Primer RNA synthesis by plasmid-specified Rep protein for initiation of ColE2 DNA replication

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Initiation of *in vitro* ColE2 DNA replication requires the plasmid-specified Rep protein and DNA polymerase I but not RNA polymerase and DnaG primase. The ColE2 Rep protein binds specifically to the origin where replication initiates. Leading-strand synthesis initiates at a unique site in the origin and lagging-strand DNA synthesis terminates at another unique site in the origin. Here we show that the primer RNA for leading-strand synthesis at the origin has a unique structure of 5'-ppApGpA. We reconstituted the initiation reaction of leading-strand DNA synthesis by using purified proteins, the ColE2 Rep protein, *Escherichia coli* DNA polymerase I and SSB, and we showed that the ColE2 Rep protein is a priming enzyme, primase, which is specific for the ColE2 origin. The ColE2 Rep protein is unique among other primases in that it recognizes the origin region and synthesizes the primer RNA at a fixed site in the origin region. Specific requirement for ADP as a substrate and its direct incorporation into the 5' end of the primer RNA are also unique properties of the ColE2 Rep protein.

Keywords: ADP requirement/ColE2 DNA replication/ColE2 Rep protein/DNA polymerase I/primer RNA synthesis

Introduction

The initiator protein (Rep; 35 kDa) of the colicin E2 (ColE2) plasmid is the only plasmid-specified *trans*-acting factor required for the initiation of plasmid replication (Horii and Itoh, 1988; Itoh and Horii, 1989; Yasueda *et al.*, 1989; Kido *et al.*, 1991). Plasmid replication also requires host DNA polymerase I (Kingsbury and Helinski, 1970; Tacon and Sherratt, 1976; Itoh and Horii, 1989) but not RNA polymerase and DnaG primase (Itoh and Horii, 1989; S.Takechi and T.Itoh, submitted). The minimal origin of the ColE2 DNA replication consists of 32 bp, where DNA replication initiates (Itoh and Horii, 1989; Yasueda *et al.*, 1989; see Figure 1). The ColE2 Rep protein binds specifically to the origin, as revealed by a filter binding assay (Kido *et al.*, 1991). In an *in vitro*

ColE2 DNA replication system with crude extracts of *Escherichia coli* cells ColE2 DNA replication starts at a unique site in the region containing the origin and proceeds unidirectionally (Itoh and Horii, 1989). We have recently shown that leading-strand DNA synthesis starts at a unique position in the origin around nucleotide position 1470 and that lagging-strand DNA synthesis terminates just at nucleotide position 1479 (S.Takechi and T.Itoh, submitted; see Figure 1). The leading-strand DNA fragment contained an RNA moiety of several residues at the 5' end and it is probably the primer RNA for initiation of ColE2 DNA replication or its remnant. Whatever mechanism might produce the primer RNA, it leaves precisely a fixed number of ribonucleotides at the 5' end of the newly synthesized leading-strand DNA fragment. We have proposed that the ColE2 Rep protein is a plasmid-encoded priming enzyme, primase, specific for the ColE2 origin and that host DNA polymerase I specifically uses the primer RNA to start DNA synthesis.

Here we show that the primer RNA for initiation of the leading-strand DNA synthesis at the origin has a unique structure of 5'-ppApGpA. We show that the Rep protein is a plasmid-specified primase, which specifically requires ADP and incorporates it as the first nucleotide.

Results

Analysis of the nucleotide sequence of the primer RNA in the *in vitro* ColE2 DNA replication system

To determine the structure of the RNA attached to the leading-strand DNA fragment at the origin in the early replicative intermediates produced in the *in vitro* ColE2 DNA replication system (S.Takechi and T.Itoh, submitted; see Figure 1), the newly synthesized DNA fragments labeled with [α -³²P]dCMP were first digested with *Bgl*I, which gave leading-strand DNA fragment ~15 nucleotides long (Figure 2, lane 1). The fragments were then digested with various RNases after heat treatment. Digestion with RNase A showed no effect on the length of the fragment (Figure 2, lane 2). Complete digestion with RNase T2 produced a fragment three nucleotides shorter than the original one (Figure 2, lane 3), whose size was identical to that of the product of alkaline treatment. Partial digestion with RNase T2 gave fragments shorter by one, two and three nucleotides (data not shown). Digestion with RNase T1 gave a fragment shorter by two nucleotides (Figure 2, lane 4). Partial digestion with RNase U2 gave fragments shorter by one and three nucleotides and a very small amount (barely visible in the figure) of a fragment shorter by two nucleotides was also produced (Figure 2, lane 5). It has been known that RNase U2 cleaves RNA specifically at the purine nucleotides and that it does so at the adenine residues much more efficiently than at the guanine residues. All these results indicated that the nucleotide

