Proc. Natl. Acad. Sci. USA
Vol. 77, No. 11, pp. 6744–6748, November 1980
Genetics

Initiation of DNA replication in a ColE1-type plasmid: Isolation of mutations in the *ori* region

(λ-mini-ColE1 hybrid phage/DNA sequence/revertants/hairpin structure)

SATOSHI NAITO AND HISAO UCHIDA

Institute of Medical Science, University of Tokyo, P. O. Takanawa, Tokyo 108, Japan

Communicated by Arthur Kornberg, August 14, 1980

ABSTRACT **We have constructed a plaque-forming hybrid phage, λSN4, which behaves as a composite replicon of the λ phage and a mini-ColE1 plasmid. From the hybrid phage, plaque-type mutants altered in the ability to replicate as a ColE1 replicon were isolated. These mutations were designated as *cer*, signifying ColE1 replication defective. One of such mutants, λSN4*cer6*, was studied further. The mutant DNA was unable to replicate *in vivo* if expression and function of its λ replicon were inhibited. The defect could not be complemented in *trans*. DNA sequence determination of the mutant phage revealed a single base pair (bp) alteration, C·G to T·A, at 160 bp upstream from the *ori* site of its ColE1 replicon. From λSN4*cer6*, revertants were obtained that had regained function of the ColE1 replicon, and they could be classified into two groups that showed a full and a partial recovery in the rates of ColE1-driven DNA synthesis. DNA sequence determination of revertant DNA indicated that the former group contained true revertants, T·A to C·G, at the *cer6* site, whereas one of the partial revertants was found to sustain a secondary-site mutation, G·C to A·T, at 187 bp upstream of the *ori* site. It was possible to construct a hairpin structure that starts by hydrogen bonding of bases at the sites −160 and −187.**

The ColE1 DNA is a plasmid of *Escherichia coli* with a molecular weight of approximately 4.2×10^6 (1, 2). The pAO3 is a small segment of the ColE1 plasmid, and its entire nucleotide sequence has been determined (3). It replicates autonomously like the original ColE1 and confers immunity to colicin E1 protein on its host cell. The segment contains the unique origin of ColE1 replication, which has been defined as the position (hereafter referred to as *ori*) at which the first deoxyribonucleotide is incorporated (4). A 436-base-pair (bp) fragment of pAO3 containing the *ori* site has been reported to carry essential information for its autonomous replication in a bacterial cell (3). For pMB1, a plasmid structurally and functionally similar to ColE1 (5), the essential region required for its replication and maintenance in bacteria has been estimated to contain no more than 580 bp (6). However, exact functions of these essential regions are unknown except that they contain the *ori* site. In order to clarify structure and functional relationships of DNA signals operating to initiate DNA replication, isolation of pertinent mutations and identification of their base changes are necessary. In this communication, we report construction of a λ-pAO3 hybrid phage, a systematic isolation of such mutations in the pAO3 segment of the phage, and a preliminary characterization of one of the mutants. We also will describe a selection and a classification of revertants of the defective pAO3 replicon and characterization by DNA sequence determination of a few such mutations.

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

Bacteria, Plasmid, and Bacteriophages. The *E. coli* K-12 strains used and their relevant genetic characteristics were as follows: 594 (*sup0*) (7); C600 (*supE44*) (8); P3478 (*sup0 polA1*) (9); 594[RI-1] (10); K802 (*sup0 grpA80*) (11); W3110Thy$^-$ (*sup0 thy*) (12); and SN27 (*supE44 polA11 ilv trp str*^{r}), which was prepared by P1 transduction of *supE44* into N611 (*polA11 ilv trp gal str*^{r}) (13). The plasmid pAO3 (3) was a gift from M. Takanami. The phage strains used were: λVIII, which was supplied by K. Murray and carrys a single *Eco*RI site (srI2) and an *amber* mutation in the *cI* gene (14); λ*ind*$^-$ (15); and λ*cI71* (16).

Media and Buffers. TY broth contained 1% tryptone (Difco), 0.1% yeast extract (Difco) and 0.8% NaCl. TY/Glc and TY/maltose were TY broth supplemented with 0.1% glucose and 0.2% maltose, respectively. TY/Glc agar was prepared by adding 1% agar to TY/Glc broth. Casamino acids medium contained 1% casamino acids (vitamin-free, Difco), 0.25% NaCl, and 1 μg of thiamine chloride (pH 7.0) per ml. TM buffer was 10 mM Tris·HCl (pH 7.4) containing 10 mM $MgSO_4$.

