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Study on the molecular mechanism of initiation of ColE2 DNA replication

by

SHINJI TAKECHI

Study on the molecular mechanism of initiation of ColE2 DNA replication

INTRODUCTION

DNA replication is controlled by on–and–off and/or frequency of its initiation. In order to reveal the control properties at initiation, it is required to identify the reaction of initiation and the factor of control. Plasmid offers useful model system to study on DNA replication. The region for autonomous replication of ColE2 plasmid (Horii & Itoh, 1988) is determined and its DNA sequence (Yasueda et al., 1989) is revealed. This region includes initiator gene (rep) and replication origin (ori). The expression of Rep protein is repressed by the antisence RNA (RNA I) that is compliment to 5' end of the Rep–mRNA. By using cell extract, in vitro DNA synthesis (Itoh & Horii, 1989) proceeds unidirectionally around ori. The initiation of replication is required host DNA polymerase I (Kingsburg & Helinski, 1970; Tacon & Sherratt, 1976) but not host RNA polymerase. The worth of the study on the initiation of replication ColE2 plasmid is that, the function of the plasmid–coded initiator protein (positive regulator) and the repression of initiation protein by antisence RNA (negative regulator), that is both of positive and negative control can be analyzed in vitro. I have ever studied about negative regulation in this system and offered the mechanism that the frequency of replication is controlled by equivalently Rep expression controlled by RNA I. In this thesis, I will describe the analysis of initiation of replication by Rep protein.

Initiation Site of Unidirectional DNA Replication at the Origin of ColE2 Plasmid

ABSTRACT

Early replicative intermediates of ColE2 plasmid were prepared by in vitro replication system using cell extracts in the presence of ddTTP, rifampicin and chloramphenicol. Short–chain DNAs produced from regions near the replication origin were analyzed by the specific radioactivity of restriction fragments. The leading–strand DNA was covalently linked to primer RNA. The transition site from primer RNA to DNA synthesis was mapped to the unique site within the ori region. The lagging–strand DNA product was also identified and its 3'end mapped to the unique site within the right side end of the ori region. In addition, the leading–strand DNA product was also observed in using several kinds of temperature–sensitive initiation mutants cell extracts. The results suggests the initiation of DNA synthesis depends on plasmid specific priming event.

INTRODUCTION

The ColE2 plasmid is small multicopy plasmid of about 7 kb . A region 1.3 kb long is necessary for its autonomous replication, and within this region encodes a plasmid–specific trans–acting Rep (replication) protein of about 300 amino acids required for autonomous replication. The region of replication of ColE2 have been located within 32 bp segment. The in vitro replication system of this plasmid consists of a crude extract of Escherichia coli cells and depends upon DNA polymerase I and other host proteins. Electron microscopic analysis shows that replication starts in a fixed region and proceeds in a fixed direction. It is not clear at molecular level, however, how and where the DNA strands made during the early stage of unidirectional replication of plasmids are synthesized on the ColE2 duplex. In this paper, using the in vitro replication system, I identify the transition point of leading strand from RNA to DNA and the termination point of lagging strand.

MATERIALS AND METHODS

Bacterial strains and plasmids

The Escherichia coli strains used is NT525 (Gellert et al., 1976). Plasmids pEC22 (Tajima et al., 1988), pTI12–6Arep, have been described.

Construction of phases

Derivatives of M13mp18 and M13mp19, containing DNA segment of either the leading or the lagging strand template of ColE2 was constructed by inserting the 100 bp BglII–XbaI segment of pTC2101 containing the origin of ColE2 between the BamHI and XbaI sites of M13mp18 and M13mp19, respectively.

Single–stranded or double–stranded DNA of the phase M13 derivative was prepared according to the method of Messing (Messing, 1983).

Other materials

Chemicals, antibiotics and most enzymes were from commercial sources.

Preparation of cell extracts

Cell extracts were prepared essentially as described (Itoh & Horii, 1989) except that bacteria were grown in Teriffic Broth.

In vitro DNA synthesis

DNA synthesis was measured essentially described ( ). The standard reaction mixture (25 μl) contained cell extracts (5 μl), 40 μg/ml template plasmid

DNA, 34 mM potassium phosphate buffer pH7.5, 12 mM $MgCl_2$, 80 mM KCl, 1 mM dithiothreitol, 2 mM ATP, 0.4 mM each of rGTP, rCTP, rUTP, 50 M each of 4 dNTPs with [α–^{32}P] dCTP (about 5000 cpm/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, 5% polyvinyl alcohol, 0.2 mg/ml chloramphenicol and 24 μ/ml rifampicin. Plasmid pEC22 were used as the template DNA. After incubation at 32°C for 60 min, incorporation of acid insoluble radioactivity was determined.

Identification of newly synthesized DNA replication intermediates of a ColE2 plasmid

The reaction was stopped by addition of 20 mM EDTA and 0.25% SLS and the mixture was treated with 0.1 mg/ml pronase at 32°C for 30 min. Nucleic acids were extracted with phenol followed by ethel and finally precipitated with ethanol. The samples were digested with a restriction enzyme and precipitated with ethanol. The precipitate dissolved in a solution containing 8 M urea, 1 mM EDTA, 0.1 % (w/v) bromphenol blue and 0.1 % (w/v) xylene cyanol. The samples were heated for 5 min at 85°C and subjected to electrophoresis on a 8% acrylamide, 7 M urea gel. Autoradiography was carried out after drying the gel.

Determination of strand orientation of DNA fragments from replication intermediates

The isolation of newly synthesized ColE2 DNA products were carried out under the assay conditions for DNA synthesis. Each band of the ^{32}P–labeled DNA fragment was eluted from crashed gel slices and purified using yeast tRNA as a carrier. The purified samples were dissolved in 40 μl of a solution

of 0.5 M NaCl and 0.1 % SDS. The samples were heated for 5 min at 95°C and subjected to hybridize with 1 μl of 200 μ/ml of single-stranded DNA of M13 derivative carrying ColE2 DNA strand for 5 min at 65°C and followed by ethanol precipitation. The precipitate dissolved in 10 μl of a solution of 50% glycerol, 10 mM Tris-Cl, pH7.5, 10 mM EDTA, 0.1 % (w/v) bromphenol blue and 0.1 % (w/v) xylene cyanol, and subjected to electrophoresis on 0.8% agarose gel in the presence of 0.5 μ/ml ethidium bromide. Autoradiography was carried out after drying the gel.

RNA sequencing

Nucleotide sequences of the RNA at the 5' terminus of the samples digested with restriction endonuclease were determined by digestion with base-specific ribonucleases. Digestion was carried out for 20 min at 55°C in separate 10 μl reaction mixture. For ribonuclease T_1 (G-specific) and ribonuclease A (pyrimidine-specific), the reactions contained 7 M urea , 1 mM EDTA and 25 mM sodium citrate, pH5.5. Ribonuclease U_2 (A-specific) was used in 7 M urea, 1 mM EDTA, 25 mM sodium citrate, pH3.5. Ribonuclease T_2 (all nucleotides) was used in 10 mM Tris-Cl,pH7.5, 10 mM EDTA. The reaction contained 0.02 unit of T_1, 0.05 unit of U_2, 8,25 μg of A, and 0.04 unit of T_2. reaction were stopped by on ice and adding 1 μl of marker dyes 0.1% (w/v) each of xylene cyanol, bromo phenol blue, Except as noted, the reaction of T_1 was stopped by adding urea to a final concentration of 7 M. Samples were heated for 5 min at 85°C immediately before loading on the gel and subjected to electrophoresis on a 8% polyacrylamide gel with 7 M urea described above.

Identification of 5'-terminal nucleotide of primer RNA

The DNA synthesis was performed under the assay conditions described above except that [α–^{32}P} ATP (10^5 cpm/pmol) was present at 20 μM. The Eco47III–generated fragment of 105 nucleotides long was extracted with phenol and, after treatment with ether, precipitated twice with ethanol. The samples was dissolved in 10 μl of a solution of 50 mM sodium acetate, pH4.5, 200 mM NaCl, 1 mM $ZnSO_4$, 0.5% glycerol and digested completely by incubation with 5 unit of nuclease S1 for 10 min at 37°C. The product was separated by HPLC and each faction was measured in a liquid scintillation counter.

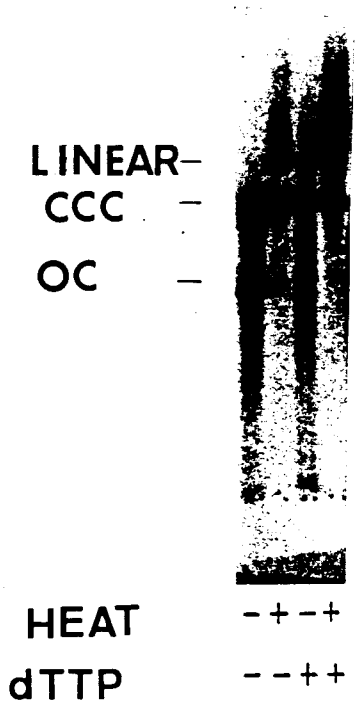

Figure 1. Early replication intermediates synthesized in vitro.
Replication products, synthesized in the standard in vitro in the presence of ddTTP or by using of dna ts mutants cell extracts. were isolated and analyzed on a 1% neutral agarose gel. Each reaction mixture contained 50 μM each of cold four dNTPs. (see Materials and Methods). Positions of open circular (Form II) and covalently closed circular (Form I) DNA molecules are indicated. The thick arrow indicates the position of replication intermediates.