Fig. 1. Nucleotide sequence of the *BglII*–*BglII* segment of plasmid Cole2 containing the origin of replication. The numbering of the nucleotide positions is as described (Yasueda *et al.*, 1989). The three-nucleotide primer RNA (asterisks) with a 5' diphosphate and the leading-strand DNA fragment (an arrow to the right) that starts at position 1472 identified in this study are indicated above the sequence. The lagging-strand DNA fragment (an arrow with a bar on the left) that terminates at position 1479 is indicated below the sequence. Direct and inverted repeat sequences which might be involved in the binding and primer RNA synthesis by the Rep protein are indicated by thin arrows. The minimal origin region is indicated by a bracket below the sequence.

Fig. 2. The newly synthesized leading-strand DNA fragment carrying RNA at its 5' end treated with various RNases. The early replicative intermediates prepared in the presence of [α - 32 P]dCTP (1000 c.p.m./pmol) as described in Materials and methods were digested with *BglII* followed by heat treatment and digestion with RNase A (lane 2), RNase T2 (lane 3), RNase T1 (lane 4), RNase U2 (lane 5). Treatment of the fragment with T4 polynucleotide kinase and alkaline phosphatase (data not shown) indicated that almost all the 5' end of the RNA possessed at least one phosphate residue. Cleavage by the RNases used here yields fragments with 5' hydroxyl ends, which migrated behind those of the same lengths with 5' phosphoryl ends in the gel electrophoresis. RNase H digestion, which yields fragments with 5' phosphate, was performed without prior heat treatment (lane 6). Lane 1 contained the *BglII*-digested fragment without RNase treatment (marked as O). The products were analyzed by electrophoresis in an 8% polyacrylamide–7 M urea gel. Only the portion of the gel containing the leading-strand DNA fragments is shown. The position of the DNA fragment obtained after alkaline treatment is indicated by an arrow. Digestion with RNase U2 was partial.

sequence of the RNA at the 5' end is 5'-AGA. This is consistent with the requirement of only ATP and GTP for initiation of leading-strand DNA synthesis (S.Takechi and T.Itoh, submitted). RNase H digestion without heat treatment produced mainly a fragment shorter by two nucleotides and a very small amount (only barely visible in the figure) of a fragment shorter by three nucleotides (Figure 2, lane 6). It has been known that RNase H only inefficiently removes a ribonucleotide attached to the 5' end of DNA (Berkower *et al.*, 1973; Darlix, 1975). The result indicated that the three-nucleotide RNA is hybridized to the complementary DNA sequence. Therefore the 5' end of the RNA is localized at the A residue (position 1469; Figure 1) and the synthesis of the leading-strand DNA starts at the T residue (position 1472).

ADP at the 5' end of the primer RNA

To determine the precise number of phosphate residues at the 5' end of the RNA, the early replicative intermediates

Fig. 3. Determination of the phosphate residues at the 5' end of the RNA moiety of the newly synthesized leading-strand DNA fragment. The early replicative intermediates were prepared with [α - 32 P]ATP (10 000 c.p.m./pmol), digested with *Eco47III* and analyzed by polyacrylamide gel electrophoresis as described in Materials and methods. The 105-nucleotide leading-strand DNA fragment was purified and digested with S1 endonuclease to completion. The products were analyzed by HPLC using a reversed phase column as described in Materials and methods. Positions of ATP, ADP and AMP added as markers determined by the absorbance at 254 nm are indicated by arrows. The histograms are for 32 P radioactivity in each fraction.

were labeled with [α - 32 P]ATP. The leading-strand fragments with the RNA at the 5' ends were purified, digested completely with S1 endonuclease and then analyzed by HPLC (Figure 3). It has been known that S1 endonuclease digests RNA as well as DNA and produces 5' phosphorylated mononucleotides. Approximately equal amounts of radioactivity were found exclusively at the positions of AMP and ADP. This was confirmed by thin layer chromatography (TLC) of the same digestion products on polyethyleneimine (PEI) cellulose (data not shown). The results indicated that almost all the 5' ends of the RNA carried two phosphate residues. Therefore we concluded that the primer RNA synthesis starts at the first A residue. As leading-strand DNA synthesis requires ATP and GTP but not UTP and CTP (S.Takechi and T.Itoh, submitted), the RNA probably is not a processed product of a longer precursor.

