Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 7639–7643, December 1982
Biochemistry

Replication of λ dv plasmid *in vitro* promoted by purified λ O and P proteins

(initiation of DNA replication/initiators/origin of λ phage/four direct repeats)

TOSHIKI TSURIMOTO AND KENICHI MATSUBARA

Laboratory of Molecular Genetics, Osaka University, Medical School, Kita-ku, Osaka 530, Japan

Communicated by Bruce M. Alberts, August 30, 1982

ABSTRACT **An *in vitro* system for replication of λ dv plasmid DNA has been constructed. This system consists of an ammonium sulfate fraction from *Escherichia coli* extract, exogenously added purified λ O and P proteins, and λ dv DNA in closed circular form. More than 85% of the added template DNA replicated semiconservatively. In the same system, another plasmid, pBR322, also replicated, but less efficiently than λ dv. Furthermore, its replication was independent of O and P proteins. Inhibitors of DNA gyrase entirely blocked the replication activity, whereas rifampicin, an inhibitor of RNA polymerase, showed a significant effect only when added prior to initiation of the DNA replication. DNA replication was initiated from a region near to or within the four direct repeats in λ origin (λ *ori*) and proceeded bidirectionally, as examined by DNA chain elongation termination with dideoxy CTP. A cloned DNA carrying a 350-base-pair region including the initiation site also initiated replication, dependent on O and P proteins, and its initiation occurred at the same position as with native λ dv DNA. An A+T-rich structure neighboring the repeats was found to be essential for λ DNA replication. Regions corresponding to *ice* and *oop* were not required for O,P-dependent initiation.**

Replication of bacteriophage λ and λ dv plasmid depends on many *Escherichia coli* replication proteins, including dnaB, dnaE, dnaG, dnaJ, dnaK, dnaZ, RNA polymerase, and DNA gyrase (1). In addition, λ encoded O and P proteins are necessary for the initiation of replication (2). O protein binds specifically to four 19-base-pair (bp) direct repeats in the λ *ori* region (3, 4). P protein interacts with O protein and with some *E. coli* replication proteins such as dnaB protein (5–8). These unique interactions (9, 10) are thought to form a replisome structure (11) at λ *ori*. The resulting replication fork moves bidirectionally (12).

Schnös and Inman (12) have demonstrated that DNA synthesis of λ phage is initiated at about 81% of the λ phage genetic map and that it proceeds bidirectionally. However, precise location of the start point has not been determined. Neither has it been made clear whether the *ori* (13, 14) or *ice* (15, 16) regions function for initiation. All these questions would be answered if λ replication could be achieved *in vitro*.

Recently, several *in vitro* systems from *E. coli* have been reported that successfully allow replication of several plasmids (17–20). Among these, the system of Fuller *et al.* (20) allowed *in vitro* replication of an *oriC* plasmid bearing the chromosomal origin (21) in the presence of an *E. coli* enzyme fraction, along with hydrophilic polymers and an ATP-regeneration system. In this paper, we report that externally added λ dv DNA can replicate in a system analogous to that of Fuller *et al.* (20) when purified O and P proteins were supplied.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* YS1 (*thr leu thi rps*L *min*A *end*) has been described (17). All the plasmids used in this study are carried in Km723 (22). λ dv021 (23) is the plasmid derivative of λ phage and is in a dimeric form. It is called λ dv in this paper for clarity. Plasmids pOri-1 (4), pOA-4, and pOri-D1 (24) are pBR322-based (25) recombinant plasmids carrying λ *ori* fragments.