Reagents. Sources of enzymes used were Bethesda Research Laboratories, Rockville, MD (*Hin*dIII, *Hae* II, and *Hin*fI); Boehringer Mannheim (*Eco*RI, *Alu* I, *Hap* II, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and T4 polynucleotide kinase); Worthington [alkaline phosphatase (*E. coli*)]; and Takara Shuzo, Kyoto, Japan (*Eco*RI). T4 ligase was a gift from M. Takanami. 5-[6-^{3}H]Bromouracil, [6-^{3}H]thymine, and inorganic ^{32}P were purchased from New England Nuclear. [6-^{14}C]Thymine was obtained from Daiichi Pure Chemicals, Tokyo, Japan.

Construction of a λ-pAO3 Hybrid Phage. The pAO3 plasmid and λVIII DNA (3, 5) were digested with *Eco*RI endonuclease, mixed, and ligated by T4 ligase. The mixture was used to transfect Ca^{2+}-treated C600 cells. When the transfectants were plated on C600, two classes of plaques were obtained: one showed turbid plaques characteristic of a lysogenic phage, and the other showed more transparent but slightly turbid plaques. Lysogenic bacteria obtained from the turbid plaques failed to show immunity to colicin E1 (67/67), and they were considered to be lysogenic with the vector phage λVIII. On the other hand, lysogenic bacteria obtained from the "slightly turbid" class always exhibited immunity to colicin E1 (97/97). One of such plaques was selected, and the phage recovered from it was named "λSN4". Plating efficiency of λSN4 on 594[RI-1] indicated that the phage has two *Eco*RI sites. *Hin*dIII endonuclease treatment of λSN4 DNA produced a novel 8.0%

Abbreviations: moi, multiplicity of infections; bp, base pair(s); TY broth, 1% tryptone/0.1% yeast extract/0.8% NaCl; TM buffer, 10 mM Tris·HCl, pH 7.4/10 mM $MgSO_4$.

λ fragment in place of the 4.6% λ *Hin*dIII fragment produced by λVIII, indicating that λSN4 DNA contains a single copy of pAO3 at the srI2 *Eco*RI site.

Detection of Phage DNA Synthesis. *Density-shift experiments.* Procedures used for preparation of labeled phages were as described (11), except that 5-[6-^{3}H]bromouracil was used in the present experiment instead of [^{3}H]thymine. Phages with a specific activity of 4000 dpm per 10^9 particles having a buoyant density of 1.54 were obtained. Analysis of intracellular phage DNA has been described (11).

[^{3}H]Thymine incorporation in the presence of chloramphenicol. Procedures which have been published (17) were generally followed with minor modifications: W3110Thy$^-$ was grown to 5×10^8 cells per ml in casamino acids medium supplemented with 5 μg of thymine per ml and 0.2% maltose. Then, 250 μg of chloramphenicol per ml was added, and aeration at 37°C was continued for 2.5 hr, when bacterial DNA synthesis was almost ceased. Cells were pelleted by centrifugation and resuspended in 0.5 vol of TM buffer containing chloramphenicol (250 μg/ml). Phages were added at a multiplicity of infection (moi) of 6, and adsorption was allowed to take place for 20 min at 37°C. Infected cells were then diluted 1:10 at time zero into prewarmed casamino acids medium supplemented with thymine (5 μg/ml), 0.1% glucose, chloramphenicol (250 μg/ml), and [^{3}H]thymine (5 μCi/ml; 1 Ci = 3.7×10^{10} becquerels). Samples were removed at intervals, and trichloroacetic acid-insoluble radioactivities were counted.

RESULTS

Properties of a λ-Mini ColE1 Hybrid Phage. The plasmid pAO3 is a small replicative element of 1683 bp derived from the ColE1 plasmid (3). λSN4 is a hybrid phage harboring one copy of pAO3 at the single *Eco*RI site (srI2) of a vector phage, λVIII (14). Construction of λSN4 *in vitro* was described in *Materials and Methods*. Plaques formed by λSN4 on a plate seeded with C600 bacteria sustaining *supE* suppressor were less turbid than those formed by the vector phage, λVIII (Fig. 1). (We will hereafter refer to the turbidity of λSN4 plaques on C600 as "slightly turbid".) The difference can be ascribed to the presence of *supE* in C600 and pAO3 within the genome of λSN4, because the phage forms clear plaques on 594*sup0* but turbid plaques on SN27 bacteria sustaining *supE* and *polA11* mutations; ColE1 replication requires DNA polymerase I coded by the *polA* gene (18).