Requirement of ADP for Cole2 DNA synthesis initiated by Cole2 Rep protein, DNA polymerase I and SSB

We then tried to carry out the initiation reaction of Cole2 DNA replication by using three purified proteins, the Cole2 Rep protein, host DNA polymerase I and single-stranded DNA binding protein (SSB). SSB was included, as it has been known to be required for discrimination between specific and non-specific template DNA species in some DNA synthesizing systems consisting of purified enzymes (Vicuna *et al.*, 1977; Itoh and Tomizawa, 1982). Only little DNA synthesis was observed (data not shown), however, when we added the purified enzymes in place of the crude cell extract in the standard reaction mixture containing 2 mM ATP, an ATP-regenerating system (creatine kinase and creatine phosphate) and 5% polyvinyl alcohol (PVA), all of which are required for Cole2 DNA replication in crude cell extracts (Itoh and Horii, 1989). Considering that the primer RNA for leading-strand DNA synthesis at the origin starts with a 5' diphosphate residue (as ADP) in crude cell extracts, we suspected possible involvement of ADP in the initiation reaction. We found

Fig. 4. Ribonucleotide requirement for initiation of ColE2 DNA synthesis by purified proteins. Standard reaction mixtures (25 μ l) containing 60 nM Rep protein, 16 U/ml DNA polymerase I and 2.5 μ g/ml SSB, 20 nM plasmid pEC22 DNA and [α - 32 P]dATP (1000 c.p.m./pmol) with varying concentrations of (A) ADP, (B) GTP or (C) ATP were incubated at 32°C for 60 min. The concentrations of the remaining two nucleotides were kept at 100 μ M. Incorporation of radioactivity into the acid insoluble fraction was determined.

Fig. 5. Kinetics of ColE2 DNA synthesis by purified proteins. A standard reaction mixture (150 μ l) was incubated at 32°C with (open circles) or without (filled circles) 100 μ M each of ADP, GTP and ATP. Aliquots (10 μ l) were withdrawn at intervals.

that omission of the ATP-regenerating system and addition of ADP to the reaction greatly stimulated DNA synthesis by the three purified proteins (Figure 4A). DNA synthesis increased as the concentration of ADP increased, and it reached its maximum at $\sim$ 40 μ M. It seems that there is a critical threshold for the required concentration of ADP.

Properties of ColE2 DNA synthesis by the three purified proteins

Besides the requirement for ADP, DNA synthesis by the three purified proteins was also dependent upon GTP and ATP (Figure 4B and C), but not upon CTP and UTP (data not shown). Other reaction conditions were set as described in Materials and methods. KCl, cAMP, NAD and PVA or PEG showed no effects or in some cases inhibitory effects on DNA synthesis. ColE2 DNA synthesis increased for as long as 80 min and then leveled off (Figure 5). In the absence of ADP and rNTPs repair DNA synthesis gradually increased. The rate of DNA synthesis was roughly proportional to the concentration of the Rep protein up to $\sim$ 50 nM (Figure 6A) and the molar ratio of the Rep protein to the template DNA at the saturation point was about three. Addition of five to six times excess amount of the Rep protein showed no effect. The rate of DNA synthesis increased with the concentration of DNA polymerase I

Fig. 6. Dependence of reconstituted ColE2 DNA synthesis on the Rep protein and DNA polymerase I. (A) Standard reaction mixtures containing 16 U/ml DNA polymerase I, 2.5 μ g/ml SSB and [α - 32 P]dATP (300 c.p.m./pmol) were incubated with varying concentrations of the ColE2 Rep protein. (B) Standard reaction mixtures containing 60 nM Rep protein and 2.5 μ g/ml SSB in the presence (open circles) or absence (closed circles) of ADP, GTP and ATP were incubated with varying concentrations of DNA polymerase I.

and reached its maximum at $\sim$ 15 U/ml (Figure 6B). DNA synthesis was dependent upon addition of supercoiled pEC22 DNA, and relaxed DNA was almost inactive (data not shown).