Assay for λ dv Replication *in Vitro*. The method of Fuller *et al.* (20) was followed. The standard assay mixture (25 μl) contained: 40 mM Hepes (pH 8.0); 80 mM KCl; 2 mM ATP; GTP, CTP, and UTP, each at 0.5 mM; bovine serum albumin at 50 μg/ml; 21.6 mM creatine phosphate; 11 mM $Mg(CH_3COO)_2$; creatine kinase at 100 μg/ml; 6% (wt/vol) polyethylene glycol (PEG) 20,000; dATP, dGTP, dTTP, and dCTP, each at 0.1 mM with [α-^{32}P]dCTP (Radiochemical Centre, Amersham, England) at 100 cpm/pmol of dCTP; λ dv DNA at 8 μg/ml in covalently closed circular form; purified O protein at 20 μg/ml; purified P protein at 16 μg/ml; and 2 μl of YS1 protein fraction (fraction II containing 50–60 mg of proteins per ml) prepared by the methods of Fuller *et al.* (20). The mixture was incubated at 30°C for 30 min, except when noted otherwise. The extent of DNA synthesis was expressed as total dNMP incorporated.

O and P Proteins. O and P proteins, each more than 95% pure, were prepared from Km723(pMY17-3) as described (5). The purified proteins were dialyzed against 10 mM potassium phosphate, pH 6.9/0.1 mM EDTA/1 mM dithiothreitol, diluted with the same buffer to contain 500 μg of O protein or 390 μg of P protein per ml, and stored at 0°C.

RESULTS

Requirement for Added O and P Proteins. An ammonium sulfate fraction (fraction II) was prepared according to Fuller *et al.* (20) from a high-speed supernatant of *E. coli* YS1 that does not carry λ or λ dv. This system, fortified with hydrophilic polymer (PEG 20,000) and the ATP-regeneration system that are essential for the successful replication of an *oriC* plasmid, was unable to replicate added λ dv DNA. However, DNA synthesis did occur upon addition of purified λ O and P proteins (Fig. 1). When one of the initiators was omitted from the assay mixture, DNA synthesis did not occur, in agreement with *in vivo* observations (2, 26). When the O protein concentration was increased, the level of DNA synthesis increased; synthesis reached a plateau at 20 μg of protein per ml, at which the molar ratio of λ dv DNA to O protein was about 1:100. Our previous observations (5) have shown that saturation of O protein binding to λ *ori* sequence occurs at a similar O protein/λ *ori* ratio. Thus,

Abbreviations: PEG, polyethylene glycol; ddCTP, 2',3'-dideoxycytidine 5'-triphosphate; bp, base pair(s).

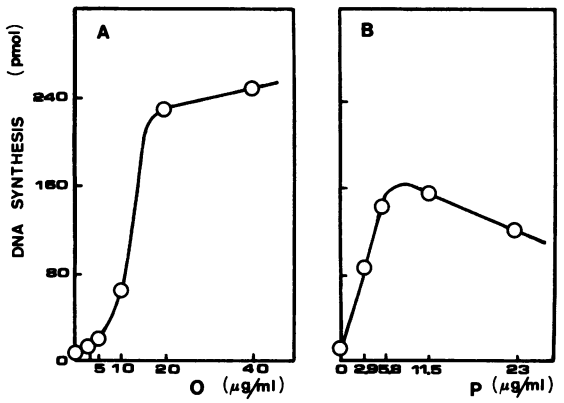


FIG. 1. Requirement for added O and P proteins in the replication of λ dv DNA *in vitro*. Reactions were carried out in the presence of λ dv021 DNA at 8 μg/ml. (*A*) DNA synthesis in the presence of P protein (9 μg/ml) and various amounts of O protein as indicated. (*B*) DNA synthesis in the presence of O protein (10 μg/ml) and various amounts of P protein as indicated.

the relationship between synthesis of λ dv DNA and concentration of O protein seems to reflect O protein binding to λ *ori*. At a P protein concentration of about 8 μg/ml, the maximal synthetic capacity of λ dv DNA was attained. Under these conditions, the molar ratio of O and P proteins is roughly unity, which suggests that in this assay system they interact in an equimolar ratio. In the following experiments, we routinely used the concentration of 20 μg of O protein and 16 μg of P protein per ml for λ dv replication. The uptake of radiolabeled precursor showed that DNA synthesis amounted to as much as 80% of the added λ dv DNA. The O,P-dependent DNA synthesis was specific for λ dv and not for pBR322 (Fig. 2).