RESULTS

1)Early replication intermediates synthesized in vitro by using cell extracts

In this study, to accumulate replication intermediates, I added chain-terminator ddTTP to the reaction mixture in addition to the four dNTPs. The concentration of ddTTP added was 50 μM, which decreased the total amount of DNA synthesis in a 60 min incubation to 25% to 30% of the standard reaction without ddTTP (data not shown). Fig. 1 shows an analysis of products synthesized in vitro in the presence of ddTTP on a neutral agarose gel. In standard condition, FormI and FormII DNA as well as high molecular weight DNA were synthesized in vitro (lane 1). In present of ddTTP, these products decreased, and new species migrating slightly above FormI started to accumulate (lane 3). When each samples heat denatured, in absent of ddTTP, instead of almost disappearing of FormII DNA , linear fragment approximately full-length size was appeared (lane 2). Otherwise, very heterologous long fragments of less than full-length size were accumulated in presence of ddTTP (lane 4).

2)Mapping of initiation of replication intermediates of ColE2 plasmid

In order to determine where replication of ColE2 plasmid is initiated, early replication intermediates were analyzed by restriction enzyme digestion. The products by DNA synthesis with [α-^{32}P] dCTP, in the absence or presence of ddTTP, were digested by Eco47III or NciI and were analyzed on a neutral polyacrylamide gel (Fig 2). In the absent of ddTTP, as described above almost products were FormI DNA, Eco47III digestion gererated one freagment of full-length long which moved hardly (3.6 kbp; lane 1). In the present of ddTTP,

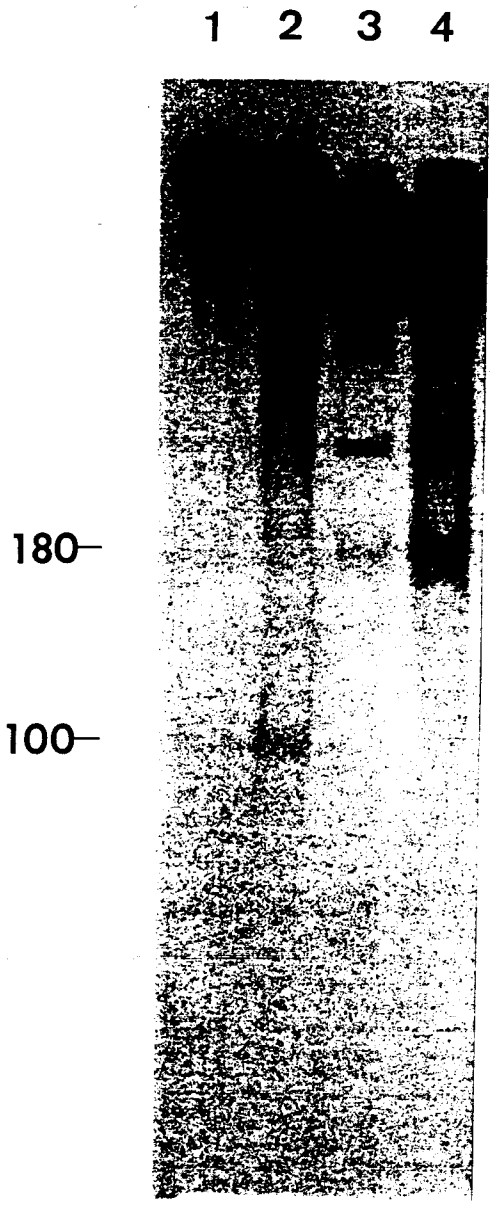

Figure 2. Mapping of initiation of ColE2 plasmid replication.
A, replication products, synthesized in vitro by using wild type or dna B ts mutant cell extracts, were isolated, digested with Eco47III or NciI, and applied on a 8% polyacrylamide gel. Fragments generated are numbered in order of size.
B, The restriction endonuclease map around ori.

Eco47III digestion generated one new fragment of approximately 100 bp (lane 2). NciI digestion generated mainly three fragments in the absent of ddTTP(717, 1170 and 1722 bp, respectively; lane 3) and two more minor fragments which were unexpected but may come from posing of replication. In addition of thses fragments, one short fragment was appeared in the present of ddTTP (lane 4). An Eco47III site was located about 150 bp down stream from the BglII site in the direction of replication and a NciI site was about 80 bp further downstream from the Eco47III site. Digestion of the replication intermediates with Eco47III or NciI generated a new band of ^{32}P-labeled fragment, about 100 or 180 bases in length respectively. Considering locations of cleavage sites for Eco47III and NciI, the ends of the DNA that gives rise to these fragments can be located at about 100 bases upstream from the Eco47III site (Fig.2).

3)5'end of the leading strand and 3'end of the lagging strand

Analysis by restriction digestion described above suggest that ColE2 plasmid replication is initiated at a specific position on the basic replicon of ColE2 plasmid. In order to locate precisely the position of the initiation site, products synthesized in vitro were digested by restriction enzyme which cuts the template DNA to the right of ori, and the single strands were analyzed by denaturing polyacrylamide gel. When they were digested by Eco47III, two fragments, 95 and 105 nucleotides long respectively, were detected (Fig.3). In order to determine the strandness of these nascent fragments, each ^{32}P-labeled fragment were subjected to hybridize with chimeric single-stranded phage DNAs carrying each strand derived ori sequence(Fig.4). As a result, 105 nucleotide long fragment hybridized only with the phage DNA containing the

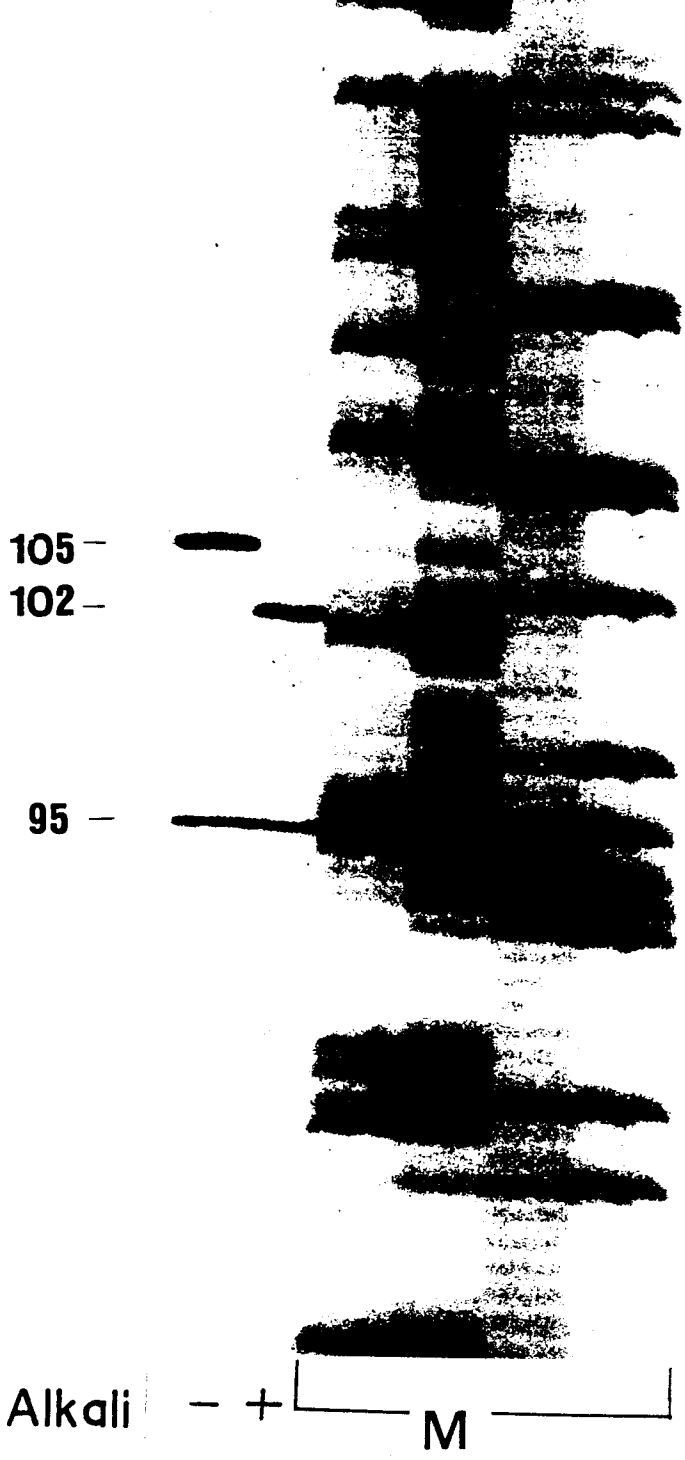

Figure 3. 5' end of the first leading strand and 3' end of the lagging strand synthesized in vitro.
Replication products, synthesized in vitro in the present of ddTTP, were digested with Eco47III(lane 1). Half of the digested materials were further treated with alkaline (lane 2). Lanes M, sequenceing ladders used as markers to determine fragment lengths. Numbers are lengths, in bases, deduced from markers.

Figure 4. Strand–specific hybridization with the nascent fragment.
Replication products, synthesized on pEC22 with substrates containing [α–^{32}P] dCTP at 10000 cpm/pmol, were digested with Eco47III and were fractionated on an 8% denaturing polyacrylamide gel. Fragments of 105 and 95 nucleotides were isolated and were hybridized with single–stranded DNA containing ori segment either the leading or the lagging strand template. as described Materials and Methods.

template strand for the leading strand and 95 nucleotide long fragment hybridized with only with the phage DNA containing the template strand for the lagging strand. Furthermore, alkaline treatment of the product resulted in decrease of the size of 105 nucleotides long fragment by about three nucleotides (Fig.3), suggesting the presence of RNA primer attached to its 5' end. This would also indicate that this nascent DNA fragment with RNA at its 5' end is generated by de novo synthesis and not processing of longer fragment. these fragments were synthesized in a very early stage of ColE2 plasmid replication, since they were observed even in the presence of 100 μM ddTTP. Based on these results, I have concluded that leading strand synthesis is initiated at 105 bases upstream of Eco47III cutting site and lagging strand synthesis is terminated at 95 bases upstream of Eco47III cutting site.