Analysis of the products synthesized by the three purified proteins

When the reaction products synthesized by the three purified proteins were analyzed by electrophoresis in an agarose gel, we found mainly replicative intermediates (data not shown) with newly synthesized fragments $\sim$ 400–450 nucleotides long (Figure 7A). The structure of the products was similar to that of the early replicative intermediates produced in crude cell extracts in the presence of ddTTP (Itoh and Horii, 1989; S.Takechi and T.Itoh, submitted). When the products digested with *Eco*47III were analyzed by a sequencing gel before (Figure 7B, lane 1) or after (Figure 7B, lane 2) alkaline treatment, DNA fragments identical to the newly synthesized leading-strand DNA fragments (105 and 102 nucleotides, respectively) produced in crude cell extracts (Figure 7B, lanes 3 and 4) were found. The strand specificity was confirmed by specific hybridization with the template strand for the leading strand (Figure 7C, lanes 1 and 2). These results demonstrated that the ColE2 Rep protein, DNA polymerase I and SSB can initiate leading-strand DNA synthesis at the site in the origin identical to that in the reaction with crude cell extracts and that the identical primer RNA is attached to the 5' end of the leading-strand DNA fragment. The reaction products synthesized in the absence of SSB gave an identical leading-strand DNA component (data not shown), demonstrating that SSB is not essential for the initiation of the leading-strand DNA synthesis.

ADP at the 5' end of the primer RNA synthesized in the reconstituted system

Treatment of the leading-strand DNA fragment with alkaline phosphatase and T4 polynucleotide kinase and analysis by a sequencing gel showed that there was more than one phosphate residue at the 5' end (data not shown), suggesting that the 5' residue of the primer RNA is ADP or ATP. By using [β - 32 P]ADP as a substrate we found that

Fig. 7. Analysis of the products of ColE2 DNA synthesis by purified proteins. (A) A standard reaction mixture containing 60 nM Rep protein, 16 U/ml DNA polymerase I and 2.5 µg/ml SSB with [α - 32 P]dATP (10 000 c.p.m./pmol) was incubated at 32°C for 60 min. The products were isolated and analyzed by electrophoresis in 5% polyacrylamide gel after heat treatment. Numbers on the right-hand side of the panel are approximate sizes (in nucleotides) of DNA fragments deduced from the size markers (pBR322 DNA digested simultaneously with *Hind*III and *Hinf*I). (B) A standard reaction as in (A) was run (lanes 1 and 2). A standard reaction mixture (25 µl) containing 5 µl and 1 µl of crude cell extracts of NT525 and NT525 (pT112-6Arep), respectively, 20 nM pEC22 DNA, 50 µM ddTTP and [α - 32 P]dATP (10 000 c.p.m./pmol) was incubated at 32°C for 60 min (lanes 3 and 4). The products were purified, digested with *Eco*47III and analyzed by 8% PAGE before (lanes 1 and 3) or after (lanes 2 and 4) alkaline treatment. The 95-nucleotide lagging-strand DNA fragment contains three times as many dAMP residues as the 105- or 102-nucleotide leading-strand DNA fragment. (C) The 102-nucleotide fragment shown in lane 2 of (B) was purified and hybridized with bacteriophage M13 DNA carrying the template strand for the lagging-strand (lane 1) or the leading-strand (lane 2) at 65°C for 5 min. Samples were analyzed by 1% agarose gel electrophoresis. The position of M13 single-strand DNA is marked.

Fig. 8. Incorporation of ribonucleotides into the newly synthesized leading-strand fragment and analysis of ribonucleotide residues in the RNA portion of the leading-strand fragment. (A) A standard reaction mixture with purified proteins containing [β - 32 P]ADP (100 000 c.p.m./pmol; lane 1), [α - 32 P]GTP (100 000 c.p.m./pmol; lane 2), [α - 32 P]ATP (100 000 c.p.m./pmol; lane 3) or [α - 32 P]dATP (4000 c.p.m./pmol; lane 4) was incubated at 32°C for 60 min. The products were isolated, digested with *Eco*47III and analyzed by 8% PAGE containing 7 M urea. (B) The 105-nucleotide fragment produced with [α - 32 P]ATP in (A) was purified, digested with S1 endonuclease in completion and analyzed by PEI cellulose TLC with 0.75 M KH_2PO_4 pH 3.4. Positions of AMP, ADP and ATP added as markers are indicated. The origin (O) of development is also marked.