Characteristics of the λ dv Replication System. Table 1 shows the requirements for the reaction. ATP and Mg^{2+} were absolutely required. The ATP-regeneration system and PEG 20,000 greatly increased the activity, as discussed by Fuller *et al.* (20). Nalidixic acid and novobiocin, inhibitors of DNA gyrase, produced significant inhibition whereas chloramphenicol, an inhibitor of protein synthesis, did not. Rifampicin, an inhibitor

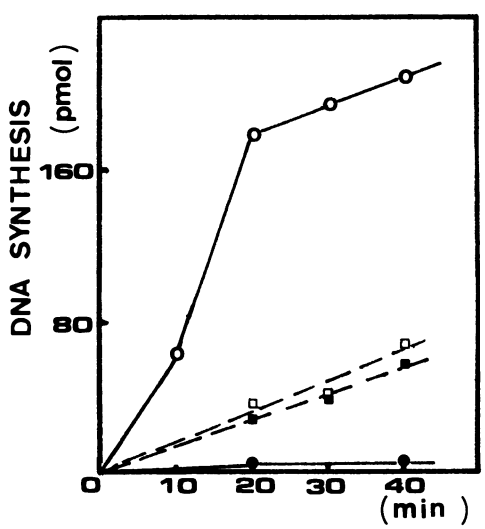


FIG. 2. Kinetics of λ dv and pBR322 DNA synthesis with or without O and P proteins. Reactions were carried out in the presence of 8 μg of λ dv021 DNA (○, ●) or pBR322 DNA (□, ■) per ml. Open symbols represent the activities with a combination of O protein (20 μg/ml) and P protein (16 μg/ml); solid symbols represent activities without the proteins.

Table 1. Requirements for the reaction

Reaction mixture	DNA synthesis pmol	DNA synthesis %
Complete	221.2	100
Without:		
$Mg(CH_3COO)_2$	4.4	2.0
ATP, creatine phosphate, and creatine kinase	0.6	0.3
Creatine phosphate and creatine kinase	99.8	45.1
PEG 20,000	28.5	12.9
With added inhibitor:		
Nalidixic acid (500 μg/ml)	18.8	8.5
Novobiocin (20 μg/ml)	8.0	3.6
Chloramphenicol (200 μg/ml)	187.2	84.9
Rifampicin (20 μg/ml)	42.4	19.2

Inhibitors were added just prior to the start of the reaction.

of RNA polymerase, had an intermediate effect. In this case, the reaction was started by addition of the initiators and rifampicin without preincubation. However, when the drug was added 10 min prior to initiation (i.e., addition of the initiators and dNTPs), the level of DNA synthesis was similar to that seen when O and P proteins were not added. Addition of this drug at or after initiation exerted practically no effects (data not

