase. For example, several *E. coli* promoters have been reported to have the sequence TTTACG in the −35 region (for a review, see reference 11). In addition, our preliminary results indicate that a DNA fragment (49 bp long) carrying the nucleotide sequence identical to that of the top strand in the model shown in Fig. 6 possesses a low but significantly detectable level of promoter activity in vivo (unpublished data). Further studies, including mutational analyses of the minus-strand origin, may shed light on the relationship between the priming reaction and general transcription.

In this paper, we described a new method for estimating the function of the minus-strand origin in vivo, i.e., the double-origin plasmid assay (Fig. 4). The assay seems to provide a more sensitive way of detecting the in vivo activity of the minus-strand origin than measurements of phage yield or plaque morphology. The assay provides a direct measurement of origin function with less involvement of phage physiology and may be useful in detecting *cis*-acting elements with low levels of initiation activity on single-stranded templates in vivo.

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