it was incorporated into the reaction product. When the product synthesized in the presence of [β - 32 P]ADP was digested with *Eco*47III and then analyzed by polyacrylamide gel electrophoresis (Figure 8A, lane 1), the labeled fragment of 105 nucleotides identical to that labeled with

Fig. 9. Oligonucleotide synthesis by the ColE2 Rep protein. Standard reaction mixtures containing 50 nM Rep protein, 20 nM pEC22 DNA and [β - 32 P]ADP (100 000 c.p.m./pmol) were incubated at 32°C for 60 min with the following additions or omissions: -Rep protein +4rNTP (lane 1), -template DNA +4rNTP (lane 2), +GTP (lane 3), +GTP and ATP (lane 4), +GTP, ATP and CTP (lane 5), +GTP, ATP and UTP (lane 6), and +4rNTPs (lane 7). The reaction products were purified by chromatography with DEAE cellulose and analyzed by a 24% PAGE. The arrow indicates the position of ADP.

[α - 32 P]dAMP (Figure 8A, lane 4) was found, demonstrating that ADP is incorporated at the 5' end of the primer RNA. As expected, the radioactivity from [α - 32 P]GTP or [α - 32 P]ATP was also incorporated into the identical DNA fragment (Figure 8A, lanes 2 and 3). A roughly equal amount of radioactivity was present in the 105-nucleotide fragment of each sample with ADP, GTP or ATP as a labeled substrate, consistent with the nucleotide sequence, 5'ppApGpA, of the primer RNA. No radioactivity was incorporated into the product by using [γ - 32 P]ATP, [α - 32 P]UTP or [α - 32 P]CTP. The *Eco*47III-digested DNA fragment labeled with [α - 32 P]ATP as a substrate was purified, digested with S1 endonuclease to completion and analyzed by TLC using PEI cellulose (Figure 8B). Radioactivity was found only at the position of AMP and only little radioactivity, if any, was detected at the position of ADP or ATP. The result demonstrated that ATP was incorporated into the primer RNA as the third residue, AMP, and not as the first residue.

RNA synthesis by ColE2 Rep protein

We examined the RNA molecules synthesized on the ColE2 DNA by the Rep protein alone in the presence of [β - 32 P]ADP as described in Materials and methods. We used a DEAE cellulose column to separate the reaction products from the unincorporated ADP and eluates were analyzed by PAGE. The eluates with 0.2 M and 0.3 M TEAA (triethyl ammonium acetate) buffers contained large amounts of unincorporated ADP and the reaction products were not visible (data not shown), whereas the 0.5 M eluate contained the reaction products (Figure 9). The Rep protein synthesized an oligonucleotide depending upon ATP and GTP (Figure 9, lane 4). Requirement of ADP for the oligonucleotide synthesis was confirmed by using [α - 32 P]ATP and unlabeled ADP (data not shown). Although we have not sequenced the oligonucleotides, these results are consistent with the synthesis of ppApGpA by the Rep protein. Depending upon UTP in addition to ADP, ATP and GTP the Rep protein also synthesized another oligonucleotide (Figure 9, lane 6), probably ppApGpApU, which is consistent with the nucleotide

sequence (see Figure 1). Even in the presence of all four rNTPs, however, no longer oligonucleotides were synthesized (Figure 9, lane 7). Synthesis of ppApG in the presence of ADP and GTP was not detected (Figure 9, lane 3). The Rep protein might require ATP not only as a substrate but also as a cofactor or the dinucleotide might have been washed off from the column by the 0.2 M and 0.3 M buffers.

Discussion

We showed that leading-strand DNA synthesis in *in vitro* ColE2 DNA replication starts with the primer RNA (5'-ppApGpA) at a unique position in the origin and that the ColE2 Rep protein is a plasmid-specified priming enzyme, primase, which is specific for the ColE2 origin.