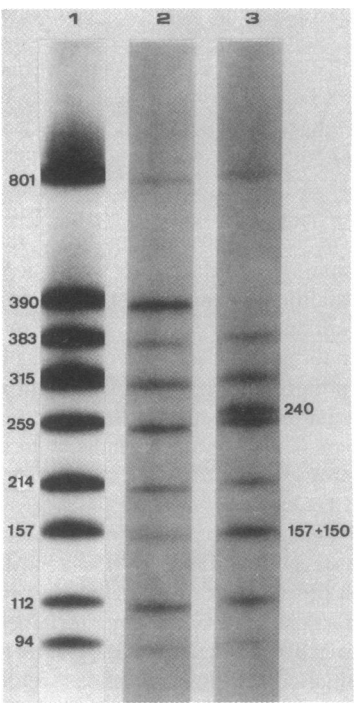


FIG. 3. Determination of the initiation site in λ dv DNA by the ddCTP method: Distribution of newly incorporated radioactivity among various cleavage fragments from λ dv DNA. The 25-μl standard reaction mixture contained [α-^{32}P]dCTP at 500 cpm/pmol and 25 μM (lane 1) or 100 μM ddCTP (lanes 2 and 3). Note that 25 μM ddCTP was ineffective in our assay system because incorporated radioisotopes distributed almost uniformly throughout the λ dv genome. The reaction was stopped by addition of 5 μl of 0.25 M EDTA and 2.5% $NaDodSO_4$, followed by incubation at 37°C for 1 hr with 15 μl of proteinase K (5 mg/ml). DNA was extracted with phenol and precipitated with 70% ethanol three times; then it was digested with *Dde* I (lanes 1 and 2) or *Dde* I/*Eco*RI (lane 3). The digested fragments were electrophoresed in 8% polyacrylamide gel with Tris borate buffer containing 8 M urea and autoradiographed. Numbers indicate size of the bands in bp. Locations of these fragments along the λ dv genome are shown in Fig. 4.

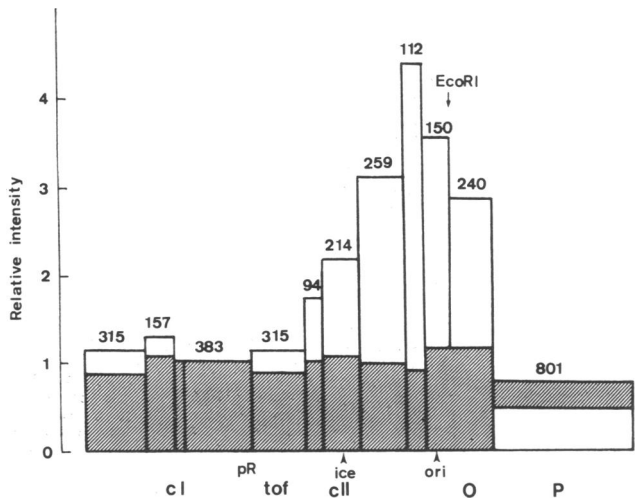


FIG. 4. Relative intensities of radioactivity in bands in Fig. 3 were measured by scanning and divided by the size of the fragment, and then each value was normalized by taking the value for the 383-bp fragment, located around *cI* and *pR*, as 1.0. Hatched bars, 25 μM ddCTP; open bars, 100 μM ddCTP. In Fig. 3, two 315-bp bands overlap. The relative intensities of these two fragments were taken to be equivalent. Lane 3 shows 150-bp and 240-bp fragments generated by *Eco*RI cleavage at the 390-bp fragment in lane 2. The 150-bp fragment overlaps with a 157-bp fragment. Its intensity was determined by subtraction of the value of the 157-bp fragment in lane 2 from that of the overlapped bands in lane 3. The sites of the genetic markers cI, *tof*, cII, *pR*, *ice*, *ori*, *O*, and *P* are shown according to ref. 28.

shown). In contrast, DNA synthesis of pBR322 was inhibited by addition of rifampicin at any stage, as previously shown with ColE1 (17). These observations demonstrate that RNA synthesis by RNA polymerase is required for λ dv replication and suggest strongly that its role in replication is different between λ dv and pBR322. This problem will be discussed elsewhere.

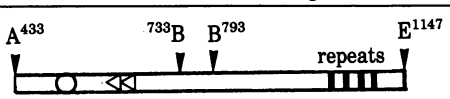
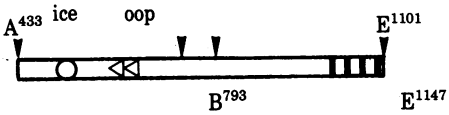
Product of λ dv Replication. Products were analyzed by gel electrophoresis in both pBR322 and λ dv DNAs (data not shown). Most of the molecules were found in open circular form and a limited amount was in covalently closed circular form. A small fraction migrated behind the covalently closed circular form and presumably represents replicative intermediates.