4)Analysis of primer RNA

As described above, leading strand has RNA about three nucleotides long at its 5' end, then I analyzedt the structure of the primer RNA. Fig.5 shows the results of replication intermediates cleaved by BglI were treated with sequence specific RNase. RNase T2 generated three bases shorter fragment (lane 3). Partial digestion with RNase T1 (G–specific: lane 4) generated new fragment which was two bases shorter than non–RNase digested leading fragment. And partial digestion with RNase U2 (A–specific: lane 5) generated new two fragments which were one and three bases shorter than original leading fragment. But, RNase A did not make any effect (lane 2). Furthermore, DNA–RNA hybridized RNA specific RNase,RNase H (lane 6) digestion

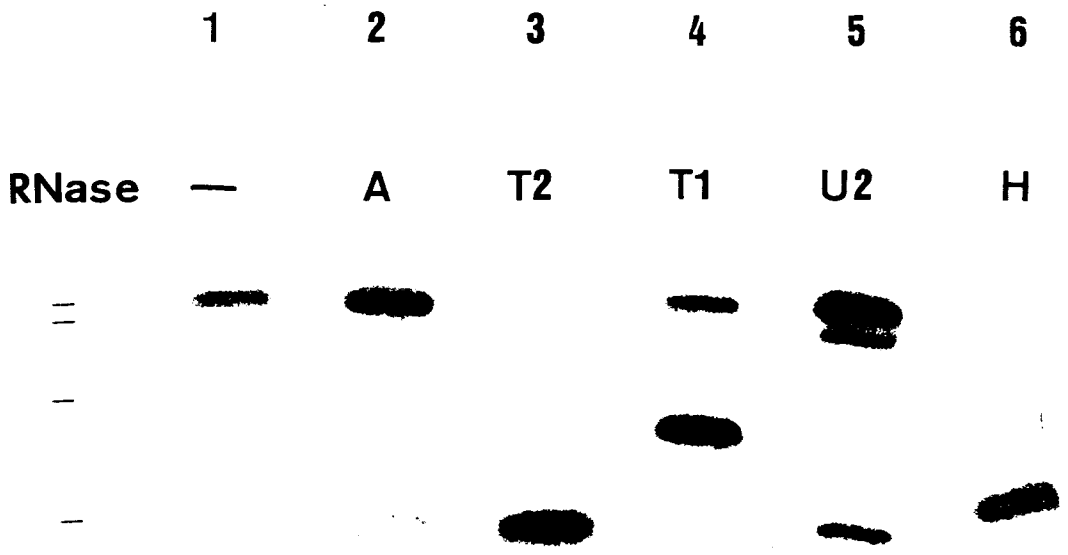

Figure 5. Sequence of primer RNA.
Replication products, synthesized on pEC22 with substrates containing [α–^{32}P] dCTP at 10000 cpm/pmol, were digested with Bgl I (lane 1), and subjected to digested by RNases (lane 2, 3, 4, 5 and 6 are digested with RNase A, T2, T1, U2 and H, respectively), as described Materials and Methods. In all cases samples were analyzed on a 20% denaturing polyacrylamide gel.

generated shorter fragment that of RNase T1 digestion. This result shows the sequence of the RNA at 5' end of the leading strand is AGA, which is complementary to the leading strand template DNA. The position of the leading fragment with triribonucleotides was slightly lower than that of expected fragment in size. This might be the effect of the 5' end of primer RNA.

The leading strand fragment with triribonucleotides was not phosphorylated at the 5' terminus with T4 polynucleotide kinase(data not shown). This suggests the presence of at least one phosphate residue at the 5' end of triribonucleotides. In order to determine the number of phosphate residue at the 5' end of the triribonucleotides, the leading fragment labeled with [α–^{32}P] ATP was digested completely by nuclease S1 and the products were analyzed with HPLC. Fig.6 shows the ^{32}P–labeled monoribonucleotide were almost equal of AMP and ADT, and the AMP was derived from the third base of the triribonucleotides and the ADP was derived from the 5' terminus of that. This results strongly suggests that the initiation of this primer RNA is the first base of A.

5)Ribonucleotide dependency of the leading strand synthesis

Analysis of the RNA at 5' end of the leading strand described above suggest that the primer RNA of the leading strand is started from A which is located three bases upstream of the initiation site of the leading DNA chain, and the sequence of it is AGA. In order to determine whether it is necessary more than three bases long could initiate DNA chains, ribonucleotide dependency on the DNA synthesis of the leading strand was examined. To neglect the effect of ribonucleotides derived from cell extracts, these were filtrated by Sephadex G–25. Table 1 shows the result of ribonucleotide

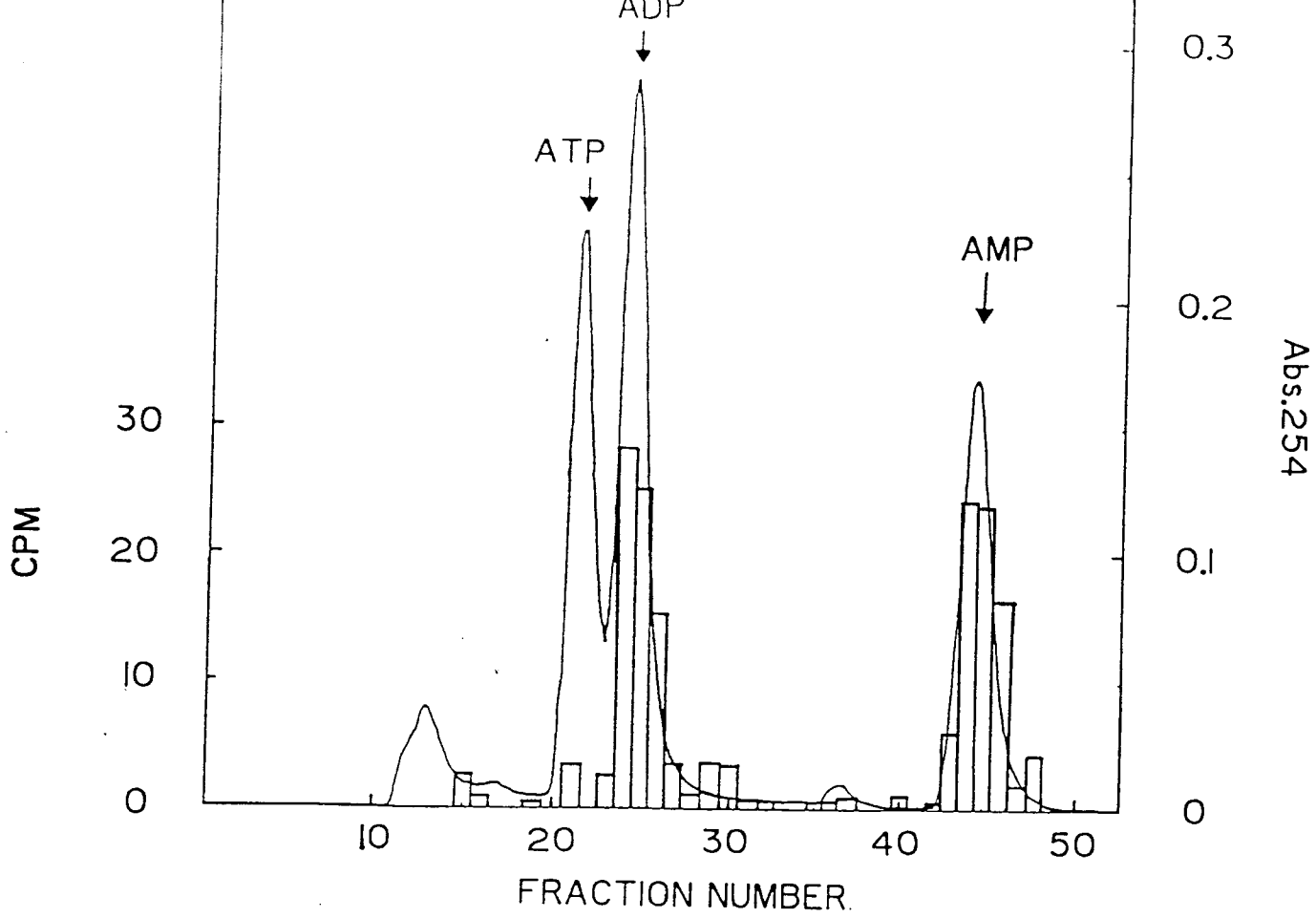

Figure 6. Initiation residue of primer RNA.
Replication products, synthesized on pEC22 with substrates containing [α–^{32}P] ATP at 10000 cpm/pmol, were digested with Eco47III and were fractionated on an 8% denaturing polyacrylamide gel. Fragment of 105 5 nucleotides was isolated, and subjected to be digested by nuclease S1. The sample was applied with cold AMP, ADP and ATP, as a marker, and analyzed by HPLC, as described Materials and Methods. The line describes UV absorbance. The bars are ^{32}P radioactivities of each fraction.

dependence on incorporation of dAMP. When ATP or GTP was omitted, DNA synthesis was reduced about 25%. When UTP and/or CTP was omitted, DNA synthesis was slightly effected. Fig.7 shows the fragment of the replication intermediates at these conditions described above on denatured gel. When ATP or GTP was omitted, the fragment of the leading strand was almost disappear. When UTP and/or CTP was omitted, the appearance of that was almost not effected. These results suggest that the synthesis of the leading strand depend on ATP and GTP but does not depend on UTP and CTP. These results also imply that the leading strand synthesis can be initiated by the synthesis of triribonucleotides (AGA).

component omitted	dCMP incorporation pmol	(%)
None	3.0	(100)
ATP	0.7	(23)
GTP	0.7	(23)
CTP	2.0	(67)
UTP	3.0	(100)
CTP,UTP	2.5	(83)
Rep protein	(0.6)	

Table 1. Effect of rNTP omission on ColE2 DNA synthesis.
Cell extracts were filtrated by Sephadex G25 (see Materials and Methods) to remove internal nucleotide. DNA synthesis were performed as standard (see Materials and Methods), but one or two components were omitted, and measured DNA synthesis by ^{32}P counting, as described Materials and Methods.

