A DNA fragment from the origin of single-strand to double-strand DNA replication of bacteriophage fd

(Initiation of DNA replication/RNA nucleotidyltransferase binding site/hairpin structures in single-stranded DNA/protein-DNA interaction)

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Communicated by Arthur Kornberg, October 8, 1975

ABSTRACT A specific complex is formed between fd DNA, Escherichia coli DNA unwinding protein, and RNA polymerase (EC 2.7.7.8; nucleosidetriphosphate:RNA nucleotidyltransferase) during the first steps in the conversion of the single-stranded viral DNA to the double-stranded replicative form. In this complex a unique DNA fragment of about 120 nucleotides is protected against nuclease digestion. Both the requirements for its isolation and its position on the map of the phage genome indicate that the fragment contains the origin of single-strand to double-strand DNA replication. The isolated DNA fragment possesses double-strand-like characteristics, which protect it from being covered by the DNA-unwinding protein and thus indirectly positions the RNA polymerase to the origin of replication.

Single-strand to double-strand (SS → RF) replication of filamentous bacteriophage (M13, fd) DNA requires as an early step the function of the host RNA polymerase (1–3). This function most likely is the transcription of a short specific segment of the DNA template, the position of which has been identified as a specific gap in a replicative form (RF) II intermediate (4). Experiments with purified proteins showed that the site-specificity of the initiation reaction depended on the presence of Escherichia coli DNA unwinding protein (3), a DNA binding protein with high affinity for single-stranded DNA (5). This suggested that the origin region is specified by a hairpin structure, which, due to its secondary structure, remains unmasked by the DNA unwinding protein, and is thus specifically accessible to the RNA polymerase. This notion is also supported by the fact that several short fragments with hairpin characteristics can be isolated from single-stranded fd DNA (6).

In this study we report the isolation and some characteristic properties of the DNA segment that is bound by the RNA polymerase in the fd DNA-unwinding protein-RNA polymerase complex and thus protected against nuclease digestion.

MATERIALS AND METHODS

Most materials and methods have been described previously: fd [³H] and [³²P]DNA (10), pyrimidine tract analysis and base composition (9), venom phosphodiesterase and E. coli exonuclease I (6), RNA polymerase (EC 2.7.7.6; nucleosidetriphosphate:RNA nucleotidyltransferase) (7), E. coli DNA unwinding protein (5, 8), S1 nuclease (11). DNase I grade I and Neurospora crassa endonuclease were from Boehringer Mannheim.

Isolation of a DNA Fragment (ori-DNA) from the Origin of SS → RF Replication. Reaction mixtures contained 20 mM Tris·HCl (pH 7.5), 80 mM NaCl, 20 mM mercaptoethanol, 3 mM MgCl₂ 0.2 mM EDTA, 0.2 mg/ml of bovine serum albumin, and 10% glycerol. In a typical preparative experiment (1.0 ml) fd [³²P]DNA (10⁶ cpm, 17 μg), DNA unwinding protein (180 μg), and RNA polymerase (50 μg) were added at 0° in this order, followed by DNase I (180 μg), N. crassa endonuclease (18 μg), and exonuclease I (200 units). The mixture was incubated at 30° for 30 min; 2.7% of the DNA remained acid-insoluble. EDTA was added to 10 mM and sodium dodecyl sulfate to 0.5%, and proteins were extracted with phenol. Phenol and salt were removed from the aqueous phase by gel filtration on Sephadex G-50 in 10 mM ammonium carbonate, pH 8.6. Fractions from the void volume were pooled and lyophilized. The DNA was dissolved in 50 μl of formamide and purified by electrophoresis on a 12% polyacrylamide gel in the presence of 7 M urea.

Random fd DNA Fragments. [³²P]-Labeled fd DNA was degraded with DNase I to 10% acid-soluble radioactivity. The DNA was concentrated by ethanol precipitation, dissolved in formamide, and fractionated by electrophoresis on a 12% polyacrylamide gel in the presence of 7 M urea. Fragments migrating relative to the bromphenol blue marker with RF values of 0.29, 0.34, 0.38, 0.43, and 0.50 (corresponding to chainlength of approximately 130, 115, 105, 90, and 70 nucleotides, respectively (7)) were recovered and used as references for electrophoretic mobility and nuclease resistance.

Hpa II-Fragmented fd RF DNA was prepared as described (10). fd DNA minus-strand fragments were isolated by A. Klaas from a preparation that carried bromouracil as a density label in its minus strand using equilibrium centrifugation in an alkaline CsCl/Cs₂SO₄ gradient (12).