λSN4 behaves as a composite replicon consisting of both λ and ColE1 replicational units. Thus, λSN4 forms a plaque on a plate seeded with *polA* mutant cells. On the other hand, λSN4 DNA is able to replicate when expression of λ replicon is inhibited, as evidenced by the following experiments: 594 cells lysogenic with λ*ind*$^-$, or nonlysogenic 594 cells in the presence of chloramphenicol, were infected with λSN4 labeled with 5-[^{3}H]bromouracil and incubated at 37°C for 20 min, and the DNA that was extracted from the infected cells was fractionated by a CsCl density gradient equilibrium centrifugation. The results (Fig. 2) indicate that λSN4 DNA replicates within a lysogenic cell harboring a homoimmune prophage or within a nonlysogenic cell in the presence of chloramphenicol. It has been demonstrated that proteins encoded by the ColE1 DNA are not required for its replication *in vitro* and *in vivo* (17, 19, 20), whereas replication of a λ replicon requires functions of the λ*O* and λ*P* genes, which do not express in a homoimmune cell or in the presence of chloramphenicol. λSN4 does not grow in *polA* cells lysogenic with *imm*λ prophage.

When λSN4 infects a C600 cell, the phage is able to integrate into the bacterial chromosome and produce active *imm*λ repressor. However, the cell will not survive probably because of

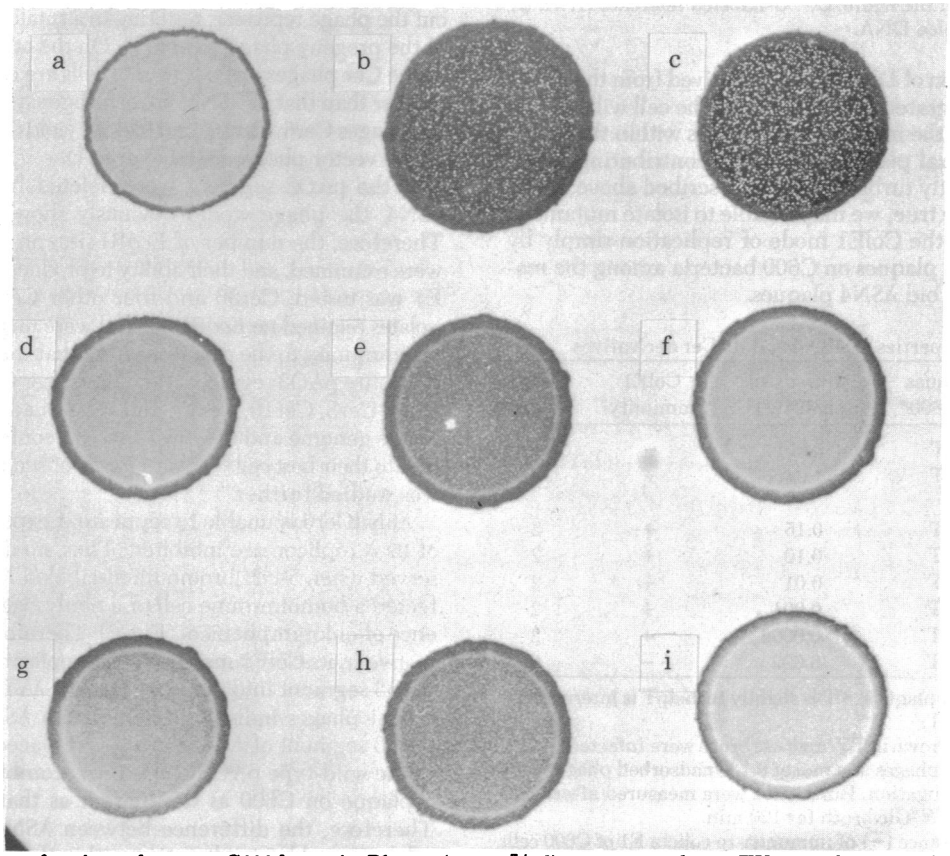

FIG. 1. Lysis zones of various phages on C600 bacteria. Phages (5×10^7/ml) were spotted on a TY agar plate containing 0.25% NaCl and seeded with C600. The plate was incubated overnight at 37°C. (*a*) λVIII; (*b*) λSN4; (*c*) λcI71; (*d*) λSN4Cer6; (*e*) λSN4Cer8; (*f*) λSN4Cer10; (*g*) λSN4Cer17; (*h*) λSN4Cer34; (*i*) λSN4Cer36.