The primer RNA molecules in the ColE2 DNA replication systems both with crude cell extracts and with purified enzymes were exclusively ppApGpA. On the other hand the Rep protein alone synthesized ppApGpApU along with ppApGpA in the presence of ADP, GTP, ATP and UTP, although addition of UMP seems to be inefficient. In the reaction coupled with DNA synthesis the tetranucleotides might not be synthesized. DNA polymerase I might take over the 3' hydroxyl end very efficiently immediately after the synthesis of the trinucleotides. Alternatively the tetranucleotides might be synthesized. They might not be used by DNA polymerase I for an unknown reason or DNA polymerase I might remove the UMP residues and then use trinucleotides as a primer.

The ColE2 Rep protein is unique as compared with most of the other known primases. It binds to double-stranded DNA containing the ColE2 origin in a sequence-specific manner as revealed by a filter binding assay (Kido *et al.*, 1991) and synthesizes a unique primer RNA at the origin. The gene α protein of bacteriophage P4 required for phage replication (Gibbs *et al.*, 1973; Krevolin and Calendar, 1985) binds to double-stranded DNA containing the *cis*-acting regions required for replication in a sequence-specific manner (Ziegelin *et al.*, 1993) and possesses primase and helicase activities (Krevolin and Calendar, 1985; Strack *et al.*, 1992; Ziegelin *et al.*, 1993). Although synthesis of oligoribonucleotides on single-stranded fd DNA or on synthetic oligodeoxyribonucleotides has been demonstrated (Ziegelin *et al.*, 1993), primer RNA synthesized on double-stranded DNA containing the origin has not been examined. All other known primases bind only to single-stranded DNA and unwinding of double-stranded DNA by other proteins are prerequisites for loading of them on to the double-stranded template DNA to initiate primer RNA synthesis (for a review see Kornberg and Baker, 1992).

Requirement for ADP in the primer RNA synthesis and its direct incorporation into the 5' end of the primer RNA are unique properties of the ColE2 Rep protein as a primase. Intact primer RNA molecules so far identified in both prokaryotic and eukaryotic DNA replication systems start with a triphosphate residue at the 5' end (for a review see Kornberg and Baker, 1992). Purified primases, such as the *E. coli* DnaG primase (Bouché *et al.*, 1978; Ogawa *et al.*, 1983), bacteriophage T7 gene 4 protein (Scherzinger *et al.*, 1977) and bacteriophage T4 gene 41 and gene 61 proteins (Liu and Alberts, 1981) initiate primer RNA

synthesis by using ATP as the first residue and ATP can be recovered from the 5' end of the primer RNA. Although ADP can substitute for ATP in the priming reaction by the DnaG primase for complementary DNA strand synthesis of bacteriophage G4, it is not incorporated into the primer RNA (Wickner, 1977; Rowen and Kornberg, 1978).

The common sequence motif, bhhhhEGYhDhh-h (b, h and - represent basic, hydrophobic aliphatic and variable amino acid residues, respectively), among prokaryotic DNA primases proposed (Pansegrau and Lanka, 1992) was not found in the ColE2 Rep protein. Furthermore a comparison of amino acid sequences of the ColE2 Rep protein and other primases including the *E. coli* DnaG primase (Smiley *et al.*, 1982), T7 gene 4 protein (Dunn and Studier, 1983), T4 gene 61 protein (GenBank accession number K03113), P4 α protein (Flensburg and Calendar, 1987) and *Saccharomyces cerevisiae* PRI1 protein (Lucchini *et al.*, 1987), and the initiator proteins (Rep proteins) of other plasmids including IncFII (Rosen *et al.*, 1980), ColIb (Hama *et al.*, 1990), R6K (Germino and Bastia, 1982), F (Murotsu *et al.*, 1981), pSC101 (Vocke and Bastia, 1983) and P1 (Abeles *et al.*, 1984) revealed no significant localized sequence homology conserved between the ColE2 Rep protein and any of these primases and Rep proteins. Nevertheless there is apparently a common feature in primer RNA syntheses by the ColE2 Rep protein and by the *E. coli* DnaG primase. It has been known that the DnaG primase preferentially recognizes the 3'-GTC-5' sequence in template DNA around the bacteriophage λ origin and the *oriC* of *E. coli* and synthesizes primer RNA molecules starting with 5'-AG (Yoda *et al.*, 1988; Yoda and Okazaki, 1991). The primer RNA (5'-AGA) for initiation of ColE2 DNA replication is synthesized using 3'-GTCT-5' as a template. There might be a common mechanism in recognition of the template nucleotide sequence by these proteins.