Gel Electrophoresis. Slab gels 120 mm long, 1.5 mm thick were run in 40 Tris-acetate (pH 7.8), 1 mM EDTA at 140 V, 30 mA. Gels (12%) contained 0.2% bisacrylamide, and if run with 7 M urea, 0.08% bisacrylamide. Gels with 4% acrylamide contained 1% bisacrylamide. For denaturation, samples were heated in 80–100% formamide to 100° for 2 min and quenched in ice. Bands of [³²P]-labeled DNA were located by autoradiography. If desired, they were cut and eluted from the gel by soaking in 10 mM Tris·HCl (pH 8.0), 0.1 mM EDTA, 0.1 M LiCl.

Annealing of Fragments from fd Plus-Strand to Hpa-Fragmented fd Minus-DNA. [³²P]-Labeled ori-DNA or random fd [³²P]DNA fragments of 110 nucleotides (about 1000 cpm, 10⁻⁵ pmol chains) and a mixture of unlabeled fd minus-DNA Hpa-fragments (0.1 pmol of each fragment) in 5 μl of 10 mM tris·HCl (pH 8), 0.1 mM EDTA, 0.1 M NaCl...
were heated in sealed capillaries to 100° for 2 min, and slowly cooled in a water-bath from 80° to 30° (total time about 4 hr).

RESULTS

Isolation of ori-DNA fragment

RNA polymerase bound to promoter regions of double-stranded phage fd replicative form protects the binding sites against degradation with pancreatic DNase (7). An analogous approach was used to isolate the initiation site for SS → RF conversion of fd DNA. Preinitiation complexes were formed by mixing fd DNA, DNA unwinding protein, and RNA polymerase in the absence of the ribonucleoside triphosphates. The DNA covered by unwinding protein was fragmented by the combined action of DNase I and Neurospora endonuclease. The fragments produced were degraded further with E. coli exonuclease I, whose action is not impaired by the DNA unwinding protein. Little acid-soluble material was found if exonuclease I was omitted or replaced by venom phosphodiesterase.

The kinetics of degradation of 32P-labeled fd DNA (Fig. 1) demonstrates that RNA polymerase protects a small fraction of the DNA from being digested: a background level of 0.5–1% of acid-insoluble, nuclease-resistant radioactivity was increased by about 2% when the enzyme was present. As demonstrated in Fig. 2A, optimal protection of fd DNA was achieved at low polymerase/fd DNA ratios and could not be increased by further addition of the enzyme. Protection was rapidly abolished if the four ribonucleoside triphosphates were added during nuclease digestion (Fig. 1). This indicates that the RNA polymerase binding site is a site for efficient initiation of RNA synthesis.

Size of ori-DNA

Samples of the reaction mixtures were analyzed by electrophoresis under denaturing conditions on polyacrylamide gels that had been calibrated previously with short nucleic acids of defined chainlength (7). In samples taken from the complete system, i.e., with DNA unwinding protein and polymerase being present, a strong band was always observed with an RF of about 0.35 (Fig. 3a–c). This main band sometimes split into two or three closely spaced subspecies with RF values of 0.32–0.37 (Fig. 3e). In addition, several larger DNA species of lower mobility, which were sometimes detected, were apparently converted to the main band during the course of the nuclease digestion. No defined large DNA

FIG. 2. Specific and random protection of fd DNA by RNA polymerase at different polymerase/DNA ratios. (Curve A) Protection at the origin of replication. Assayed with fd [32P]DNA (2 × 10⁶ cpm, 2.7 µg), DNA unwinding protein (40 µg), RNA polymerase as indicated, DNase I (100 µg), exonuclease I (100 units), and N. crassa endonuclease (10 µg) for 1-ml reaction mixture. (Curve B) Randomly protected fd DNA: as for curve A, but minus DNA unwinding protein and exonuclease I, plus venom phosphodiesterase (60 units/ml). (Curve C) Degradation of randomly protected fd DNA by exonuclease I: as for curve B, but in the presence of exonuclease I (100 units/ml). Acid-insoluble radioactivity was determined after 30 min at 30°. The results were corrected for background measured in the absence of RNA polymerase (1.7% for specific, 0.6% for unspecific conditions).

