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Multiple initiation sites of DNA replication flanking the origin region of λdv genome

(mapping of RNA-DNA transition sites/*ori* sequence/λ phage/plasmid replication *in vitro*)

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ABSTRACT **Early replicative intermediates of λdv plasmid were prepared by an *in vitro* replication system in the presence of 2',3'-dideoxycytidine 5'-triphosphate, an inhibitor of DNA chain elongation. Short-chain DNAs produced from regions near the replication origin were purified from the intermediates. A fraction of the DNAs was covalently linked to primer RNA. The transition sites from primer RNA to DNA synthesis were mapped along the nucleotide sequence of the genome, by eliminating the RNA by alkaline hydrolysis and labeling the freshly exposed 5' ends of DNA with ^{32}P. The transition sites were found to be located on both sides of the *ori* region, which includes four 19-base-pair repeats where one of the λ specific initiator proteins, O, binds. No transition arose within the *ori* region. The transition sites are multiple on both sides of the *ori* region and are clustered in one of the two strands in such a way that DNA syntheses from the two sides converge. The frequency of the "leftward" DNA synthesis is several times higher than that of "rightward" synthesis, reflecting the asymmetric bidirectional replication of λdv DNA.**

The mechanism controlling initiation of DNA synthesis involves key reactions by which RNA primer synthesis is initiated and by which a transition is made from RNA to DNA synthesis. Several replicons, all of which are single-stranded phage genomes, have unveiled the key reactions (1). ColE1 plasmid is unusual among double-stranded replicons, and its key reactions are well understood (2, 3). It is not, however, certain that the general idea we now have of the key reactions of these replicons can be extended to the majority of bacterial plasmid replicons. We must ask for each individual plasmid replicon how and where the primer RNA is made and under what control mechanism.

We have been studying the replication of λdv plasmid, which has a double-stranded circular genome, in *Escherichia coli*. The genome and the sites related to replication are well understood (4). The replication initiation of this plasmid depends on two phage λ-encoded initiator proteins, O and P (5). The replication proceeds bidirectionally and is initiated at or around a unique DNA region (6, 7) called *ori* (for replication origin), which has been defined by some *cis*-dominant replication defective mutations (8, 9). The *ori* sequence is located at the center of the *O* gene (10, 11) and has four tandemly arranged 19-base-pair (bp) sequences (*ori* repeats), an A+T-rich segment reminiscent of the primer RNA coding sequence for replication initiation of bacteriophage G4 (12), and a 28-bp palindromic sequence that can form a stem-and-loop structure. The *ori* repeats are the sites of binding of one of the initiation factors, O protein (13). The roles of the other sequences are unknown.

An *in vitro* system has been developed that allows replication of λdv (14). In this system, any DNA that carries the λ DNA around *ori* can initiate a round of replication in the presence of *E. coli* extract (15) and λ-encoded O and P proteins. The DNA synthesis is initiated at or around the *ori* repeats (14). Further work has narrowed the λ DNA region essential for initiation to a 165-bp fragment from the *Ava* II site (position 983, as numbered in Fig. 1) to the *Eco*RI site (position 1147), just covering all of the sequence described above except for a part of the palindromic sequence. We define this essential region for the *in vitro* replication-initiation as the "*ori* region" (26). Its nucleotide sequence is shown in Fig. 4.

To study the first step of initiation of DNA synthesis, we attempted to locate the transition sites between primer RNA and DNA and to find in which direction the DNA synthesis

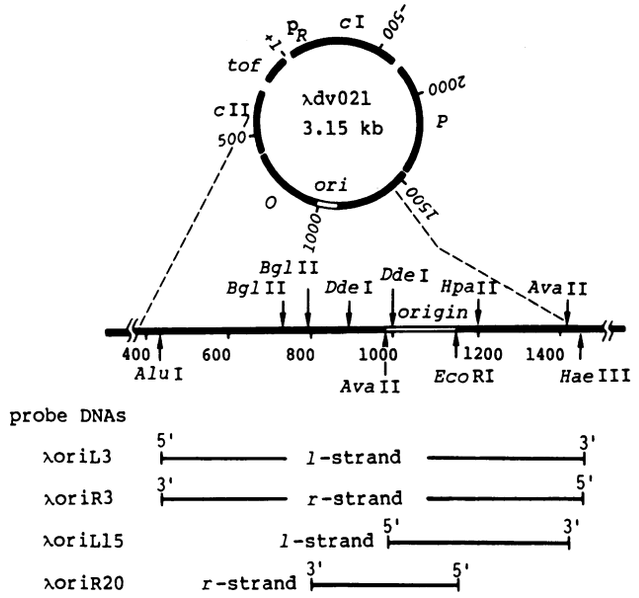

FIG. 1. Structure of the template DNA, λdv, and restriction sites in the relevant region of λ genome around the *ori* region (open box). Genes *cI*, *tof*, *cII*, *O*, and *P* are shown by thick lines. Numbers outside the circle indicate nucleotide numbers from the mRNA starting point (+1) of p_R. Cleavage sites by the restriction enzymes used in this study are shown. To purify short-chain DNAs produced from regions around λ *ori*, single-strand-specific probes were constructed by cloning part of λdv DNA in single-strand M13 phage vectors, mp8 and mp9 (16). Horizontal lines represent the DNA regions and strands cloned in M13 phage DNAs. λoriL3 and λoriR3 carry the sequences of *l* and *r* strands covering the *Alu* I (432) to *Hae* III (1439) region, respectively. λoriL15 carries the *l* strand spanning the two *Ava* II sites (983 to 1416). λoriR20 carries the *r* strand of the region *Bgl* II (793) to *Eco*RI (1147). These single-stranded phage DNAs were purified as described in ref. 17, except that BND-cellulose chromatography was omitted.