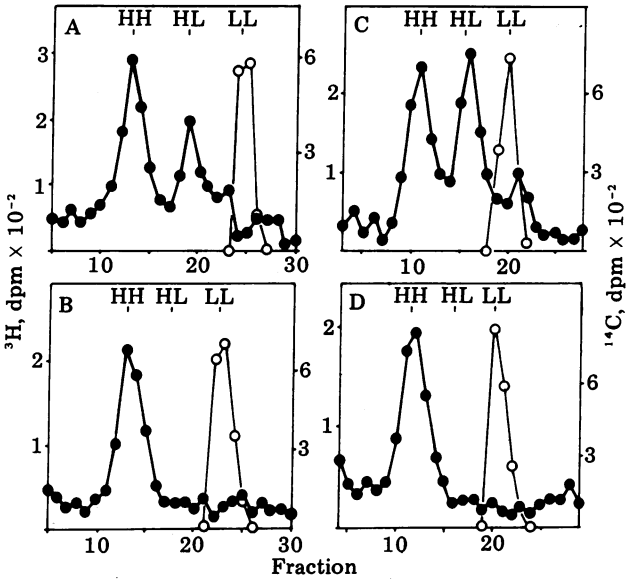

FIG. 2. Density-shift experiments. (*A*) 594(λ*ind*$^-$) cells were infected with 5-[6-^{3}H]bromouracil-labeled λSN4 at a moi of 1.5 phages per cell. After incubation for 20 min at 37°C, cells were collected by centrifugation, and DNA was extracted. (*B*) The same as *A* except that the labeled phage used was λSN4Cer6. (*C*) Cells (594) were incubated with chloramphenicol (50 μg/ml) for 20 min at 37°C, and infected with 5-[6-^{3}H]bromouracil-labeled λSN4 at a moi of 1.5 per cell. Further incubation was continued for 20 min at 37°C in the presence of chloramphenicol (50 μg/ml), and DNA was extracted. (*D*) The same as *C* except that the labeled phage used was λSN4Cer6. Each DNA sample was mixed with an appropriate amount of [^{14}C]-thymine-labeled light λ phage DNA and was analyzed by CsCl density gradient centrifugation using a Spinco SW50.1 rotor at 32,000 rpm for 44 hr at 20°C. The average density was adjusted to 1.750 g/cm^3. The positions of fully heavy (HH), half-heavy (HL), and light (LL) DNAs are indicated in the figure. ○, ^{14}C-Labeled reference DNA; ●, [^{3}H]bromouracil-labeled DNA.

the aberrant initiation of DNA synthesis derived from the pAO3 replicon of the integrated phage (11, 21). The cell will survive and multiply when the infecting λSN4 exists within the cell in an extrachromosomal plasmid state (20), contributing to the formation of "slightly turbid" plaques described above. If the interpretation were true, we may be able to isolate mutants of λSN4 defective in the ColE1 mode of replication simply by choosing for turbid plaques on C600 bacteria among the majority of slightly turbid λSN4 plaques.

Table 1. Properties of λSN4 and its Cer derivatives

Phage	Plaques on C600*	Burst size on 594(λ)†	ColE1 immunity‡	*Eco*RI sites§
λVIII	TT	0.001	−	1
λSN4	<T	1.04	+	2
λSN4				
Cer34	T	0.15	+	2
Cer8	T	0.10	+	2
Cer17	TT	0.01	+	2
Cer10	TT	0.001	+	2
Cer6	TT	0.0006	+	2
Cer36	TT	0.002	−	0

* TT indicates turbid plaques; <T is slightly turbid; T is intermediate between TT and <T.

† Cells (594; λ*ind*$^-$) grown in TY/maltose broth were infected in TM buffer with various phages at a moi of 0.1. Unadsorbed phages were removed by centrifugation. Burst sizes were measured after incubation at 37°C in TY/Glc broth for 120 min.

‡ Presence (+) or absence (−) of immunity to colicin E1 of C600 cells lysogenic with indicated phages.

§ Number of *Eco*RI sites as estimated by plating efficiency of phages on 594[RI-1] bacteria.

Table 2. Mixed infection of 594(λ*ind*$^-$) cells by λSN4 and λSN4Cer6

Infecting phages	moi	Phage yields per cell λSN4	Phage yields per cell λSN4Cer6
λSN4	5	3.6	—
	10	3.4	—
λSN4Cer6	5	—	0.05
	10	—	0.02
λSN4 plus λSN4Cer6	5	4.99	0.11

λSN4 and λSN4Cer6 phages were individually or as a mixture infected to 594(λ*ind*$^-$) at moi as indicated. Phage growth was stopped at 180 min, and progeny phages were plated on C600. λSN4 and λSN4Cer6 form slightly turbid and turbid plaques, respectively.