FIG. 3. Gel electrophoresis of ori-DNA. Samples (5 µl) from the experiment of Fig. 1 were taken from the complete system at (a) 20 min, (b) 30 min, and (c) 40 min and applied in 50% formamide to a 12% acrylamide gel in 7 M urea. (d) As gel e but 4 NTPs added at 30 min; (e) main band material fe-electrophoresed; (f and g) ori-DNA after digestion with nuclease S1 for 5 and 10 min, respectively; (h) randomly protected DNA obtained in the absence of DNA unwinding protein (see Fig. 2B). B indicates the position of the bromphenol blue marker.
species, but only low-molecular weight material, was observed if RNA synthesis was allowed during digestion (Fig. 3d), or if the RNA polymerase or the DNA unwinding protein were omitted from the reaction mixture (not shown).

Thus, a family of related DNA fragments of similar size seems to be protected against nuclease digestion in the specific preinitiation complex for SS → RF conversion. The size of the main band material, as judged from its electrophoretic mobility, is about 120 nucleotides. This is a low estimate since it is not certain that the ori-DNA fragment is fully single-stranded in the electrophoresis buffer (7 M urea). It agrees well with the fraction of the fd DNA molecule protected (2% of 6000 nucleotides), and suggests that a single DNA segment has been selectively protected and isolated. This was substantiated by fingerprint analysis (see below). The material of the main band(s) ($R_F$ 0.33–0.38) was, therefore, pooled from a large-scale experiment without further resolution into subspecies and used for structural analysis of the ori-DNA.

Non-specific binding of RNA polymerase to fd DNA

In the absence of the DNA unwinding protein less than 1% of fd DNA resisted the standard nuclease treatment (Fig. 1), even at high polymerase/ DNA ratios, and no defined bands were detected on the gels. Binding sites could be isolated, however, if exonuclease I was omitted from the reaction mixture or was replaced by venom phosphodiesterase. Under these modified conditions the polymerase-protected DNA showed no specific bands, but a broad size distribution from 30 to 60 nucleotides (Fig. 3b). Its yield strictly depended on the amount of polymerase present (Fig. 2b). At a ratio of 20 polymerase molecules per fd DNA, about 10% of the DNA was protected, which corresponds to more than ten sites per DNA molecules. This randomly complexed DNA was rapidly degraded in the presence of exonuclease I (Fig. 2c).

Base composition of ori-DNA

Analysis of the constituting 5'-mononucleotides after hydrolysis with DNase I and venom phosphodiesterase indicated a base composition of ori-DNA as follows: G 26%, T 32%, A 17%, C 25%. This differs substantially from the composition of the total fd DNA: G 20.2%, T 34.8%, A 24.5%, C 20.5% (6), and does not agree with a perfectly base-paired hairpin structure.

Polypyrrimidine fingerprints

Fingerprint analysis of oligopyrimidine tracts was used to determine the complexity of the DNA fragments. As shown in Fig. 4, ori-DNA gives rise to a simple fingerprint, which is characterized by the unusually strong spots T$_5$C, T$_4$C, T$_3$, T$_2$, T$_1$, and T$_0$, whereas the very common spots T$_2$ and T$_C$ are missing. A quantitative evaluation of the data (Table 1) shows that most nucleotides are present in stoichiometric amounts and indicates a total of about 120 constituting nucleotides. Several oligopyrimidines, e.g., T$_3$C and T$_C$, were absent from an otherwise identical fingerprint of a short subspecies, ($R_F$ 0.40, n = 100), whereas several additional spots were observed in the pattern of a large precursor band ($R_F$ = 0.22) (data not shown). These results indicate that the ori-DNA fragments are all derived from the same RNA polymerase binding site by variable extents of nuclease degradation, which occurs at specific sites of the protected region.

In contrast, fingerprints from DNA fragments that were protected by the polymerase in the absence of the DNA unwinding protein showed complexities similar to total fd DNA (Table 1). This result was obtained even at low enzyme/ DNA ratios and at a variety of binding conditions. It demonstrates that there is no preferential binding site for RNA polymerase on the fd DNA single-strand under these conditions.