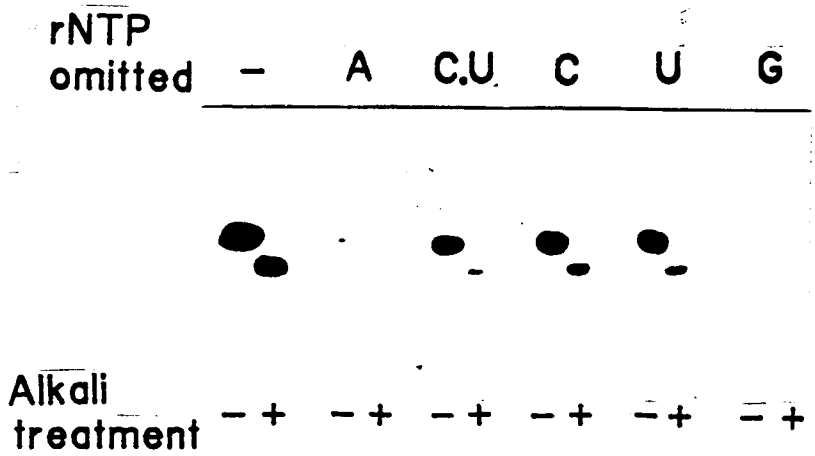

Figure 7. Effect of rNTP omission on product of ColE2 DNA synthesis.
The samples were prepared as described in Table 1., and subjected to be digested with Eco47III and were fractionated on an 8% denaturing polyacrylamide gel.

DISCUSSION

I found that the site of initiation of ColE2 DNA replication in vitro by using cell extract.

The leading strand synthesis was initiated at a specific site within ori. The leading strand DNA products have triribonucleotides (ppApGpA) at their 5' ends. Furthermore, leading strand synthesis did not required host dnaG primase by using dna mutant extracts. These results support the notion that leading strand synthesis of ColE2 plasmid is mediated by plasmid specific priming machinery including Rep protein at a specific site within ori.

The lagging strand synthesis was terminated at a specific site downstream of ori. The position of the termination coincides with the 3' edge of the region where Rep protein interacts with ori sequence. Rep protein staying bound to ori even after initiation of replication of replication may simply sterically interfere with the DNA polymerase and cause termination.

My data have suggested the scheme of ColE2 plasmid replication. After assembly at ori of an initiation complex including Rep, the leading strand synthesis is initiated by triribonucleotides and proceeded by DNA polymerase I. Lagging strand synthesis, initiated at multiple sites, is terminated at a specific site, thus preventing further fork movement into the other direction. Once initiated, the leading strand will be synthesized continuously and the lagging strand discontinuously. Okazaki fragments generated by multiple primings appear to be efficiently ligated, because no specific 5' end for a nascent lagging strand was detected by the method employed in this work.

Primer RNA Synthesis on ColE2 Plasmid DNA in a Purified Protein System

SUMMARY

By using an in vitro system for ColE2 plasmid replication dependent on a plasmid–coded protein (Rep) and host DNA polymerase I, the nascent leading DNA strand was synthesized from the unique position within the origin (ori). The priming of the leading DNA chain occurred at three bases upstream of the initiation site of it. The unique primer at 5'end of leading strand was trirbonucleotides (ppApGpA). This reaction required ADP, GTP and ATP.

INTRODUCTION

I have shown above in vitro replication of ColE2 plasmid, by using cell extract, depends on the plasmid coded Rep protein. This replication starts at a unique site within the origin (ori), and the primer of the leading strand is ppAGA. The lagging strand synthesis is terminated at the right end of ori. The synthesis of leading strand does not depend on host RNA polymerase and dnaG primase. However, mechanism of priming of the leading strand synthesis is unknown.

In this paper, I have used ColE2 DNA as template to characterize the leading strand synthesis carried out by Rep and DNA polymerase I.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages

The Escherichia coli strains used are as follows:

Plasmids pEC22 have been described. Derivatives of M13mp18 and M13mp19, containing DNA segment of either the leading or the lagging strand template of ColE2 have been described. Single–stranded or double–stranded DNA of the phase M13 derivative was prepared according to the method of Messing.

Assay of DNA synthesis with purified protein

DNA synthesis was assayed in 25 μl volume containing 20 to 60 μg/ml template DNA, 85 mM potassium phosphate buffer, pH7.5, 15 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM spermidine, 100 μM each ATP, GTP, ADP, 50 M each four dNTPs with [α–^{32}P] dATP (300 to 1000 cpm/pmol), 0.1 mg/ml bovine serum albumin, 0.4 unit of DNA polymerase I, 2.5 mg/ml of SSB and approximately 1 pmol of Rep protein. Incubation was at 32°C for 60 min. Incorporation of [α–^{32}P] dATP was measured by using a liquid scintillation counter.

Isolation of synthesized DNA

The reaction was stopped by addition of phenol. Nucleic acids were extracted with phenol followed by ethel and finally precipitated with ethanol. The samples were digested with a restriction enzyme and precipitated with ethanol. The precipitate dissolved in a solution containing 8 M urea, 1 mM EDTA, 0.1 % (w/v) bromphenol blue and 0.1 % (w/v) xylene cyanol.

Other materials

Chemicals, antibiotics and most enzymes were from commercial sources. ColE2 Rep protein was prepared in our laboratory from heat-induced NT525 cells carrying pTI12-6Arep. E.coli SSB was prepared in our laboratory from heat-induced HB101 cells carrying pNT204 and pNI6 (Simamoto et al.,1987; obtained from N. Shimamoto).

Determination of strand orientation of DNA fragments from replication intermediates

The method has described above.

Identification of 5'-terminal nucleotide of primer RNA

As noted above, the ^{32}P labeled sample was digested completely by S1, and separated by PEI celrose.

Preparation of [β-^{32}P] ADP

The reaction mixture in 250 μl contained 50 mM Tri-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 5 mM AMP, 3.6 units of myokinase (Boehringer Mannheim) and 0.23 nmol of [γ-^{32}P] ATP (7000 Ci/mmol). The mixture was incubated at 37°C for 60 min and subjected to treat with phenol chloroform (1:1). [β-^{32}P] ADP was isolated by DEAE-sephadex A25.

RESULTS

Characterization of ColE2 DNA synthesis by three purified enzymes

ColE2 DNA synthesis by purified enzymes was not observed in the conditions for DNA synthesis by using cell extracts. Several modifications were to enhance the DNA synthesis. Some of them were to decrease the concentration of ATP and to omit ATP regeneration system, that is creatine kinase and creatine phosphate(data not shown). Therefore, it was expected that those changes caused allowance of residual ADP in the reaction mixture. I examined the effect of ADP, in addition to several other components, on DNA synthesis(Fig. 1).

I found rather height concentrations of KPi buffer and $MgCl_2$ with optimal synthesis around 85 mM and 15 mM, respectively (data not shown). Addition of spermidine (2 mM) had a slight stimulation. DNA synthesis increased as concentration of ADP, GTP and ATP was increased to 100 μM, but had no requirement on UTP and CTP.

The kinetics of DNA synthesis by Rep and DNA polymerase I with or without are shown in Figure 2. DNA synthesis with SSB is about half of that without SSB. This inhibition of SSB on DNA synthesis examined more detail in the next section. The effect of varying the concentration of Rep, DNA polymerase I and SSB are shown in Figure 3, 4 and 5, respectively.