Isolation of Mutants Defective in ColE1 Mode of DNA Replication. By visual inspection, 24 turbid plaques were found among 1.2 × 10^6 plaques formed by plating a λSN4 stock on C600. The turbid character was named "Cer" for ColE1 replication defective, and the mutation responsible for the Cer character was designated as "*cer*". Each isolate was referred to by the isolate number. Although no mutagen was used in the isolation, the occurrence of the Cer phages was rather frequent, suggesting that they were derived from λSN4 by single mutations. As shown in Fig. 1, turbidities of lysis zones formed by different Cer phages on C600 lawn were not the same; Cer6, Cer10, Cer17, and Cer36 were as turbid as the vector phage, λVIII, but others were more transparent. Nevertheless, all of them formed clear plaques on 594 bacteria because the *cI* gene of these phages sustain an *amber* mutation.

Since λSN4 DNA is able to replicate in a 594 cell lysogenic with *imm*λ prophage, the phage DNA will eventually titrate out the phage repressor, resulting in a small but significant burst of the progeny phages (Table 1). On the other hand, burst sizes of the Cer phages on 594(λ*ind*$^-$) cells are expected to be much smaller than that of λSN4, if Cer phages are unable to replicate; for phages Cer6, Cer10, and Cer36, yields were as small as that of the vector phage, λVIII (Table 1).

If the pAO3 segment were deleted from the genome of λSN4, the phage would obviously show the Cer character. Therefore, the number of *Eco*RI sites on Cer phage genomes were examined, and their ability to produce immunity to colicin E1 was tested; Cer36 and four other Cer phages among 24 isolates retained no *Eco*RI site and were unable to confer colicin E1 immunity to the cell, indicating that they had probably lost the entire pAO3 segment. Whereas, other Cer phages including Cer6, Cer8, Cer10, Cer17, and Cer34 had two *Eco*RI sites per phage genome and they were able to confer colicin E1 immunity to their host cells (Table 1). One of such phages, λSN4Cer6, was studied further.

λSN4Cer6 is unable to replicate if expression and function of its λ replicon are inhibited. Thus, no density-shift was observed when 5-[^{3}H]bromouracil-labeled λSN4Cer6 phage infected a homoimmune cell or a nonlysogenic cell in the presence of chloramphenicol (Fig. 2). Therefore, λSN4Cer6 is defective in its ColE1 mode of DNA replication. Recloning of its pAO3 segment into the *Eco*RI site of λVIII produced recombinant phages indistinguishable from λSN4Cer6. When the pAO3 segment of λSN4Cer6 was replaced *in vitro* by a copy of the wild-type pAO3 DNA, the recombinant phage formed a plaque on C600 as transparent as that formed by λSN4. Therefore, the difference between λSN4 and λSN4Cer6 is solely caused by an alteration within the pAO3 segment of the latter.

When 594(λ) cells were infected with a mixture of λSN4 and

λSN4Cer6 phages, the majority of the progeny phages formed "slightly turbid" plaques on C600, indicating that the defect in λSN4Cer6 could not be complemented in *trans* by superinfecting λSN4 phages (Table 2). A slight increase of λSN4Cer6 observed in the results may be explained by a delayed replication of the λSN4Cer6 genome, which is possible after titration of the phage repressor by multiplied copies of the λSN4 DNA. If the site altered by *cer6* is needed in *cis* for the ColE1 mode of DNA replication, we may suppose that the alteration has occurred within the essential segment of pAO3 needed for its autonomous replication.

Sequence Analysis of the pAO3cer6. It has been noted that the 436-bp fragment of ColE1, though it is capable of autonomous replication, was less stable than longer fragments or the whole of the ColE1 plasmid (3). For pMB1, a plasmid structurally and functionally very similar to ColE1 (5), the essential region required for its replication and maintenance in bacteria has been estimated to contain no more than 580 bp (6). The 580-bp sequence is very homologous to the 436-bp sequence of ColE1, except that the latter is devoid of an adjacent region transcribing an RNA about 110 nucleotides in length (3, 6). Therefore, DNA sequence of a part of the pAO3 segment of the *cer6* mutant phage that corresponds to the 580-bp segment of pMB1 was determined by the method of Maxam and Gilbert (22). Strategy used is presented in Fig. 3, and the results indicated that the *cer6* alteration is a 1-bp change. Thus, at 160 bp upstream from the *ori* site, a C·G pair of pAO3 was changed to T·A in the *cer6* mutant. No other change was detected within a range of −570 to +20. As a control, we also have determined the sequence of a part of the parental λSN4 DNA, and we found that the site corresponding to the *cer6* was, in fact, a C·G pair as is in the wild-type pAO3.