Double-strand characteristics of ori-DNA

Two types of criteria were used to assay for DS-like properties in ori-DNA: electrophoretic mobility under native and denaturing conditions, and susceptibility to the SS-specific

<table>
<thead>
<tr>
<th>Pyrimidine tract</th>
<th>ori-DNA</th>
<th>Random sites</th>
<th>fd DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>T$_r$C$_r$</td>
<td>0.8</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>T$_r$C$_1$</td>
<td>0.9</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>T$_1$</td>
<td>0.95</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>T$_{C_r}$</td>
<td>1.95</td>
<td>1.3</td>
<td>1.5</td>
</tr>
<tr>
<td>T$_{C_1}$</td>
<td>1.9</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>T$_{C_2}$</td>
<td>2.15</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>T$_1$</td>
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</tr>
<tr>
<td>T$_{C_4}$</td>
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<td>0.05</td>
</tr>
<tr>
<td>T$_{C_5}$</td>
<td>1.4</td>
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<td>0.4</td>
</tr>
<tr>
<td>T$_{C_6}$</td>
<td>0.1</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>T$_C$</td>
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<tr>
<td>P$_1$</td>
<td>3.8</td>
<td>5.9</td>
<td>5.8</td>
</tr>
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</table>

Samples of ori-DNA (about $10^4$ cpm) and randomly protected binding sites (RNA polymerase/fd DNA ratio 3, about $10^6$ cpm) were analyzed as described in the legend to Fig. 4 and in Materials and Methods. The values for fd DNA were taken from a detailed determination of the oligopyrimidine distribution in fd $^{32}P$DNA (unpublished results). Yields were normalized to a chainlength of 120 nucleotides.

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Fig. 4. Oligopyrimidine fingerprint of $^{32}P$-labeled ori-DNA. For details and for a fingerprint from total fd DNA see refs. 7 and 9.
nuclease S1. Both experiments included as standards DNA fragments of similar size from SS fd DNA and from fd RF DNA.

The electrophoretic mobilities of ori-DNA and of some references are presented in Table 2. They show that under non-denaturing conditions ori-DNA migrates relatively fast with an $R_F$ of 0.52 which corresponds to the mobility of an SS DNA reference of about 90 nucleotides, and to that of a DS DNA fragment of about 350 base pairs. Under the same conditions an SS marker of ori-DNA size migrated significantly more slowly ($R_F$ 0.45), whereas DS markers with 120 and 60 base pairs migrated well ahead. These data suggest for ori-DNA a secondary structure that is more compact than that of SS fragments from other parts of the fd DNA molecule, but less ordered than that of a perfectly base-paired DNA duplex.

The S1 experiments (Fig. 5) showed that ori-DNA is about 10 times more resistant to this SS-specific enzyme than full-length fd DNA or random DNA fragments of ori-DNA size. On the other hand, ori-DNA is slowly degraded, whereas Hpa-L, a DNA duplex of 60 base pairs, is stable. Gel electrophoresis of the reaction products (Fig. 3f and g) indicates the formation of distinct intermediates during ori-DNA degradation. These migrate in urea gels as diffuse bands with a size of about 55, 35, and 15 nucleotides. Thus the DS segments in the ori-DNA appear to be separated by SS regions which are preferentially attacked by the nuclease.

Mapping of ori-DNA on the fd DNA genome

The origin of SS $\rightarrow$ DS DNA replication in filamentous bacterial phage has been located within or close to fd Hpa-H (4), a restriction fragment of about 400 nucleotides which maps in the vicinity of the junction of genes II and IV (10). It was therefore of interest to find out whether ori-DNA also mapped within the same region on the fd genome. To this end $^{32}$P-labeled ori-DNA was annealed to a mixture of Hpa-fragmented, unfragmented fd minus-strand DNA. The resulting hybrids were then analyzed for size distribution on a 4% polyacrylamide gel. Random fd $^{32}$P-labeled DNA fragments of ori-DNA size were annealed and analyzed in a parallel control experiment. In addition, Hpa-fragmented, $^{32}$P-labeled fd minus-DNA served as a marker.

The result of such an experiment is presented in Fig. 6. It shows that $^{32}$P label from ori-DNA migrated as a single band in the position of the single-stranded Hpa-H reference. In the control, label from random DNA fragments was distributed between several more diffuse bands which migrated with the mobility of the large Hpa fragments A to E, whereas no defined $^{32}$P-labeled bands were visible on the autoradiograms in the range of Hpa-F to -H. Therefore, a direct correlation of the hybrid bands was not possible. However, since fragment Hpa-H separates well in this system from any other Hpa fragment, it seems safe to use the single-stranded Hpa-H marker as a reference and to conclude that ori-DNA maps within Hpa-H.