Specific requirement of ADP for the ColE2 primer RNA synthesis and apparent existence of a threshold for the concentration required might suggest some regulatory role for ADP in the initiation of ColE2 DNA replication. Intracellular concentration of ATP is quite high (~3 mM) and ADP concentration is somehow kept at a significantly high level (~250 μ M) in *Salmonella typhimurium* cells (Neuhard and Nygaard, 1987). Assuming that their concentrations in *E. coli* cells are at a similar level, the ADP concentration seems to be high enough to allow the primer RNA synthesis of plasmid ColE2, as 40 μ M ADP is enough for maximum activity in the reconstituted system. ColE2 DNA replication in crude extracts of *E. coli* cells does not require addition of ADP and moreover addition of ADP showed no effect (unpublished results). The reaction requires a high concentration of ATP and an ATP-regenerating system, indicating the existence of very high ATPase activity producing ADP in the cell extract, which might be sufficient for synthesis of the primer RNA at the origin.

The DNA polymerase III holoenzyme is incapable of substituting for DNA polymerase I, as a crude extract of *E. coli* *polA* cells containing the ColE2 Rep protein is completely inactive for initiation of the ColE2 DNA synthesis (Itoh and Horii, 1989). The large fragment of DNA polymerase I is also inactive in the ColE2 system

(unpublished results). None of the temperature-sensitive *polA* mutants tested can support ColE2 DNA replication at the permissive temperature, whereas some can support ColE1 DNA replication (Kingsbury and Helinski, 1970; Tacon and Sherratt, 1976; unpublished results). The efficient capacity of intact DNA polymerase I to displace a non-template strand without the assistance of other factors such as an energy-driven helicase(s) (Kornberg and Baker, 1992) might be required for elongation of the DNA chain from the primer. Alternatively DNA polymerase I might interact specifically with the complex containing the ColE2 Rep protein, primer RNA and template DNA.

Materials and methods

Bacterial strains, plasmids and phages

The *E. coli* K12 strains used are W3110 (Lederberg, 1950), HB101 (Sambrook *et al.*, 1989) and NT525 *pnp rna endA* (Gellert *et al.*, 1976). Plasmids pEC22 (Horii and Itoh, 1988), pT112-6Arep (Kido *et al.*, 1991) and pTC2101 (Yasueda *et al.*, 1989) have been described. Plasmids pNT204 and pNI6 (Shimamoto *et al.*, 1987) were obtained from N.Shimamoto. A derivative of bacteriophage M13mp18 or M13mp19 (Yanisch-Perron *et al.*, 1985) containing a DNA segment of either the leading- or the lagging-strand template of ColE2 DNA has been described (S.Takechi and T.Itoh, submitted).

Purified proteins

The ColE2 Rep protein was purified from heat-induced W3110 cells carrying pT112-6Arep to near homogeneity by the method described (Kido *et al.*, 1991) with some modifications (H.Matsui and T.Itoh, in preparation). The purity was >95% as judged by SDS-PAGE. *Escherichia coli* DNA polymerase I was obtained from Takara Shuzo Co. Ltd. The purity of the enzyme was ~90%. *Escherichia coli* SSB was prepared from heat-induced HB101 cells carrying pNT204 and pNI6 and was a gift from S.Tateishi. The purity of SSB was >98%. No proteins with a molecular mass corresponding to that of *E. coli* DnaG primase (60 kDa) were detected in these purified proteins.

Preparation of [β - 32 P]ADP

[β - 32 P]ADP was prepared as described (Furuichi and Shatkin, 1977).

Other materials

Chemicals, antibiotics and enzymes were from commercial sources. Terrific broth was prepared as described (Sambrook *et al.*, 1989).

In vitro DNA synthesis using cell extracts

Cell extracts of NT525 and NT525 carrying plasmid pT112-6Arep were prepared essentially as described (Itoh and Horii, 1989; Kido *et al.*, 1991) except that bacteria were grown in Terrific broth. The standard reaction mixture (25 μ l) contained cell extracts (5 μ l), 20 nM plasmid pEC22 DNA, 34 mM potassium phosphate buffer pH 7.5, 12 mM MgCl₂, 80 mM KCl, 1 mM dithiothreitol (DTT), 2 mM ATP, 0.4 mM each of GTP, CTP, UTP, 50 μ M each of four dNTPs with [α - 32 P]dCTP (100–1000 c.p.m./pmol), 0.3 mM cAMP, 0.3 mM NAD, 10 mM creatine phosphate, 0.1 mg/ml creatine kinase, 0.1 mg/ml bovine serum albumin (BSA), 5% PVA or 5% PEG, 0.2 mg/ml chloramphenicol and 24 μ g/ml rifampicin. Incubation was at 32°C for 60 min. To prepare early replicative intermediates, 50 μ M ddTTP was added to the reaction mixture.