Reversion of the Defective ColE1 Replicon. K802 cells sustaining a *grpA* mutation at the *dnaB* locus do not support replication of λ phages (11). On the other hand, the hybrid phage λSN4, harboring an extra ColE1 replicon (i.e., pAO3), forms plaques on K802 at a high efficiency of 0.8–1.0. In principle, therefore, revertants of λSN4*cer6* that have regained functions of the ColE1 replicon can be selected by plating λSN4Cer6 on K802. However, because certain *reg*-type mutants altered in the λ*P* gene are known to plate on K802 (11), this class of mutations has to be discriminated from those that occur within the pAO3 segment of the phage.

When a stock of λSN4Cer6 was plated on K802, spontaneous plaque formers were detectable at a frequency of about 1×10^{-7}. Fifty-three plaque formers were isolated and tested for plaque formation on 594(λ*cI857*) at 37.5°C. [At this temperature, λ*reg90* (11), λVIII, and λSN4*cer6* did not form plaques on the lysogen, whereas λSN4 formed normal-sized plaques at full efficiency.] Twelve isolates among 53 failed to form plaques, and they were discarded as *reg*-type mutants. The remaining 41 isolates were tentatively named λSN4*cer6*Rev, followed by the isolate number.

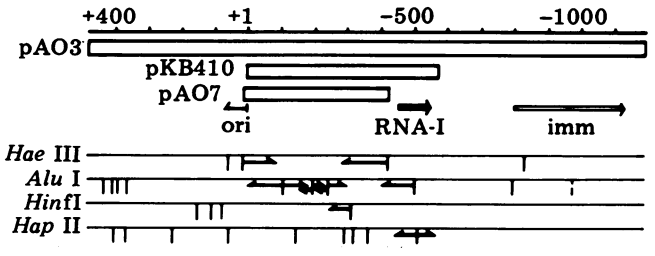

FIG. 3. Restriction enzyme maps of the pAO3 plasmid (3) and sequencing strategy employed to identify the *cer6* mutation. The arrows below each restriction map show the portions with sequences determined as described (22). Regions essential for replication of pKB410 (12) and pAO7 (3) are indicated. The nucleotides are numbered according to the position from the *ori* site.

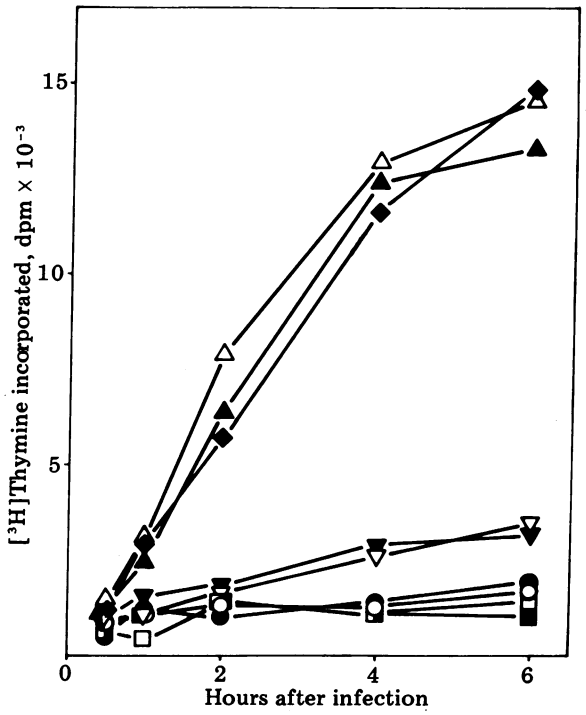

FIG. 4. [³H]Thymine incorporation in the presence of chloramphenicol by W3110Thy⁻ cells infected with various phages. ○, Uninfected cells; □, λVIII; ◆, λSN4; ●, λSN4*cer6*; ▲, λSN4*cer6*Rev3; △, λSN4*cer6*Rev31; ▽, λSN4*cer6*Rev11; ▼, λSN4*cer6*Rev16; ■, λSN4*cer6reg352*. (*reg352* is one of *reg*-type reversions. See text.)