Analysis of product by reconstitution system

As described above, DNA synthesis in a cell extract initiates at specific site with unique primer. I examined the initiation of DNA synthesis by three

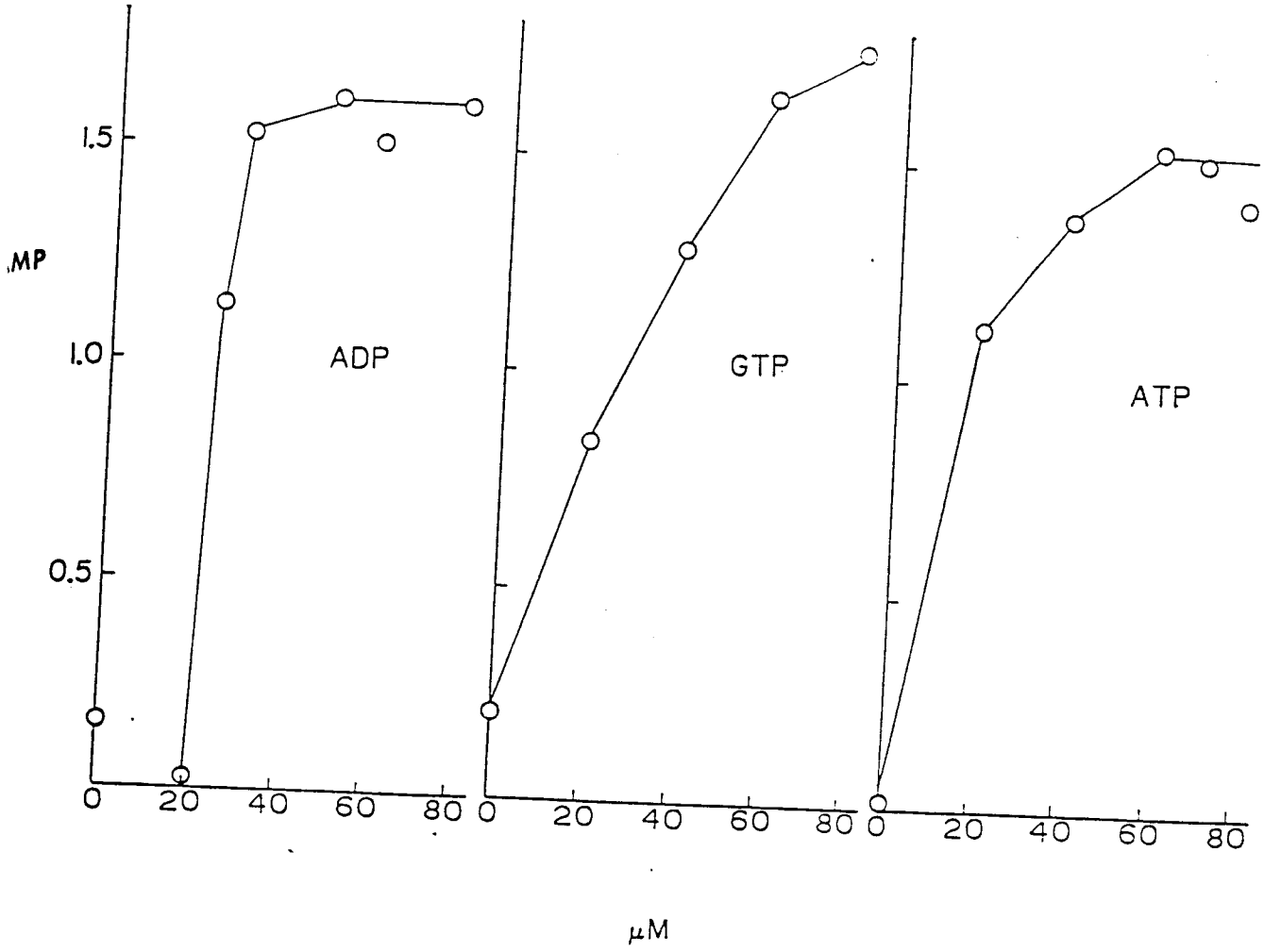

Figure 1. Influence of ribonucleotide concentrations on ColE2 DNA synthesis by purified protein system.
DNA synthesis assay were performed as described under Materials and Methods, except that in A, ADP was added to the indicated concentrations in the presence of 100 μM each of GTP and ATP, in B, GTP was added to the indicated concentrations in the presence of 100 μM each of ADP and ATP, in C, ATP was added to the indicated concentrations in the presence of 100 μM each of ADP and GTP. Each reaction mixture contained 50 μM each of cold four dNTPs, with [α-^{32}P] dATP (1000 cpm/pmol). Incubation were for 60 min at 32°C.

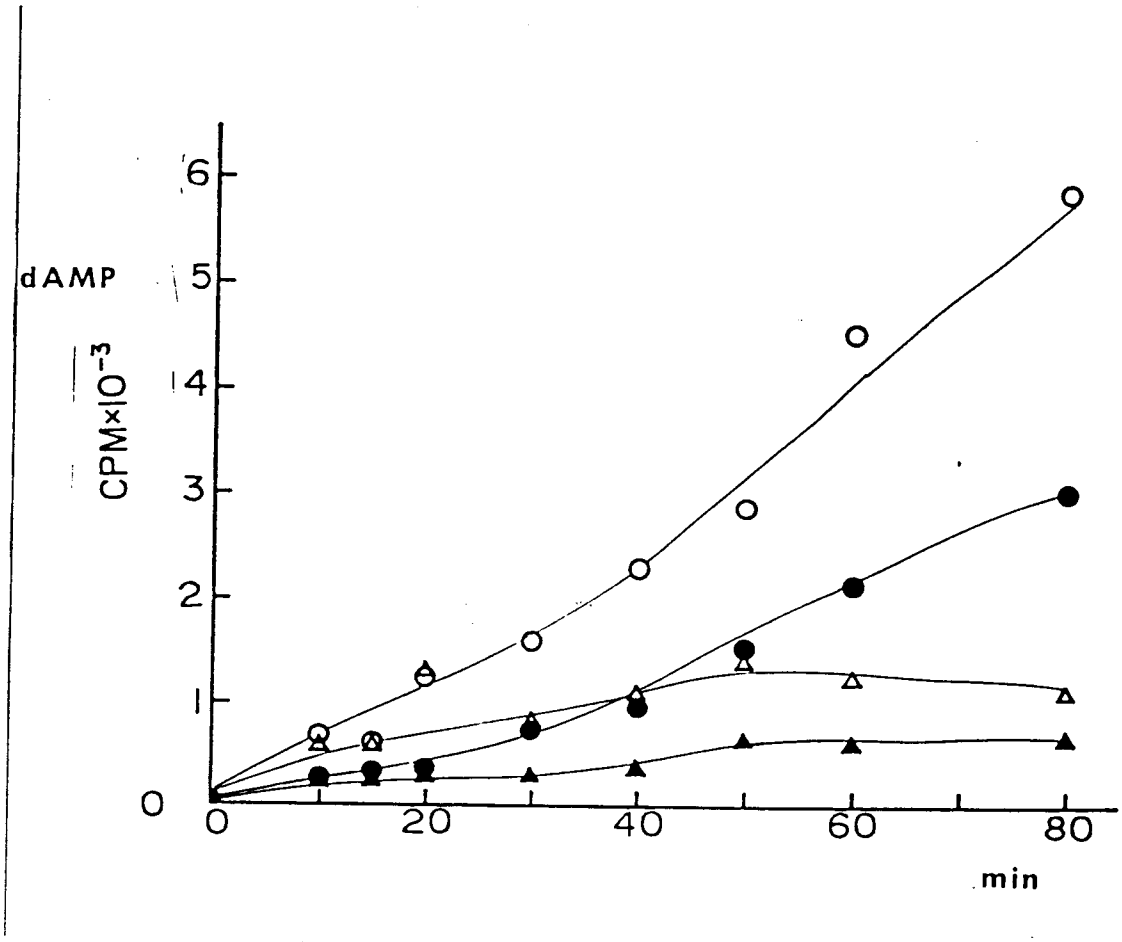

Figure 2. Kinetics of ColE2 DNA synthesis by Rep and DNA polymerase I in the absence or presence of SSB. DNA synthesis assay were performed as described under Materials and Methods, except that in the absence (o,Δ) or presence (•,▲) of SSB. DNA synthesis without ribonucleotides (•,▲) were as background for standard, containing 50 μM [α–^{32}P] dATP (1000 cpm/pmol). Incubation were at 32°C.

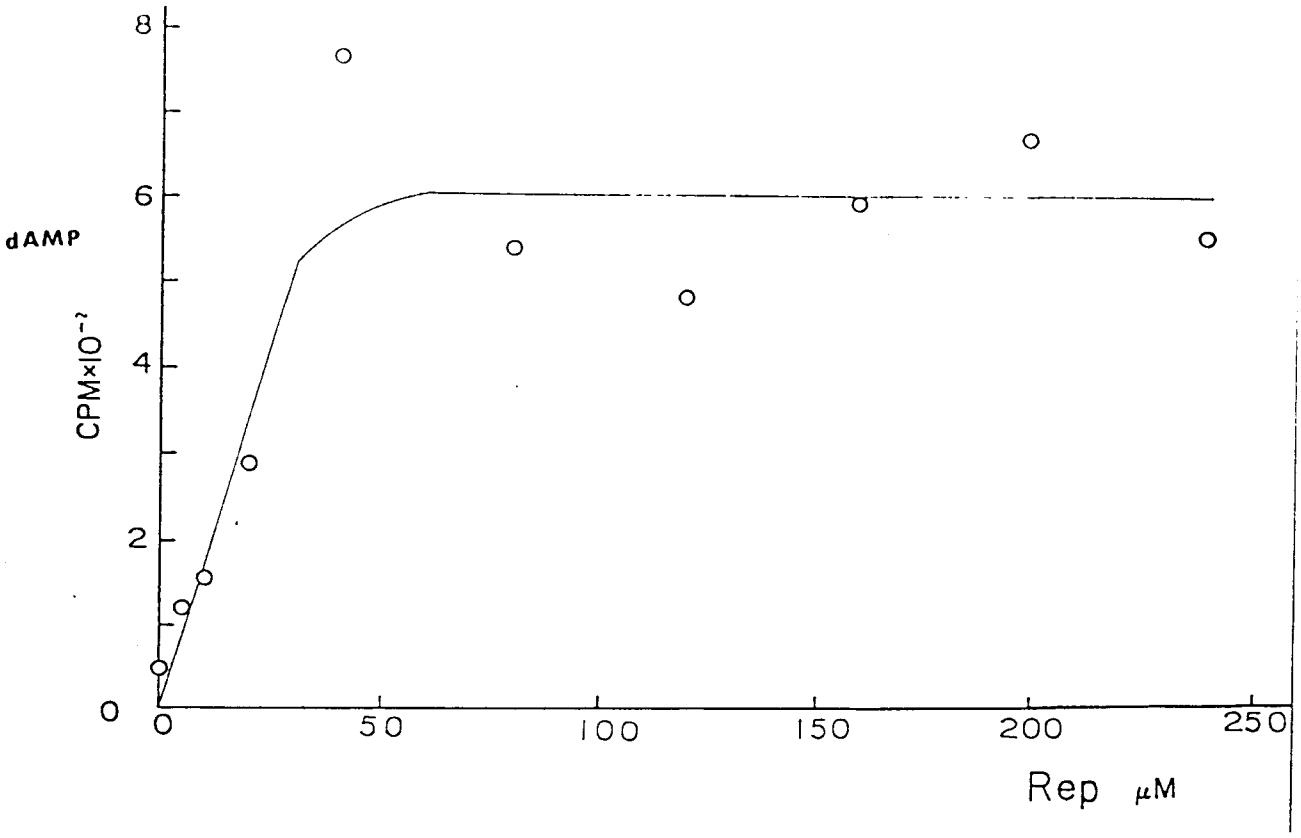

Figure 3. ColE2 DNA synthesis with purified enzymes; effect of Rep on ColE2 DNA synthesis. Various amounts of Rep were added as indicated, containing 50 μM [α-^{32}P] dATP (1000 cpm/pmol). Incubation were for 60 min at 32°C.