^{3}H-Labeled light λ dv DNA was subjected to *in vitro* replication in the presence of [α-^{32}P]dCTP and BrdUTP for density labeling. Analysis of the reaction product in neutral CsCl gradients showed that approximately 86% of the added template, ^{3}H-labeled λ dv DNA, was converted to a half-heavy molecule. ^{32}P-Labeled fully heavy DNA did not appear (data not shown). The half-heavy DNA consisted of a ^{32}P-labeled heavy strand and a ^{3}H-labeled light strand. These results indicate that the DNA synthesis represents the replication of λ dv plasmid. Most of the template DNA replicated within 30 min.

Initiation Site of λ dv Replication. In order to investigate the initiation site of λ dv DNA, we tried the dideoxy CTP (ddCTP) method which blocks chain elongation at an early phase of replication (27). λ dv DNA was synthesized in the presence of [α-^{32}P]dCTP and ddCTP. The DNA was purified, cleaved with *Dde* I or *Dde* I/*Eco*RI, and then analyzed by electrophoresis in polyacrylamide gel. The result of autoradiography is shown in Fig. 3 and the fragment locations on λ dv are shown in Fig. 4. With 25 μM ddCTP, the isotope was incorporated almost uniformly into each fragment. However, with 100 μM ddCTP, some fragments were labeled specifically. Relative radioactivities among these fragments were measured by scanning the intensity of radioactivity, dividing by the number of nucleotides, and normalizing as described in the legend to Fig. 4.

The results clearly demonstrate that some fragments located in the *O* gene are preferentially labeled at 100 μM ddCTP. Because DNA elongation is halted randomly by ddCTP, the relative amount of a fragment should reflect its distance from the starting point of DNA synthesis. Thus, we can conclude that DNA synthesis of λ dv starts at a point in or very close to the 112-bp fragment which is located left of the four direct repeats that define λ *ori* (13, 14, 29, 30). It is not possible to pinpoint the position of initiation of DNA synthesis through analyses of incorporation into restriction fragments. Analyses of primer RNA and polarity of newly synthesized DNA strands are necessary in future studies. The gradual decreases of radioactivity on both sides from the starting point indicate that the DNA synthesis proceeds bidirectionally. However, the relative radioactivity was markedly low in the 801-bp fragment which covers the *P* gene. Thus, the bidirectional synthesis is asymmetric. The

Table 2. Structures of λ *ori* fragment containing hybrid plasmids and their activities in the replication system *in vitro*

Plasmid	Structure of cloned λ fragment	DNA synthesis, pmol: Without O and P	DNA synthesis, pmol: With O and P
pOA-4		74.4	150.4
pOri-D1		80.8	89.6
pOri-1		58.4	134.4
pBR322		72.0	80.8
λ dv021		1.1	248.0

λ fragments as indicated were inserted in pBR322 by substitution in the *Bam*HI-*Eco*RI region. A, B, and E represent cutting sites of *Alu* I, *Bgl* II, and *Eco*RI, respectively, on the λ fragment. Numbers at cleavage sites are the distance in nucleotides from the transcriptional start point (*pR*). DNA structures of *ice*, *oop*RNA-encoding region, and four direct repeats are shown as ○, ◁◁, and ▮▮▮, respectively. DNA synthesis was measured with 8 μg of each plasmid DNA per ml in the presence or absence of O and P proteins. Results are expressed as total dNMP incorporated.

significance of this behavior in comparison with *in vivo* replication of λ is not clear at present.

Minimal DNA Structure for Initiation of λ dv Replication. To narrow down the essential region for replication, we took the following pBR322-based recombinant plasmids that carry part or all of the replication origin: pOri-1, carrying the complete *ori* sequence (the four repeats and A+T-rich region); pOA-4, the *ice* and *oop*RNA regions in addition to the pOri-1; pOri-D1, similar to pOA-4 but lacks the A+T-rich region. pBR322 was used as a control. These DNAs were added to the *in vitro* replication system in the presence or absence of O and P proteins. The results are summarized in Table 2. Without O and P proteins, λ dv was not able to synthesize DNA, but the pBR322-based recombinant plasmids allowed incorporation to some extent. Addition of O and P proteins stimulated DNA synthesis in the cases of pOA-4 and pOri-1 but not with pBR322 or pOri-D1.

