Selective Cloning of a DNA Single-Strand Initiation Determinant from ϕX174 Replicative-Form DNA

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Received 28 July 1983/Accepted 27 September 1983

An M13 phage deletion mutant, M13ΔE101, developed as a vector for selecting DNA sequences that direct DNA strand initiation on a single-stranded template, has been used for cloning restriction enzyme digests of ϕX174 replicative-form DNA. Initiation determinants, detected on the basis of clear-plaque formation by the chimeric phage, were found only in restriction fragments containing the unique effector site in ϕX174 DNA for the Escherichia coli protein n′ dATPase (ATPase). Furthermore, these sequences were functional only when cloned in the orientation in which the ϕX174 viral strand was joined to the M13 viral strand. A 181-nucleotide viral strand fragment containing this initiation determinant confers a ϕX174-type complementary-strand replication mechanism on M13 chimeras. The chimeric phage is converted to the parental replicative form in vivo by a mechanism resistant to rifampin, a specific inhibitor of the normal RNA polymerase-dependent mechanism of M13. In vitro, the chimeric single-stranded DNA promotes the assembly of a functional multiprotein priming complex, or primosome, identical to that utilized by intact ϕX174 viral strand DNA. Chimeric phage containing the sequence complementary to the 181-nucleotide viral strand sequence shows no initiation capability, either in vivo or in vitro.

The single-stranded (SS) viral DNA of ϕX174 is distinguished from that of the SS phages G4 and M13 by the presence of a specific DNA sequence that serves as an effector for the Escherichia coli protein n′ (Y factor) dATPase (ATPase) activity. This interaction between protein n′ and the effector sequence initiates the assembly of a multiprotein priming complex, the primosome, that once formed moves along the DNA laying down RNA primers for complementary strand synthesis (1, 3). In vitro analysis of SS fragments of ϕX174 viral DNA has localized the protein n′ effector activity to a 55-nucleotide sequence within the intergenic space between the ϕX174 genes F and G (22).

One purpose of this work was to determine whether such sequences might also be present in the ϕX174 complementary strand to allow a discontinuous mode of viral strand synthesis. Continuous synthesis of the ϕX174 viral strand has been observed in an in vitro system with purified gene A protein, rep helicase, single-strand-binding protein (SSB), and pol III holoenzyme (4, 6). Synthesis is initiated by gene A protein nicking at a specific site on the viral strand, followed by continuous elongation by a rolling-circle mechanism. In contrast, recent in vivo experiments have indicated that viral strand synthesis can also occur by a discontinuous process with initiations at sites other than the gene A nicking site (14). Such internal initiations might be directed by n′ effector sequences in the complementary strand. We have, therefore, analyzed the entire ϕX174 genome for the presence of such initiation determinants, using the M13 deletion mutant ΔE101 as a vector to allow selection of DNA sequences that direct initiation on an SS template (15, 19).

The M13ΔE101 vector contains a 105-nucleotide deletion spanning the normal M13 complementary strand origin and consequently gives a 20-fold reduced phage yield and forms very turbid plaques (13). The insertion of a DNA sequence capable of directing DNA initiation on an SS template restores phage growth to a normal level and causes the production of a clear plaque.

By in vitro directed mutagenesis of the cloned initiation determinant, it will be possible to define more precisely the n′ recognition sequence and to identify possible contiguous sequences that might be required for primosome assembly or movement but not for n′ recognition per se. The chimeric phages constructed here represent a first step in the genetic analysis of the ϕX174 initiation determinant.

MATERIALS AND METHODS

Bacterial and phage strains. The bacterial strains used are derived from E. coli K-12. The strains used were K37 (Hfr supD), W1485 (F′ supE), RL108 (F′ Tet" leu met t\textsuperscript{r} m\textsuperscript{r} recA56). The construction of bacteriophages M13blu61 and M13ΔE101 has been described previously (10, 13). Bacteriophages M13 and ϕX174am3 were laboratory stocks.