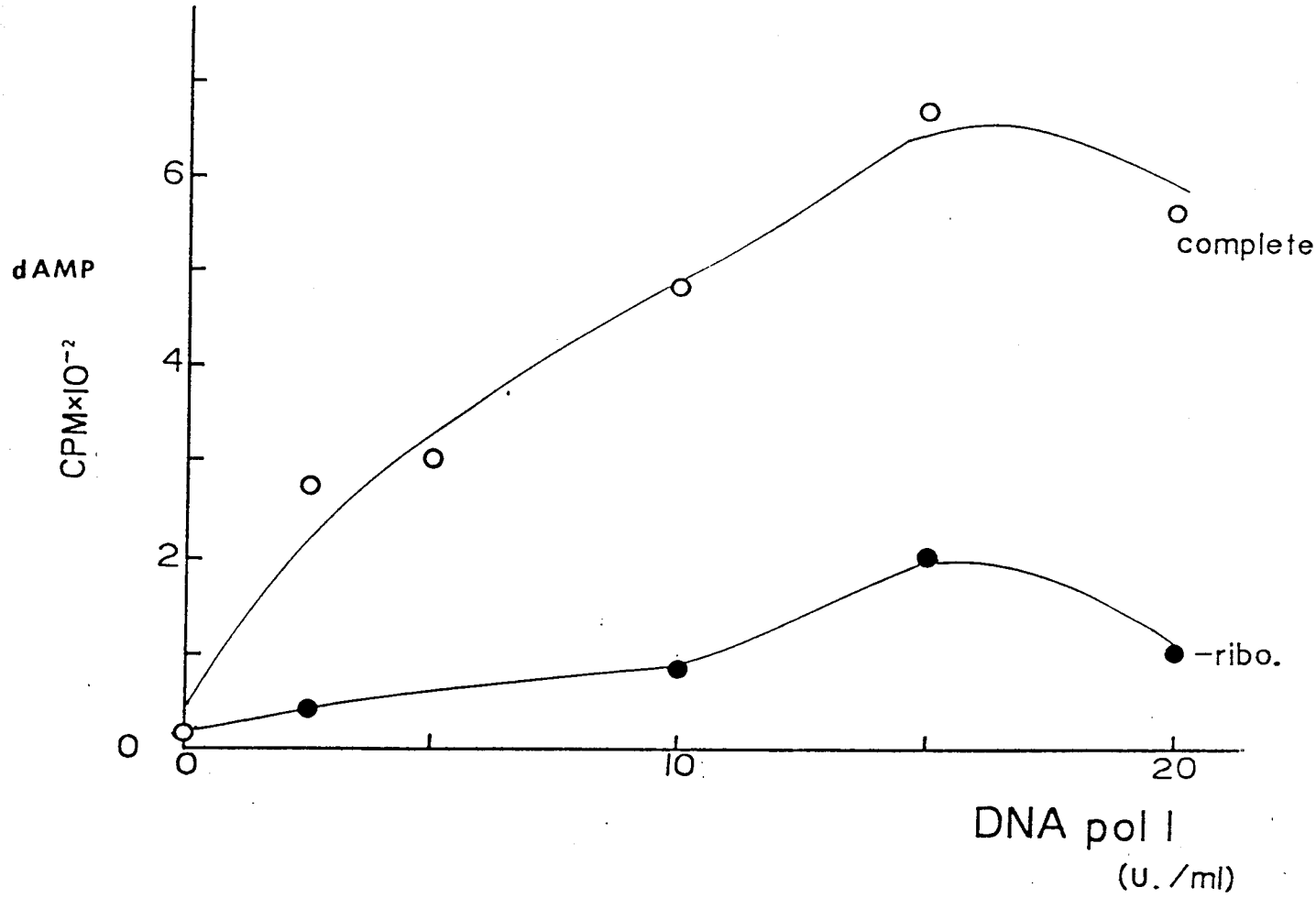

Figure 4. ColE2 DNA synthesis with purified enzymes; effect of DNA polymerase I on ColE2 DNA synthesis. Various amounts of DNA polymerase I were added as indicated. DNA synthesis without ribonucleotides (●) were as background for standard. Containing 50 μM [α-^{32}P] dATP (1000 cpm/pmol). Incubation were for 60 min at 32°C.

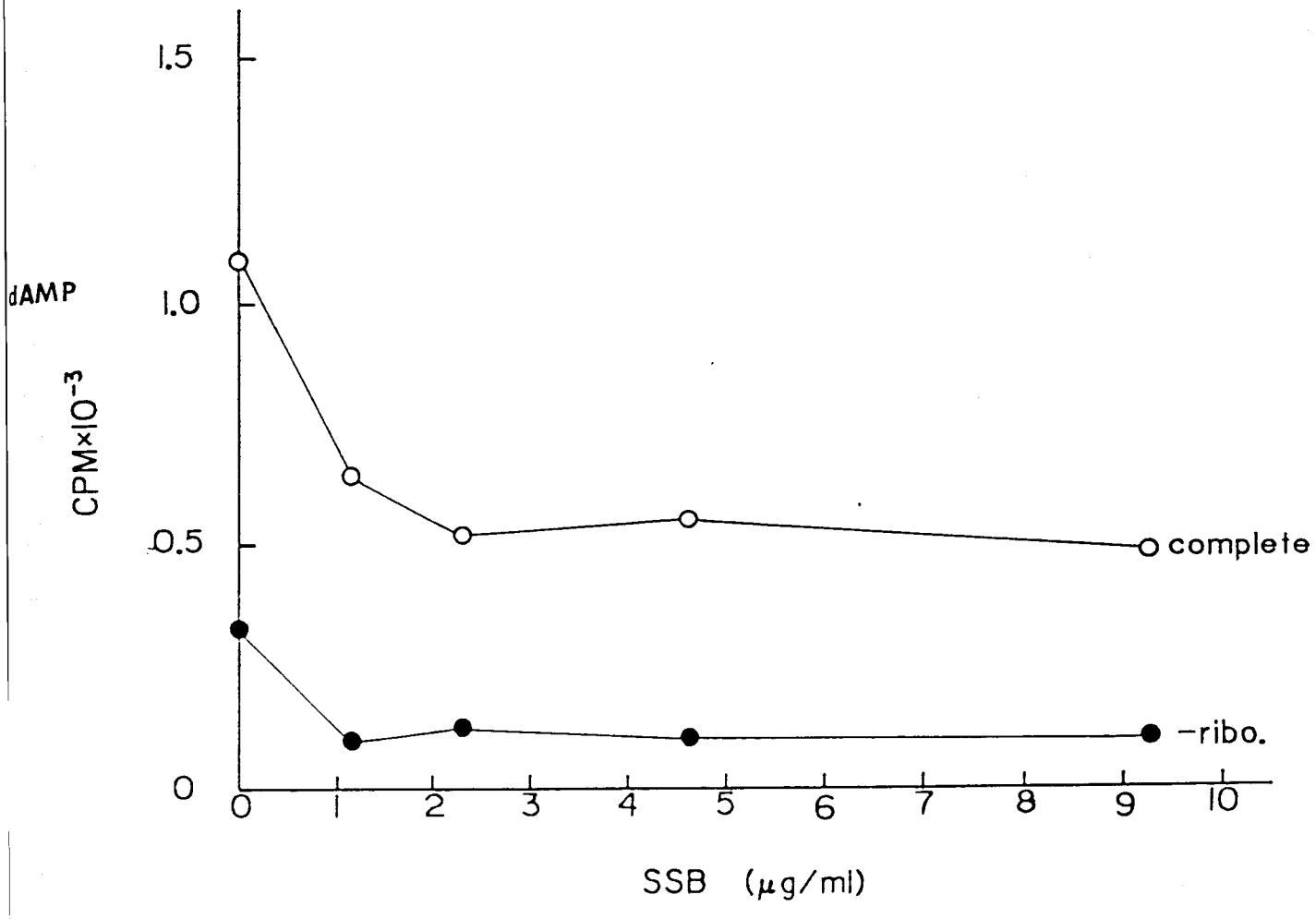

Figure 5. ColE2 DNA synthesis with purified enzymes; effect of SSB on ColE2 DNA synthesis. Various amounts of SSB were added as indicated. DNA synthesis without ribonucleotides (●) were as background for standard, containing 50 μM [α–^{32}P] dATP (1000 cpm/pmol). Incubation were for 60 min at 32°C.

enzymes. Figure 6. shown the result on denature gel, the products in each condition were uniformly labeled with [α–^{32}P] dATP, and digested by Eco47III.