These revertants could be classified into two groups; the first group to which 27 revertants belong showed lysis zones on C600 as transparent as that of λSN4. Thymine incorporation of cells infected in the presence of chloramphenicol by eight revertant strains randomly chosen from this group was measured individually. All of them showed DNA synthesis to the same extent as that observed for λSN4-infected cells (Fig. 4). The second group includes the remaining 14 revertants, which showed lysis zones on C600 with turbidities intermediate between those of λSN4 and λSN4*cer6*. Thymine incorporation in the presence of chloramphenicol was positive but very low as compared with that of λSN4 (Fig. 4). If we consider the first group as full revertants, the second group may be called partial revertants.

DNA sequences of typical revertants were determined by the method of Maxam and Gilbert (22). One of the full revertants, λSN4*cer6*Rev3, was a true revertant of the *cer6* mutation in the sense that the T·A pair of *cer6* at 160 bp upstream from the *ori* site was changed to a C·G pair, and this was the only change detectable within the range of −570 to +20. In another full revertant, λSN4*cer6*Rev31, the *cer6* mutation was also found to be mutated back to a C·G pair. On the other hand, in one of the partial revertants, λSN4*cer6*Rev16, the *cer6* mutation stayed unchanged, but there was a secondary-site mutation of G·C to A·T at 187 bp upstream from the *ori* site. No other alteration was found within the range of −570 to +20.

DISCUSSION

A ColE1 derivative, pAO3 (3), was joined *in vitro* to bacteriophage λVIII (14) to obtain a hybrid with both plasmid and phage properties. The entire nucleotide sequence of pAO3 has been published (3). From this hybrid phage, λSN4, a series of mutants defective in the ColE1 mode of DNA replication were isolated.

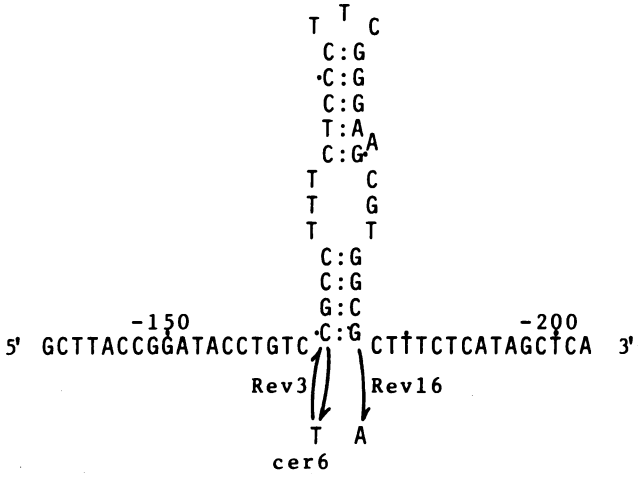

FIG. 5. Possible secondary structure of DNA near the *cer6* mutation.

To select such defective mutants, we have utilized a distinct property of the λSN4: it forms on C600 indicator bacteria a plaque less turbid than that of the vector phage (Fig. 1). Defective mutants of λSN4 were detected as turbid plaque formers. The behavior of λSN4 is analogous to that of a clear mutant, λ*c17* (23). The *c17* mutation creates a new promoter downstream to the termination site, t_{R1}, and λ genes *O* and *P* needed for initiation of λDNA synthesis are expressed constitutively from the new promoter. When λ*c17* is integrated into the bacterial chromosome, the cell will be killed by an aberrant DNA synthesis initiated within the phage genome, which explains the formation of a clear plaque by the phage. Since λSN4 is able to exist and multiply in a plasmid state (20), the plaque formed by λSN4 is slightly turbid.

Hybrid lambdoid phages containing DNA of ColE1-type plasmids have been described by Windass and Brammer (24). They observed that aberrant immunity behavior, giving clear plaques under conditions where the parental phage give turbid ones and being able to grow on homoimmune lysogens, was characteristic of phages containing both *imm21* and *ori*ColE1 and that it was not displayed by comparable *imm*λ derivatives. Because vector phages they have employed did not have the *att* site, at least some of the phenomena they have observed are different from what we are describing here.

Defective mutations we isolated were designated as *cer*, signifying ColE1 replication defective. Only one of them, *cer6*, was characterized in some detail, but it may be noted that there were many others exhibiting different degrees of defective replication (Table 1). λSN4*cer6* showed the smallest burst size among isolates when infected on 594(λ*ind*$^-$) bacteria. It did not replicate if expression and function of its λ replicon was inhibited (Figs. 2 and 4). DNA sequence determination revealed a 1-bp change within the 580-bp segment that constitutes the essential region commonly required for replication of the ColE1 and related replicons. The alteration *cer6* was found to be located at 160 bp upstream from the *ori* site. Because back mutation of the *cer6* alteration to a C·G pair fully restored DNA replication in the presence of chloramphenicol, we may infer that the alteration at 160 bp upstream from the *ori* site was the cause of the defect in the *cer6* mutation.