The initiation position of these plasmids was assayed by using the ddCTP method as in Fig. 3 (Figs. 5 and 6). The DNA synthesis of pOA-4 and pOri-1 was initiated from a position near the four direct repeats, as observed with intact λ dv. In both cases when 100 μM ddCTP was used, DNA synthesis initiated from the pBR322 origin was minimal, if there was any. On the other hand, pOri-D1 initiated its DNA synthesis at low ddCTP concentration apparently from the pBR322 origin (31) because its profile was the same as that of pBR322. These results demonstrate that a minimal λ structure necessary for the O,P-dependent initiation of replication resides in the 350-bp fragment of pOri-1 that carries the four direct repeats of λ *ori* along with an A+T-rich stretch. Deletion of a 46-bp A+T-rich region abolished the initiation ability, even though the remaining region retained the ability to bind O protein (unpublished data).

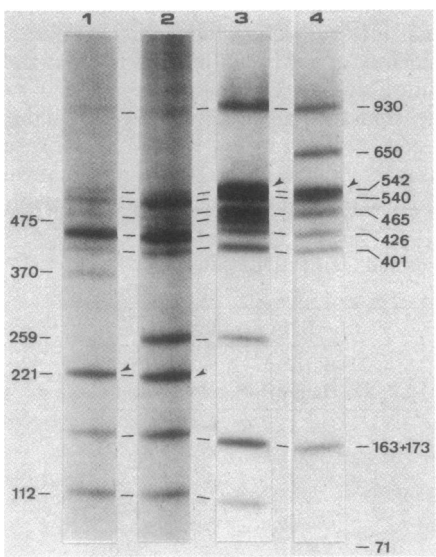


FIG. 5. Initiation site of DNA synthesis in recombinant plasmids carrying various λ fragments around *ori*. Plasmid DNAs (8 μg/ml) were incubated in 25-μl standard reaction mixture containing 100 μM (for pOri-1 and pOA-4) or 25 μM (for pOri-D1 and pBR322) ddCTP. Different concentrations of ddCTP were used because the optimal conditions for using ddCTP were different among plasmids used here. DNAs were treated as in Fig. 3, except that they were digested with *Dde* I/*Sal* I (lanes 1 and 2) or *Dde* I/*Eco*RI/*Sal* I (lanes 3 and 4). The digests were electrophoresed and autoradiographed. Lanes 1 and 2 and lanes 3 and 4 are the results obtained from independent electrophoreses. Lanes: 1, pOri-1; 2, pOA-4; 3, pOri-D1; 4, pBR322. Arrowheads, most highly labeled fragments; numbers, size of the fragments in bp. Bands 173 and 163 overlap in all the samples; in addition, bands 112 and 105 overlap in lane 3.