As noted above, two fragments were observed by using cell etract (lane 1), one was derived from the leading strand and the other was derived from lagging strand. The DNA synthesis by Rep and DNA polymerase I without or with SSB (lane 2 and 3, respectively) could produce the identical fragment which is derived from the leading strand. The strandedness of this fragment was examined by strand–specific hybridization (Fig. 7). These results shows that the leading strand synthesis can be reconstituted by Rep and DNA polymerase I. However, unexpected fragment, which was same size as the fragment derived from the lagging strand, was observed in the purified–protein system without SSB, but not observed in this system with SSB (Fig. 6).

ADP initiates RNA primer

ColE2 DNA synthesis by purified enzymes requires ADP. I then examined whthere ADP residues were incorporated into RNA primer transcripts. The products of DNA synthesis by Rep and DNA polymerase I were labeled with [α–^{32}P] ADP, [α–^{32}P] GTP and [α–^{32}P] ATP, respectively, and digested by Eco47III(Fig. 8 lane 1, 2 and 3, respectively). ^{32}P–labeled fragments were observed at the identical position . The incorporation of each ^{32}P labeled nucleotides were almost equal. Furthermore, [α–^{32}P] ATP labeled fragment was subjected to digested by nuclease S1, and recovered as ^{32}P labeled AMP (Fig.9). These results show that ADP residues are incorporated into primer RNA as the first residue and ATP residues are the third one.

Effect of single–strand DNA binding protein (SSB)

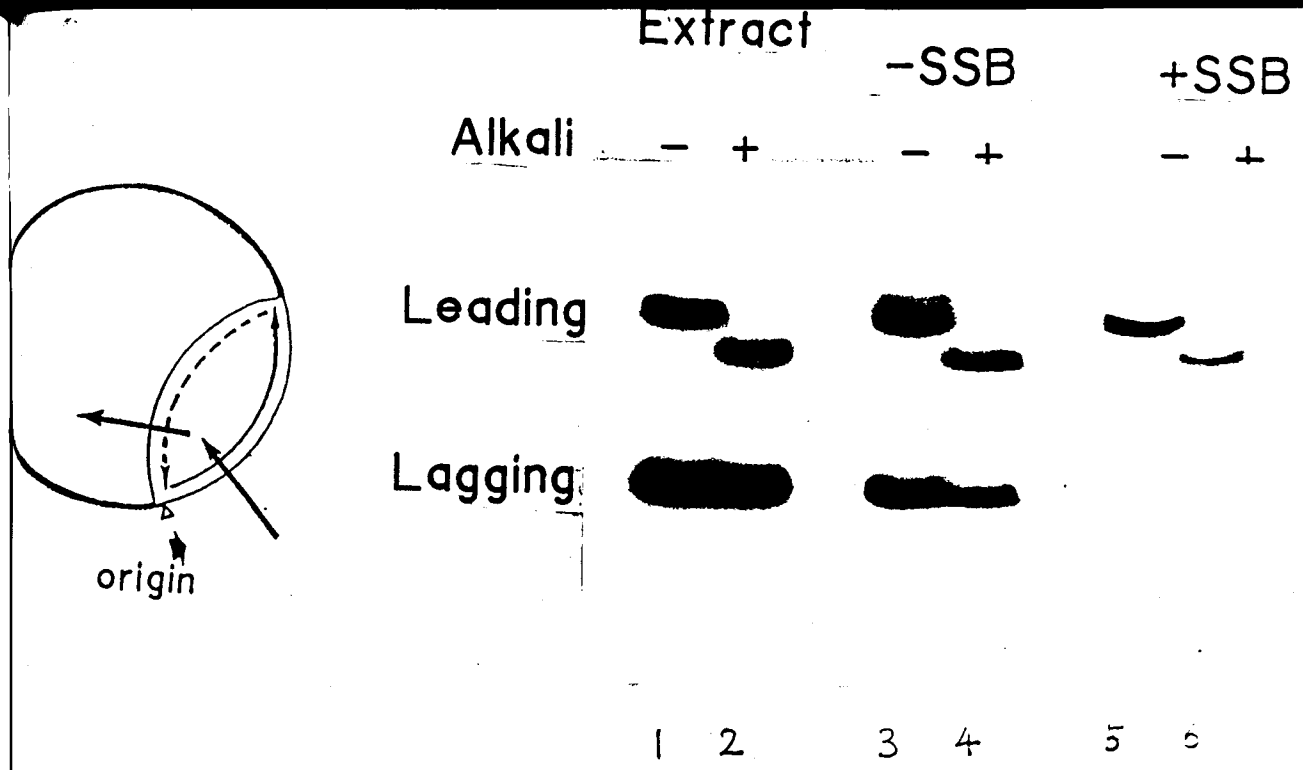

Figure 6. Product of ColE2 DNA synthesis by purified enzymes.
Replication products, synthesized in vitro by using cell extracts (lane 1,2) or by Rep and DNA polymerase I without (lane 3,4) or with (lane 5,6) SSB, were isolated, digested with Eco47III or and applied on a 8% polyacrylamide gel. Lane 1,3 and 5 were nontreated, lane 2,4 and 6 were treated with alkaline.

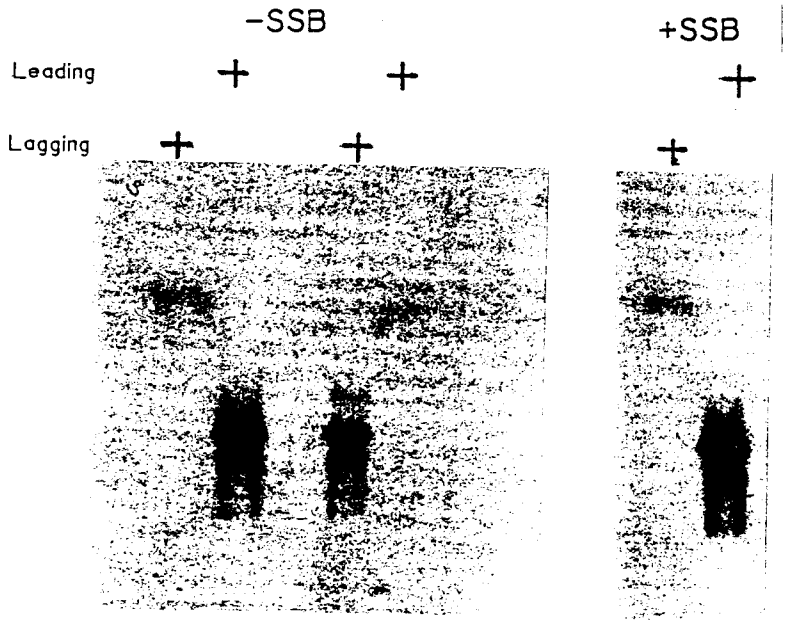

Figure 7. Strand–specific hybridization with the nascent fragment.
Replication products, synthesized on pEC22 with substrates containing [α–^{32}P] dATP at 10000 cpm/pmol, were digested with Eco47III and were fractionated on an 8% denaturing polyacrylamide gel. Fragments of 105 and 95 nucleotides were isolated and were hybridized with single–stranded DNA containing ori segment either the leading or the lagging strand template. as described Materials and Methods.

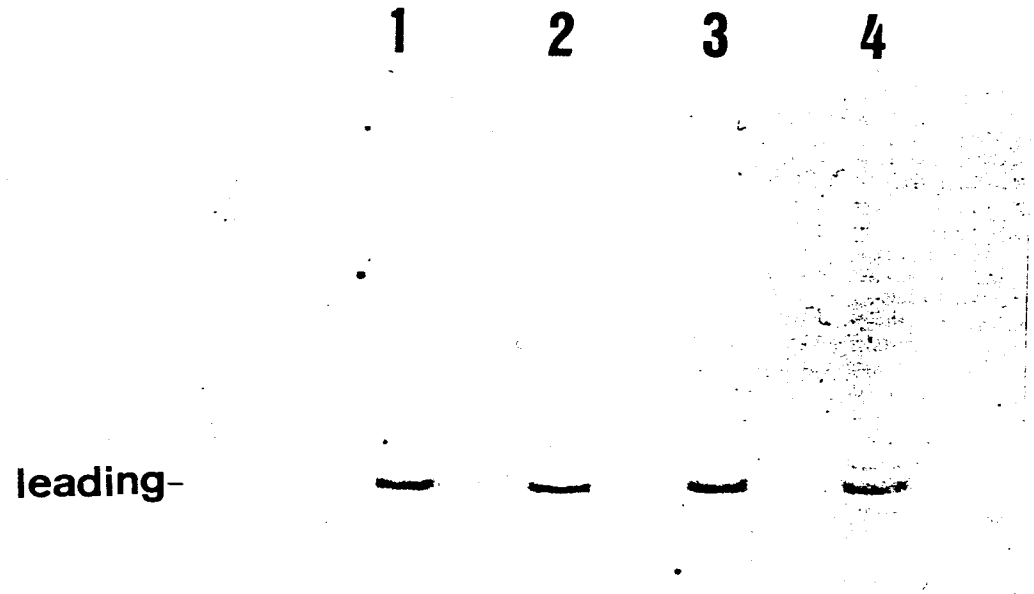

Figure 8. ADP is utilized for primer RNA.
Replication products, synthesized in vitro by Rep and DNA polymerase I with SSB, with substrates containing [β–^{32}P] ADT, [α–^{32}P] GTP, [α–^{32}P] ATP or [α–^{32}P] dATP (lane 1, 2, 3 and 4, respectively), were isolated, digested with Eco47III and applied on a 8% polyacrylamide gel.