Enzymes and chemicals. T4 DNA ligase and restriction enzymes HaeIII, HindIII, PstI, and HincII, were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). EcoRI and AluI restriction enzymes were generously provided by M. Komaromy and J. Kaguni, respectively. Bacterial alkaline phosphatase was purchased from Worthington Diagnostics (Freehold, N.J.). DNA polymerase I (E. coli) from New England Biolabs (Beverly, Mass.), [α-32P]dATP at 800 Ci/mmole and [α-32P]dCTP at 670 Ci/mmole from New England Nuclear Corp. (Boston, Mass.), and deoxyribonucleoside triphosphates and the 2′,3′-dideoxynucleoside triphosphates from P.L. Biochemicals (Milwaukee, Wis.). Avian myeloblastosis virus reverse transcriptase, EcoRI linker (CATGAAATTCAG), and the oligonucleotide primer (CGCAACGTGGTTT) were generously provided by J. W. Beard, Life Science Inc. (St. Petersburg, Fla.) and K. Itakura, City of Hope Medical Center (Duarte, Calif.), respectively. Sea Plaque agarose was purchased from Marine Colloids Division, FMC Corp. (Rockville, Md.). PstI linker (GCTGCAGC) from Collaborative Research, Inc. (Waltham, Mass.), and rifampin from Sigma Chemical Co. (St. Louis, Mo.).
Media and buffers. M9 and TYE media, TYE plates, agarose gels, electrophoresis buffer, TE, top agar, and Triton lytic mix have been described previously (10, 20).

Analysis of phage and phage DNAs. Preparation and plaque assay of M13 phages, rapid gel analysis of chimeric phage DNAs, minilysis analysis of intracellular replicative forms (RF), preparation of phage RF and plasmid DNAs (10, 12), and conditions for restriction enzyme cleavage of DNA (8, 24) have been previously described as cited.

Purification of DNA fragments. Mixtures of DNA restriction fragments were separated by electrophoresis through Sea Plaque agarose gels. The desired fragment was recovered by excising the appropriate section of the gel, melting the agarose at 65°C, bringing the NaCl concentration to 0.3 M, and extracting twice with phenol to remove agarose. The agarose-free aqueous layer was extracted twice with ether, and the DNA was recovered by precipitation with 2 volumes of ethanol at −20°C.

Phosphatase treatment. Bacterial alkaline phosphatase was used to remove the 5’-terminal phosphate from the ends of DNA fragments. The DNA was dialyzed into a buffer of 10 mM Tris, pH 8.0, and treated with 0.25 U of enzyme per µg of fragments for 30 min at 37°C. The enzyme was removed subsequently by phenol extraction.

Ligation, transformation, sequence analysis, and preparation of probe. Preparation of 32P-labeled phage (9), linker ligation (15), cohesive end ligation (5), transformation of RL108 (10), and DNA sequence analysis by the dideoxynucleotide substitution method by a modification (15) of the procedure of Sanger et al. (21) have been described previously.

RESULTS

Selective cloning of φX174 initiation determinants. To identify SS initiation determinants (ssi) that might be contained within either the viral or complementary strand of φX174 duplex DNA, we cloned restriction fragments of φX174 RF into the unique EcoRI site of M13ΔE101 RF. The M13ΔE101 phage gives a turbid plaque, whereas chimeric derivatives containing an ssi determinant give clear plaques (19).

φX174 RF was cleaved with AluI, HaeIII, or HindIII, and the resulting fragments were mixed to form a pool. EcoRI linkers were ligated to the fragment ends, followed by cleavage with EcoRI and subsequent ligation to EcoRI-cleaved, alkaline phosphatase-treated M13ΔE101 RF. The ligated DNA was used to transfect CaCl2-treated E. coli RL108 for plaque formation. Fifty-eight clear plaques were obtained on the plates in a background of greater than 1,000 turbid plaques. Fifteen of the clear-plaque isolates were selected for further study. Gel electrophoresis of viral DNA contained in culture supernatants revealed four size classes, three of which were clearly larger than the vector M13ΔE101 (data not shown).