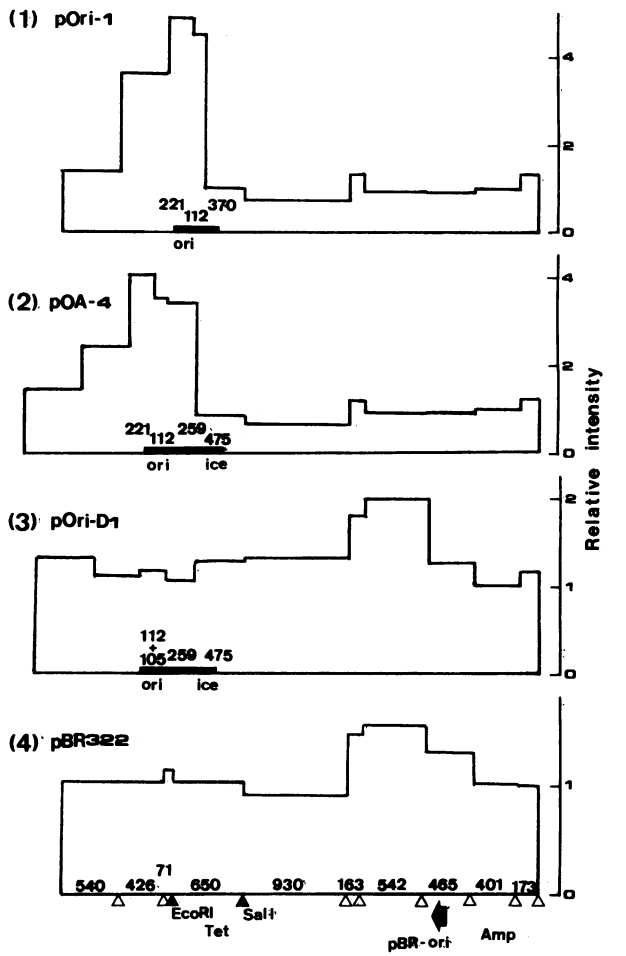


FIG. 6. Relative intensities of radioactivity calculated from Fig. 5. On the restriction map of pBR322, △ is *Dde* I and ▲ is *Sal* I and *Eco*RI cleavage sites; the origin region of pBR322 and the direction of its replication (arrow) (taken from ref. 31), and locations of tetracycline (Tet) and ampicillin (Amp) resistance genes are shown. The regions corresponding to the inserted λ fragments are indicated by thick lines. The positions of *ice* and *ori* are shown under these lines. For cleavage sites in λ DNA, see Fig. 3. Locations of the fragments in Fig. 5 are indicated by the numbers.

DISCUSSION

We described an *in vitro* system in which λ dv DNA replicates semiconservatively in the presence of purified O and P proteins. These two proteins, which have been characterized as positively acting initiators for λ replication *in vivo*, have now been shown to play the same role *in vitro*.

Most of the replication products were in open circular forms, in contrast to other systems which allow production of covalently closed circular DNA in similar quantities (17–19). This may indicate low activities of DNA ligase or DNA gyrase. In our *in vitro* replication system, most of the template DNA (about 85%) underwent a round of replication. No second round of replication was detectable.

In our system, an inhibitor of RNA polymerase was effective for DNA replication only when it was added prior to the start of DNA synthesis; it had no effect when added at or after the start. On the other hand, the same inhibitor was effective for pBR322 DNA replication, even when it was added after start of the DNA synthesis. Replication of this plasmid is known to depend upon RNA polymerase-dependent primer RNA synthesis. Apparently, RNA synthesis necessary for replication of λ dv, and pBR322 is different. There is a possibility that, in the

case of λ dv, the RNA synthesis is not directly involved in the step of primer RNA synthesis. pOA-4 and pOri-1 can carry out O,P-dependent replication, although they lack the λ promoter (*p*R), eliminating normal transcription that proceeds toward the λ *ori* region. Whether transcription into this region occurs from plasmid promoters remains to be determined.

Our studies have localized the essential DNA structure for λ replication within a 350-bp region. The initiation point of λ dv replication has also been located within the same region, near or within the four direct repeats. Note that this is the site of O protein binding (4). The DNA synthesis is likely to proceed bidirectionally. An A+T-rich region next to the repeats was found to be essential for replication *in vitro*. This A+T-rich region is similar in sequence to the priming site of G4 phage (32). In this region of λ are located several *cis*-acting *ori*$^-$ mutations (29, 30). Because the four repeats without A+T-rich sequences still retain ability to bind O protein, the A+T-rich region could function to interact with other elements such as P protein, dnaB, dnaG, etc.

Lusky and Hobom (15, 16) have argued that a region called *ice* takes part in the initiation of DNA synthesis. Our studies have shown that this region can be deleted without impairing O,P-dependent replication ability, and the *ice* region does not initiate the replication, at least in this *in vitro* system. For these analyses, we used hybrid plasmids carrying a pBR322 origin and λ fragments under conditions such that the vector origin is also active. Therefore their λ *ori* activities were expressed as the DNA synthesis additive to the vector pBR322 origin activity. However, the results shown here clearly demonstrate that the λ *ori* in the pOA-4 and pOri-1 can function. These recombinant plasmids initiate their replication from the same position as seen with λ dv.