A self-complementary DNA sequence is also indicated by the relatively high mobility of the Hpa-H minus-strand in the acrylamide gel under native conditions (Fig. 6, SS). Hybridization of the ori-DNA fragment to this region increases the contour length of the Hpa fragment, but the resulting lower mobility will be compensated by a faster migration of the now completely double-stranded section carrying the ori-DNA fragment.
DISCUSSION

Based on experiments with purified proteins, the initiation of the SS → RF conversion of filamentous bacteriophage DNA has been postulated to occur in several discrete steps (5): (i) a DNA binding protein covers all the single-stranded DNA except for a "hairpin region"; (ii) RNA polymerase attaches specifically to the unmasked region and synthesizes a short RNA chain; (iii) this RNA is utilized as a primer for DNA synthesis by the DNA polymerase III holoenzyme.

Our data provide direct experimental evidence for the first part of this scheme: by omitting the DNA-synthesizing system and the four ribonucleoside triphosphates, the reaction was arrested in the specific preinitiation complex formed by RNA polymerase and the DNA unwinding protein-covered fd DNA template. This complex turned out to be stable enough to allow the isolation of the polymerase-protected segment of the origin region by nuclease digestion.

The isolated DNA fragment has a size of approximately 120 nucleotides. It was demonstrated to encompass a unique segment of the fd DNA molecule by the congruence of size, yield, and the simplicity of its polyuridylate fingerprint, and also by its localization on a single restriction nuclease fragment. Several lines of evidence suggest that it contains—or is a part of—the origin of SS → DS DNA replication: (i) the requirements for its isolation are identical to those needed for the site-specific initiation of SS DNA → RF conversion; (ii) the site is left immediately by the RNA polymerase if RNA synthesis is allowed, i.e., it serves as an efficient promoter and contains the start point for RNA synthesis; (iii) the ori-DNA fragment maps at (or very close to) the gapped region of the RF II species that has been identified by in vitro experiments as a late intermediate in the SS → RF conversion (4).

The ori-DNA fragment showed the expected DS-like properties: high resistance to nuclease S1 and enhanced mobility on gels under native conditions. In the absence of the DNA unwinding protein, the RNA polymerase does not bind preferentially to this region, but attaches at random to many sites on the DNA single strand. The complexes formed there differ from the specific complex at the origin by a much smaller size of the polymerase-protected DNA fragment and by their high sensitivity to the action of exonuclease I.

In view of these data, the specificity of the initiation reaction appears to be caused entirely by the inability of the E. coli DNA "unwinding" protein to unwind and to cover the double-stranded origin region in the otherwise single-stranded DNA template. Thus, structural information that cannot be recognized as such by the RNA polymerase is amplified by a rather unspecific DNA binding protein, so that it becomes a specific start signal for polynucleotide synthesis. Similar auxiliary functions in regulatory processes can be assumed for many other apparently unspecific DNA binding proteins, including the histones in the eukaryotic cell.

The particular features of the origin region, by which it is discriminated by the DNA binding protein from the other 98% of the fd DNA molecules, are not completely understood. So far, size, mobility in acrylamide gels, resistance to nuclease S1, and base composition of the ori-DNA fragment definitively exclude a single completely base-paired hairpin structure comprising the total DNA segment. These data instead suggest that the origin DNA is folded similarly to tRNA into a compact structure by intramolecular base-pairing of only short runs of self-complementary nucleotide sequences. Some of the short double-stranded, looped structures of the ori-DNA appear to be present in the "core" fraction from fd DNA which resists SS-specific nucleases and which has been isolated and characterized (6). This is indicated by the overlap of the respective oligopyrimidine fingerprints and by the fact that several fd DNA core fragments have been shown to map, as the ori-DNA fragment, in restriction fragment Hpa-I (unpublished results).

It should be noted that double-stranded structures similar to ori-DNA could also be used as initiation signals in other single-stranded phage DNAs and in the SS regions of the replicative fork in chromosomes. Such sites may also account for the prolonged persistence of intermediates in DNA replication, like Okazaki fragments and phage DNA RF II. Since the secondary structure in the template cannot be covered by DNA unwinding protein, which is required for DNA polymerase III* action, DNA polymerase I is predestined to replicate those regions. This has been clearly demonstrated in the fd-like phage M13 SS → RF conversion (3).

In the life cycle of filamentous bacteriophage the self-complementary nucleotide sequences of the ori-DNA may additionally function as a recognition site in RF → RF replication, in transcription (10), and in the processing and circularization of linear SS phage DNA (6).

We thank Christina Kurz for skilful technical assistance, and the Deutsches Krebsforschungszentrum Heidelberg for the use of laboratory facilities. This work was in part supported by a grant from the Deutsche Forschungsgemeinschaft.