Restriction enzyme analysis and partial sequencing of the inserts in the RFs of these phages revealed that three different inserts had been obtained by this selection: the HaeIII-1 fragment (seven isolates), the AluI-2 fragment (four isolates), and the HindIII fragments 1 plus 9 (one isolate, fragment order as found in the genome), with three isolates containing no inserts (pseudorevertants of M13ΔE101 to clear plaque morphology). In all isolates the φX174 viral strand had been joined to the M13 viral strand. These chimeric phages were used as size markers for screening 43 additional clear-plaque isolates from the original transformation plates, and no other size classes were observed. These three fragments all contained the 55-base pair (bp) sequence previously identified as the effector for the protein n’ dATPase (22; Fig. 1).

Restoration of wild-type phage growth by cloned φX174 sequences. Figure 2 shows phage growth curves obtained for M13ΔE101 and the chimeric phages M13φX102, M13ΔX103, and M13ΔX106. The cloning vector M13ΔE101 shows an initial lag of more than 30 min in the production of phage and a yield of only 2 × 1011 PFU/ml after 20 h of growth (13). In contrast, the chimeric phages rapidly produced phage and showed final yields ranging from 5- to 40-fold greater than that of the vector.

Subcloning of the φX174 initiation determinant. We have shown previously that a 1,565-bp HaeIII fragment of φX174 RF cloned into an HaeII site within the intergenic space of M13 conferred a rifampin-resistant mechanism of initiation of SS to RF on the chimeric viral DNA (11, 18). This fragment spans the region identified here as giving clear plaques when cloned into the M13ΔE101 vector. We have used this HaeIII fragment for subcloning to define further the initiation determinant located in this region of the φX174 genome. Because this determinant confers a rifampin-resistant initiation mechanism, we term it an rri determinant (a subclass of ssi determinants) (19).

The φX174 rri determinant has been subcloned as outlined in Fig. 3. An 853-bp AluI fragment corresponding to the φX174 AluI-2 fragment was isolated from M13ΔX60 RF by electrophoresis of an AluI digest through Sea Plaque agarose. The appropriate band was excised from the gel, and the DNA was isolated as described above. This AluI fragment was cleaved with HincI, and the 3-bp overhanging end was filled in with DNA polymerase I plus all four deoxynucleotides. PstI linkers [d(G-C-T-G-C-A-G-C)] were ligated onto the blunt ends, and the resulting fragments were then treated with PstI and ligated to PstI-cleaved, alkaline phos-
phatase-treated M13bla61 RF. M13bla61 is an M13 cloning vector containing the Tn3 β-lactamase gene and a unique PstI site (10). The ligated DNA was used to transfect E. coli RL108. Forty random plaques were selected and screened for chimeric phages containing the correct size insert. Five isolates were used for preparing RF for further study. Upon treatment with PstI, all RF isolates were found to contain the same 181-bp insert. Representative isolates in which either the viral or complementary strand of this 181-bp fragment was inserted into the viral strand of M13bla61 have been termed M13bla81 and M13bla80, respectively.

The ϕX174 sequences contained in both M13bla80 and M13bla81 were determined by the procedure of Sanger et al. (21) with the synthetic oligodeoxynucleotide primer CGCAACGTGTGT (23). Both phages were found to contain only the 181-bp AluI-to-HindII fragment that spans the intergenic region between the ϕX174 genes F and G and a single PstI linker on each end of the fragment.

Rifampin-resistant RF formation in vivo. The ability of 32P-labeled M13bla80 and M13bla81 to direct initiation of complementary strand synthesis by a rifampin-resistant mechanism was carried out as previously described (11, 17). E. coli K37 cells were infected with each phage separately in either the presence or absence of 200 μg of rifampin per ml. Infected cells were harvested at 15 min after infection, lysed, and sedimented through high-salt sucrose gradients. The resulting sedimentation profiles are shown in Fig. 4. Like wild-type M13, the M13bla80 phage forms parental RF in the absence of rifampin but not in its presence. In contrast, M13bla81 is converted to the duplex form in either the presence or the absence of rifampin. These results indicate the presence of an rri determinant in the viral strand of the 181-bp fragment, but not in the complementary strand. This observation is in agreement with the finding that ϕX174 sequences capable of giving rise to clear plaques in M13ΔE101 are exclusively of viral strand origin.