This work was supported by Scientific Research Grants from the Ministry of Education, Science and Culture of Japan.

1. Skalka, A. M. (1977) *Curr. Top. Microbiol. Immunol.* **78**, 201–237.
2. Ogawa, T. & Tomizawa, J. (1968) *J. Mol. Biol.* **38**, 217–225.
3. Tsurimoto, T. & Matsubara, K. (1981) *Mol. Gen. Genet.* **181**, 325–331.
4. Tsurimoto, T. & Matsubara, K. (1981) *Nucleic Acids Res.* **9**, 1789–1799.
5. Tsurimoto, T., Hase, T., Matsubara, H. & Matsubara, K. (1982) *Mol. Gen. Genet.* **187**, 79–86.
6. Wickner, S. H. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 303–310.
7. Klinkert, J. & Klein, A. (1979) *Mol. Gen. Genet.* **171**, 219–227.
8. Tomizawa, J. (1971) in *The Bacteriophage Lambda*, ed. Hershey, A. D. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 549–552.
9. Furth, M. E., McLeester, C. & Dove, W. F. (1978) *J. Mol. Biol.* **126**, 195–225.
10. Furth, M. E., Yates, J. L. & Dove, W. F. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 147–153.
11. Kornberg, A. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 1–9.
12. Schnös, M. & Inman, R. B. (1970) *J. Mol. Biol.* **51**, 61–73.
13. Rambach, A. (1973) *Virology* **54**, 270–277.
14. Dove, W. F., Inokuchi, H. & Stevens, W. F. (1971) in *The Bacteriophage Lambda*, ed. Hershey, A. D. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 747–771.
15. Lusky, M. & Hobom, G. (1979) *Gene* **6**, 137–172.
16. Hobom, G., Grosschedl, R., Lusky, M., Scherer, G., Schwartz, E. & Kössel, H. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 165–178.
17. Sakakibara, Y. & Tomizawa, J.-I. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 802–806.
18. Inuzuka, M. & Helinski, R. (1978) *Biochemistry* **17**, 2567–2573.
19. Diaz, R., Nordström, K. & Staudenbauer, W. L. (1981) *Nature (London)* **289**, 326–328.
20. Fuller, R. S., Kaguni, J. M. & Kornberg, A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7370–7374.
21. Oka, A., Sugimoto, K., Takanami, K. & Hirota, Y. (1980) *Mol. Gen. Genet.* **178**, 9–20.
22. Matsubara, K. (1974) *J. Virol.* **13**, 596–602.
23. Chow, L. T., Davidson, N. & Berg, D. (1974) *J. Mol. Biol.* **86**, 69–89.
24. Tsurimoto, T. & Matsubara, K. (1981) in *The Initiation of DNA Replication*, ICN–UCLA Symposia on Molecular and Cellular Biology, ed. Ray, D. S. (Academic, New York), Vol. 22, pp. 263–275.
25. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Grosa, J. H. & Falkow, S. (1977) *Gene* **2**, 95–113.
26. Matsubara, K. (1976) *J. Mol. Biol.* **102**, 427–439.
27. Conrad, S. E., Wold, M. & Campbell, J. L. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 736–740.
28. Szybalski, E. H. & Szybalski, W. (1979) *Gene* **7**, 217–270.
29. Denniston-Thompson, K., Moore, D. D., Kruger, K. E., Furth, M. E. & Blattner, F. R. (1977) *Science* **198**, 1051–1056.
30. Scherer, G. (1978) *Nucleic Acids Res.* **5**, 3141–3156.
31. Sutcliffe, J. G. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 77–90.
32. Fiddes, J. C., Barrell, B. G. & Godson, N. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1081–1085.