As pointed out by other investigators (4, 25, 26), nucleotide sequences of ColE1 related plasmids are, except for one base pair, completely homologous to ColE1 for 190 nucleotides upstream from the *ori* site, suggesting that this region may play a critical role in replication of ColE1-type plasmid DNA. It is interesting to note that our *cer6* mutation has occurred within this region.

Construction of a possible hairpin structure that includes the site altered by the *cer6* mutation is shown in Fig. 5. Thus, the first C—G hydrogen bonding in this structure is broken by the *cer6* mutation, but the bond can be reformed in one of the partial revertants, λSN4*cer6*Rev16, by a secondary alteration of G to A at the site facing the *cer6* alteration. Although the hypothetical hairpin structure is depicted in Fig. 5 as a secondary structure within the DNA sequence, it is equally possible to conceive the same structure using the RNA transcript of the region. Because the *cer6* defect could not be complemented in *trans* (Table 2), a likely interpretation of the finding is that the altered bp was important in an exact disposition of a recognizing molecule on the DNA. If that is the case, the recognizing molecule acts as a positively controlling element, and the *cer6* site inclusive of its vicinity may function as the controlling site in DNA replication. Further characterization of the altered sites in other *cer* mutants described should help our understanding of the molecular mechanism of initiation of DNA replication in ColE1-type plasmids.

We are grateful to M. Takanami and T. Sekiya for advice in the determination of nucleotide sequences. A computer program used to find palindromic structures was assembled by Haruo Saito of our laboratory. This work was supported in part by a grant from the Ministry of Education, Science and Culture of the Japanese government.

1. Helinski, D. R. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 2026–2030.
2. Tomizawa, J. (1978) in *DNA Synthesis, Present and Future*, eds. Molineux, I. & Kohiyama, M. (Plenum, New York), pp. 797–826.
3. Oka, A., Nomura, N., Morita, M., Sugisaki, H., Sugimoto, K. & Takanami, M. (1979) *Mol. Gen. Genet.* **172**, 151–159.
4. Tomizawa, J., Ohmori, H. & Bird, R. E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1865–1869.
5. Betlach, M. C., Hershfield, V., Chow, L., Brown, W., Goodman, H. M. & Boyer, H. W. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 2037–2043.
6. Backman, K., Betlach, M., Boyer, H. W. & Yanofsky, S. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 69–76.
7. Campbell, A. (1965) *Virology* **27**, 329–339.
8. Appleyard, R. K. (1954) *Genetics* **39**, 440–452.
9. DeLucia, P. & Cairns, J. (1969) *Nature (London)* **224**, 1164–1166.
10. Tsujimoto, Y. & Ogawa, H. (1977) *Mol. Gen. Genet.* **150**, 221–223.
11. Saito, H. & Uchida, H. (1977) *J. Mol. Biol.* **113**, 1–25.
12. Bachmann, B. J. (1972) *Bacteriol. Rev.* **36**, 525–557.
13. Ogawa, H. (1970) *Mol. Gen. Genet.* **108**, 378–381.
14. Murray, N. E. & Murray, K. (1974) *Nature (London)* **251**, 476–481.
15. Jacob, F. & Campbell, A. (1959) *C. R. Hebd. Seances Acad. Sci.* **248**, 3219–3221.
16. Kaiser, A. D. (1957) *Virology* **3**, 42–61.
17. Kahn, M. & Helinski, D. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2200–2204.
18. Kingsbury, D. T. & Helinski, D. R. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1538–1544.
19. Tomizawa, J., Sakakibara, Y. & Kakefuda, T. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1050–1054.
20. Donoghue, D. J. & Sharp, P. A. (1978) *J. Bacteriol.* **133**, 1287–1294.
21. Eisen, H., Pereira da Silva, L. & Jacob, F. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33**, 755–764.
22. Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 560–564.
23. Packman, S. & Sly, W. S. (1968) *Virology* **34**, 778–789.
24. Windass, J. D. & Brammar, W. J. (1979) *Mol. Gen. Genet.* **172**, 329–337.
25. Bolivar, F., Betlach, M. C., Heyneker, H. L., Shine, J., Rodriguez, R. L. & Boyer, H. W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5265–5269.
26. Sutcliffe, J. G. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 77–90.