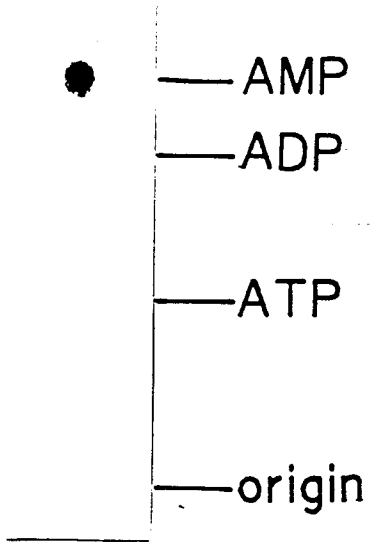

Figure 9. ATP is utilized as the third residue of primer RNA.
Replication products by purified enzymes, synthesized on pEC22 with substrates containing [α–^{32}P] ATP at 10000 cpm/pmol, were digested with Eco47III and were fractionated on an 8% denaturing polyacrylamide gel. Fragment of 105 nucleotides was isolated, and subjected to be digested by nuclease S1. The sample was applied with cold AMP, ADP and ATP, as a marker, and analyzed by PEI cellulose in a solvent system of 0.75 M KH_2PO_4, pH3.4.

As described above, in this purified-protein system, the lagging strand synthesis was occurred, and inhibited specifically by SSB. I propose the following working hypothesis as a model to explain this observation. DNA synthesis by Rep and DNA polymerase I initiates within ori and proceeds along leading strand template. Because DNA polymerase I switches template, synthesis then proceeds along the lagging strand template which becomes single-stranded DNA, and terminating near the origin. DNA polymerase I which is known to be capable of strand switching in vitro with subsequent accumulation of replicative structures including hairpins. However, in the present of SSB, single-stranded stretch are coated by SSB and lagging strand synthesis can not occur. Based on this model, I examined whether hairpins were accumulated in the product by Rep and DNA polymerase I. Therefore a low molecule weight fragment should be released upon denaturation of the product. ColE2 DNA synthesized by Rep and DNA polymerase I in the presence or in the absence of SSB, as described above. Newly synthesized DNA was detected by gel electro-phoresis after heating for 10 min at 100°C (Fig. 10). Only in the absence of SSB (lane 1), at least two short fragments were accumulated, The most intense band was detected on the fragment about 260 b.p.. Tightness of this ^{32}P labeled band suggests that the 260 b.p. fragment does not vary greatly in size. Furthermore, the S1 nuclease does reduce the size of the fragment by about 20 b.p. (in duplex DNA measurements: lane 2), and Eco47III generates two fragments about 160 b.p. and 100 b.p., respectively (lane 3). This result shows that the fragment which has a hairpin-like structure is produced by Rep and DNA polymerase I, and this fragment is not produced in the presence of SSB.

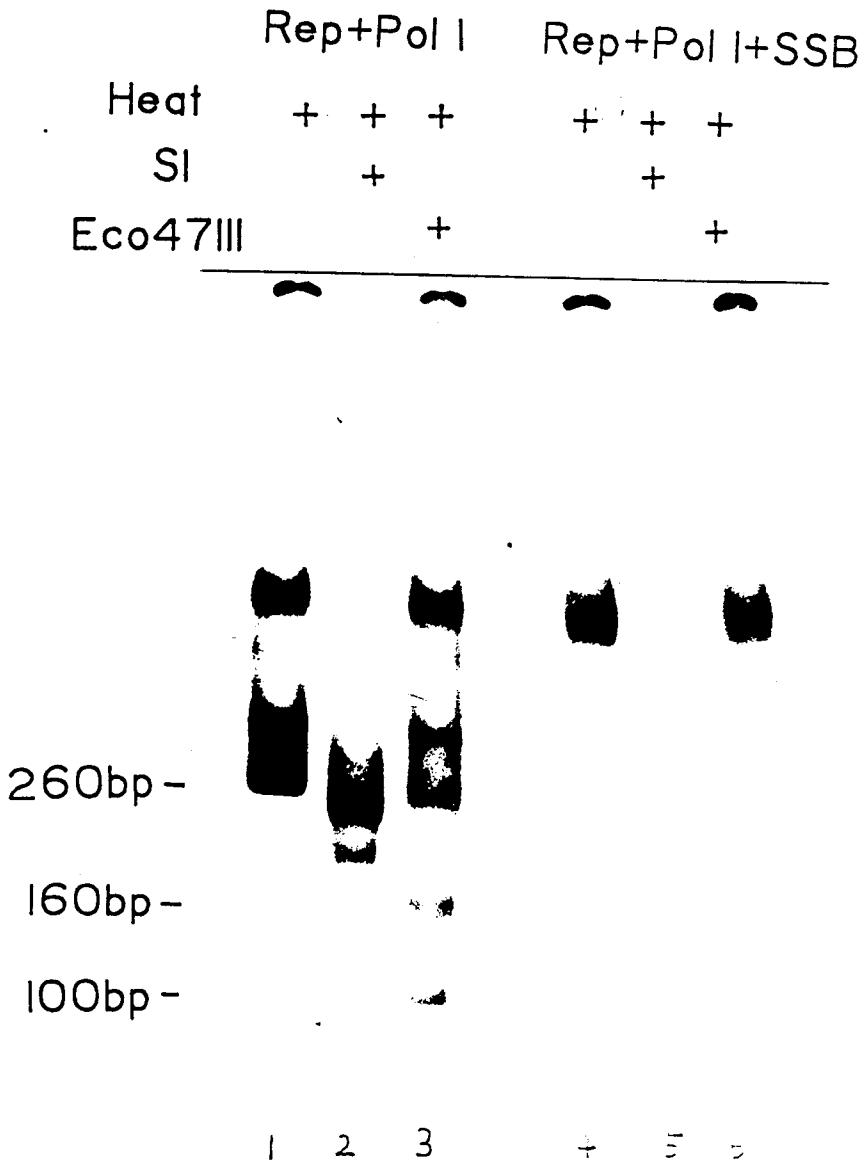

Figure 10. Effect of SSB on ColE2 DNA synthesis by Rep and DNA polymerase I.

Replication products by Rep and DNA polymerase I, synthesized on pEC22 with substrates containing [α–^{32}P] dATP at 10000 cpm/pmol, without (lane 1, 2, 3) or with (lane 4, 5, 6) SSB, were isolated and heat denatured, and subjected to be digested with nuclease S1 (lane 2 and 5) or restriction endonuclease Eco47III (lane 3 and 6). The samples were applied on a 8% polyacrylamide gel. Approximate size of fragments are based on the cold marker fragments (not shown) made by pBR322 digested with HindIII and HinfI. In without SSB, several fragments were observed (lane 1), and the most intense fragment of approximately 260 b.p. was observed, but not in with SSB (lane 4). Treatment with nuclease S1 detectable increases the mobility of 260 b.p. fragment (lane 2). By Eco47III digestion, in stead of almost disappearing 260 b.p. fragment, two nascent fragments (160 and 100 b.p.) were appeared (lane 3). In each case, the slowest mobility of rather smeared fragment was single stranded DNA because of disappearing with nuclease S1 treatment.

DISCUSSION

I have found that Rep and DNA polymerase I synthesized the ColE2 leading strand DNA. In this reaction, newly synthesized DNA products have RNA which initiated by ADP. These results strongly suggest that Rep protein synthesizes the primer RNA (ppApGpA) started with ADP.

Rep has unique character differ from other well-known primase, that is, Rep primase binds to specific sequence on double strand DNA, and does not require other enzyme in unwinding, utilizes ADP as the first residue of primer.

A search for possible homology with prokaryotic and eukaryotic DNA primase such as the E.coli dnaG protein (Smiley et al., 1982), the T7 gene 4 protein (Dunn et al., 1983), the T4 genes 41 and 61, the yeast S.cerevisiae PRI1 (Plevani et al., 1987) protein did not reveal any significant homology conserved.

REFERENCES

Bazaral, M. & Helinski, D. R. (1968). J.Mol. Biol. **36**, 185–194.

Dunn, J. J. & Studier, F. W. (1983). J.Mol. Biol. **166**, 477–535.

Gellert, M., O'Dea, M., Itoh, T. & Tomizawa, J. (1976). Proc. Nat. Acad. Sci., U.S.A. **73**, 4474–4478.

Herschman, H. R. & Helinski, D. R. (1967). J Bacteriol. **94**, 691–699.

Horii, T. & Itoh, T. (1988). Mol Gen Genet. **212**, 225–231.

Itoh, T. & Horii, T. (1989). Mol Gen Genet. **219**, 249–255.

Kingsbury, D. T. & Helinski, D. R. (1970). Biochem Biophys Res Commun. **41**, 1538–1544.

Messing, J. (1983). Methods Enzymol. 101, 20–78.

Plevani, P., Francesconi, S. & Lucchini, G. (1987). Nucl. Acids. Res. **15**, 7975–7989.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). Proc. Nat. Acad. Sci., U.S.A. **74**, 5463–5467.

Simamoto, N., Ikushima, N., Utiyama, H., Tachibana, H. & Horie, K. (1987). Nucl. Acids. Res. **15**, 5241–5250.

Smiley, B. L., Lupski, J. R., McMacken, R. & Godson, G. N. (1982). Proc. Nat. Acad. Sci., U.S.A. **79**, 4550–4554.

Tacon, W. & Sherratt, D. J. (1976). Mol Gen Genet. **147**, 331–335.

Tajima, Y., Horii, T. & Itoh, T. (1988). Mol Gen Genet. **214**, 451–455.

Yasueda, H., Horii, T. & Itoh, T. (1989). Mol Gen Genet. **215**, 209–216.