Strand specificity of the ϕX174 primosome loading site. M13bla80 and M13bla81 viral DNAs were assayed for their ability both to function as effectors of the dATPase activity of the purified n’ protein and to mediate the assembly of a functional primosome. The results (Fig. 5) indicate that M13bla81 and ϕX174 viral DNAs coated with SSB are equally good effectors of the n’ dATPase, whereas M13 and M13bla80 DNAs are not. Similarly, M13bla81 DNA, but not M13bla80 DNA, was found to direct SS to RF synthesis in

![Diagram](http://jvi.asm.org/)

**FIG. 2.** Restoration of normal phage growth by the cloned ϕX sequences. E. coli K37 cells were grown in M9 medium containing 0.2% glucose and 0.5% Casamino Acids. At a cell density of 2 × 10^8 cells per ml, samples were infected with M13ϕX102, M13ϕX103, M13ϕX106, or M13ΔE101 phage at a multiplicity of infection of 1. Samples were removed from each culture at intervals and assayed for PFU.

**FIG. 3.** Outline of the construction of M13bla80. M13bla61 carries a ϕX174 HaeII fragment (1,565 bp) that contains the intergenic space between genes F and G. M13bla60 RF was cleaved with AluI, and the resulting fragments were separated by agarose gel electrophoresis. The 853-bp AluI fragment was extracted from the agarose and treated with HindII, giving rise to four restriction fragments. The cohesive ends produced by HindII were filled by using DNA polymerase I (as outlined in the text), and the fragments were ligated to PstI linkers which were subsequently cleaved with PstI to produce cohesive ends. The vector DNA, M13bla61 RF (10), was cut at its single PstI site and treated with bacterial alkaline phosphatase to prevent self-closure. After ligation of the AluI-HindII fragments into the PstI-cut vector, the ligated DNA was used to transfect CaCl2-treated E. coli K37 cells. Phage DNAs from individual plaques were screened by agarose gel analysis (10) to identify chimeric phages containing a single copy of the cloned 181-nucleotide sequence.
RF ATPase found of the assembly of the X174 holoenzyme. By omitting initiations of n' complementary strand of viral DNA. Thus, the 4X174 complementary strand is indeed the template. The initiation of viral strand synthesis occurs at a unique nick introduced into the viral strand of the RF by the action of the 4X174 gene A protein. In contrast, in vivo pulse-labeling experiments are consistent with a discontinuous mechanism of viral strand synthesis. Based on an analysis of the sequence of the 4X174 complementary strand, Matthes et al. (14) have identified three sites in the complementary strand that have some homology to the single n' site in the viral strand. These sites could possibly be involved in a discontinuous synthesis of the viral strand in vivo by a mechanism involving the primosome. Alternatively, such initiations might occur by still other mechanisms, such as a dnaB-primase reaction (2).

The phage M13ΔE101 is a viable deletion mutant of M13 from which the complementary strand origin has been deleted (13). The mutant is extremely defective in the conversion of the viral DNA to a duplex RF. Phage DNA synthesis is reduced 20-fold, and consequently, the phage forms very turbid plaques. We have found that DNA sequences capable of directing initiation on an SS template will rescue this defect when cloned into the mutant genome (14). We have, therefore, made use of this property of M13ΔE101 to search for initiation determinants that could possibly be located within the 4X174 complementary strand and which would restore normal phase growth and lead to the formation of a clear plaque (15).

By cloning HaeIII, AluI, and HindII digests of 4X174 RF, we were able to construct chimeric phage that give a clear plaque and a high phage yield. However, in all cases, the cloned fragment overlapped the known n' recognition site within the untranslated region between genes F and G. Furthermore, in all cases, the 4X174 strand contained within the M13 viral strand was the viral strand and not its complement. These results imply, but do not prove, an absence of primosome loading sites in the 4X174 complementary strand. The successful cloning of other primosome loading sites from each of the two strands of duplex ColE1 DNA (15, 16) support this conclusion. We therefore propose that if 4X174 viral strand synthesis is indeed discontinuous in vivo, the mechanism differs significantly from that of the