DNA and RNA syntheses by purified proteins

DNA synthesis was carried out in a standard reaction mixture (25 μ l) containing 85 mM potassium phosphate buffer pH 7.5, 15 mM MgCl₂, 1 mM DTT, 2 mM spermidine, 100 μ M each ATP, GTP, ADP, 50 μ M each of four dNTPs, 0.1 mg/ml BSA, 16 U/ml of DNA polymerase I, 2.5 μ g/ml of SSB, 60 nM of ColE2 Rep protein and 20 nM pEC22 DNA. The products were labeled with [α - 32 P]dATP (300–10 000 c.p.m./pmol), [α - 32 P]ATP, [α - 32 P]GTP or [β - 32 P]ADP (100 000 c.p.m./pmol). After incubation at 32°C for 60 min incorporation of radioactivity into the acid insoluble fraction was measured. RNA synthesis by ColE2 Rep protein was carried out under the same conditions as described above with [β - 32 P]ADP or [α - 32 P]ATP (100 000 c.p.m./pmol) except that DNA polymerase I and SSB were omitted.

Analysis of the products of in vitro DNA and RNA syntheses

The reaction products of DNA synthesis in crude cell extracts or those with purified proteins were analyzed as described (S.Takechi and T.Itoh, submitted). The reaction products of RNA synthesis by the ColE2 Rep protein were analyzed similarly except that they were purified by chromatography with DEAE cellulose (DE52). The reaction mixtures were applied on a DEAE cellulose column equilibrated with 0.2 M triethyl ammonium acetate (TEAA) pH 7.0. The column was washed successively by 0.2, 0.3 and 0.5 M TEAA. The 0.5 M eluate was analyzed by a 24% polyacrylamide gel electrophoresis.

Determination of strand specificity of DNA fragments from replicative intermediates

Determination of strand specificity of DNA fragments by hybridization with derivatives of bacteriophage M13 and electrophoresis in an agarose gel has been described (S.Takechi and T.Itoh, submitted).

DNA sequencing

The 264-nucleotide *Eco*47III–*Xho*I fragment containing the ColE2 origin region labeled at the 5' *Eco*47III end was sequenced by the chemical modification method as described (Maniatis *et al.*, 1982; Sambrook *et al.*, 1989) and used as size marker.

RNA sequencing

The nucleotide sequence of the RNA at the 5' end of the DNA fragment digested with an appropriate restriction endonuclease was determined by digestion with base-specific RNases (RNase A, RNase T1, RNase T2 and RNase U2) after heating at 95°C for 5 min. Digestion with RNase H was carried out without heat treatment. Samples were heated for 5 min at 85°C and immediately subjected to electrophoresis in a 20% polyacrylamide–7 M urea gel.

Identification of the 5'-terminal nucleotide of primer RNA

A standard reaction for *in vitro* DNA synthesis was performed with 20 μ M [α - 32 P]ATP (100 000 c.p.m./pmol) and 5% PEG. The products were analyzed by electrophoresis in a polyacrylamide gel. The DNA fragment with RNA at its 5' end was eluted from the gel slice. The purified DNA fragment was digested completely with 5 U of S1 endonuclease for 10 min at 37°C. The products were fractionated by HPLC or TLC with AMP, ADP and ATP as markers. A pre-packed column (0.8×10 cm) of a carboxyl-containing silica-based matrix (Waters NOVA-PAKTMC₁₈) was used for HPLC with a Waters M600 instrument. The column was equilibrated with 50 mM NH₄H₂PO₄ pH 6.0 and the elution was done by the same buffer (90 ml) at a flow rate of 1 ml/min with a back pressure of ~1300 p.s.i. The elution profile was monitored by absorbance at 254 nm and 1 ml fractions were collected. Thin layer chromatography was performed on strips (3×12 cm) of PEI cellulose in a solvent system of 0.75 M KH₂PO₄ pH 3.4 at room temperature.

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