**FIG. 4.** Effect of rifampin (rifampicin) on parental RF formation in vivo by M13ΔX80 and M13ΔX81. E. coli K37 cells were grown with aeration at 37°C in glucose minimal medium to an absorbance at 595 nm of 0.4 and were infected with 32P-labeled phage in the presence or absence of 400 μg of rifampin per ml. Fifteen minutes after infection, the cells (5 ml) were poured over 5 ml of ice-cold TENCN buffer, centrifuged, and washed three times with TENCN buffer at 0°C. The washed pellets were suspended in 0.5 ml TENCN buffer, and lysozyme was added to 400 μg/ml. After a 10-min incubation at 37°C, sarkosyl was gently mixed into the lysate to a final concentration of 1% (wt/vol). After another 10-min incubation at 37°C, Sarkosyl was gently mixed into the lysate to a at -20°C or layered directly onto 5 to 20% sucrose gradients in TE buffer containing 1.0 M NaCl. Sedimentation was for 4 h at 5°C at 40,000 rpm in a SW40 rotor. Samples of fractions were spotted on Whatman no. 1 paper and counted.

**FIG. 5.** dATPase activity of the n' protein with different DNAs as effectors. Assay mixtures (25 μl) contained 240 pmol (as nucleotide) of the indicated SSB-coated SS DNAs. Production of [3H]dADP was measured by polyethyleneimine-cellulose thin-layer chromatography.

**DISCUSSION**

An exonuclease VII-resistant, 55-nucleotide fragment of 4X174 viral DNA located in an untranslated region of the 4X174 genome between genes F and G has been shown to contain the single recognition site for the E. coli n' protein within the 4X174 viral strand (22). Recognition of this specific sequence in 4X174 DNA is accompanied by n' ATPase (dATPase) activity and the destabilization of a fraction of the SSB molecules bound to the viral DNA. Binding of n' to its recognition sequence is followed by the assembly of a multiprotein primosome which moves progressively around the circular SS viral DNA to prime repeated initiations of the complementary strand (1, 3)

Synthesis of the 4X174 viral strand occurs by a rolling-circle mechanism (7) in which the viral strand of the duplex RF is continuously synthesized with the circular complementary strand as the template. The initiation of viral strand synthesis occurs at a unique nick introduced into the viral strand of the RF by the action of the 4X174 gene A protein.
TABLE 1. Requirement for M13xX81 chimeric phage SS to RF synthesis in vitro

<table>
<thead>
<tr>
<th>Missing enzyme</th>
<th>Incorporation (pmol)</th>
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<tbody>
<tr>
<td>None</td>
<td>137</td>
</tr>
<tr>
<td>dnaB protein</td>
<td>3</td>
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<tr>
<td>dnaC protein</td>
<td>4</td>
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<tr>
<td>Primase</td>
<td>10</td>
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<td>i'</td>
<td>4</td>
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<td>n'</td>
<td>11</td>
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<tr>
<td>n</td>
<td>6</td>
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<tr>
<td>pol III holoenzyme</td>
<td>0.3</td>
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</table>

* In vitro DNA replication assay was carried out as described by Nomura et al. (16), using M13xX81 as a template for SS→RF synthesis. Trichloroacetic acid-precipitable counts were measured after incubation for 10 min at 30°C.

complementary strand and probably does not involve the primosome.

The cloned viral strand primosome assembly site functioned as efficiently as φX174 viral DNA both in vivo and in vitro. Recombinants containing the complement of the active n' site were obtained by plaque hybridization techniques and were shown to give a turbid plaque and to have no significant n' dATPase effector activity or primosome assembly capability. Similarly, a synthetic 55-mer corresponding to the complementary copy of the 55-mer n' site is also inert as an dATPase effector (R. L. Low, R. Crea, and A. Kornberg; unpublished data). The lack of activity by the sequence complementary to that of the primosome assembly site on the viral strand suggests that specific sequence information as well as nucleic acid secondary structure is required for a functional site. This is also true of the primosome assembly sites in CoIE1 DNA (15, 16).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI 10752 from the National Institutes of Health. M.S. was a trainee on a cell and molecular biology training grant GM 07185 from the National Institutes of Health.

We thank Arthur Kornberg, in whose lab the primosomal proteins were purified, for encouragement.

LITERATURE CITED