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Abbreviations: bp, base pair(s); kb, kilobase pair(s); ddCTP, 2',3'-dideoxycytidine 5'-triphosphate; BND-cellulose, benzoylated naphthoylated DEAE-cellulose.

proceeds. For this purpose, the earliest replicative intermediates of λdv were forcibly accumulated by addition of 2',3'-dideoxycytidine 5'-triphosphate (ddCTP) to the reaction mixture, and RNA-DNA joint molecules were isolated and analyzed. The results showed that λdv has multiple transition sites on both sides of the *ori* region, but no major transition sites within the *ori* region.

MATERIALS AND METHODS

Preparation of λdv Replicative Intermediate *in Vitro*. λdv021 (18) DNA [3.15 kilobase pairs (kb); see Fig. 1], here called λdv, was used as a template DNA in our *in vitro* replication system. The reaction conditions were essentially the same as described previously (14), except that 300 μl of reaction mixture contained 20 μg/ml of λdv DNA, 20 μM of each dNTP with 4.2 mCi/μmol of [*methyl*-^{3}H]TTP (New England Nuclear; 1 Ci = 37 GBq) and 40 μM ddCTP, and was incubated at 30°C for 10 min. A higher ddCTP-to-dCTP ratio (40:20) was adopted than previously (100:100; ref. 14) to minimize the possibility of excessive chain elongation that might lead to new Okazaki fragment synthesis for general chain elongation. During incubation, approximately 30% of the template DNAs initiated DNA synthesis (active templates), and short-chain DNAs of 60–160 nucleotides were synthesized. The reaction was stopped by addition of 100 μl of 0.5 M EDTA and 100 μl of 5% $NaDodSO_4$, followed by incubation at 37°C for 150 min with Pronase E (Kaken Kagaku, Tokyo, Japan) at 1 mg/ml. The DNA was extracted with phenol and chloroform (1:1) and chromatographed on Sepharose 2B (0.8 × 15 cm) with 10 mM Tris·HCl, pH 7.9/1 mM EDTA/50 mM NaCl/0.1% $NaDodSO_4$ (TENS). The DNA in the excluded fraction was precipitated with ethanol, dissolved in 0.2 ml of 10 mM Tris·HCl, pH 7.9/1 mM EDTA (TE), and applied to a column of 50 μl of benzoylated naphthoylated DEAE-cellulose (BND-cellulose; Boehringer Mannheim). The column was washed successively with 1 ml each of TE containing 0.3 M NaCl, TE containing 0.65 M NaCl, and TE containing 1 M NaCl and 2% caffeine. Approximately 50–60% of the ^{3}H incorporated during the incubation was recovered in the final caffeine fraction, which contained replicative intermediate DNAs as well as single-stranded DNAs (17, 19). The sample was then passed through a Sephadex G-100 column with TENS to remove caffeine.

Analysis of the Transition Sites. To locate the transition sites of primer RNA to DNA, the 5' ends of nascent DNA were determined as follows (20). The purified replicative intermediate of λdv DNA in 0.2 ml of TE was heated at 95°C for 3 min, then loaded on a Sepharose CL-4B (0.6 × 15 cm) column with TENs. Fractions with relative retention (K_d) values of 0.4 to 0.8, containing approximately 60% of applied ^{3}H, were pooled. In this fraction, short-chain DNAs of 60–160 nucleotides were recovered. The short-chain DNAs were precipitated with 70% (vol/vol) ethanol, and their 5' ends were masked by incubation with T4 polynucleotide kinase (P-L Biochemicals) at 50 units/ml in 200 μl of 60 mM Tris·HCl, pH 8.3/10 mM $MgCl_2$/12 mM 2-mercaptoethanol/0.1 mM ATP at 37°C for 30 min. (At this stage, the sample was estimated to contain about 0.7 pmol of short-chain DNA by phosphorylation with T4 polynucleotide kinase and [γ-^{32}P]ATP of the phosphatase-treated sample.) The masked DNA was precipitated with ethanol, dissolved in 150 μl of 0.15 M NaOH/4 mM EDTA, and incubated at 37°C for 14 hr to expose the 5'-OH group that had been covalently linked with primer RNA. The sample was neutralized and passed through a Sephadex G-100 column. The DNA was precipitated with ethanol, and the freshly exposed 5'-OH group was labeled by incubating with T4 polynucleotide kinase at 20 units/ml in 60 mM Tris·HCl, pH 8.3/10 mM $MgCl_2$/12 mM 2-mercaptoethanol/1.3 μM [γ-^{32}P]ATP (5000 Ci/mmol; Amersham) at 0°C for 60 min. Approximately 10% of total short pieces (0.07 pmol) were end labeled. Note that this figure does not represent recovery, but the fraction of DNA pieces that had been linked with primer RNA. The labeled DNA was divided into two equal portions, I and II, and one was mixed with 30 μg of λoriL3 DNA, the other with λoriR3 DNA (see Fig. 1). The DNA in each sample was precipitated with ethanol, denatured in 90 μl of TE containing 0.1% $NaDodSO_4$ at 95°C for 3 min, and renatured in TE containing 0.1% $NaDodSO_4$ and 0.5 M NaCl at 65°C for 6–14 hr (first hybridization). They were then chromatographed on Sepharose CL-4B (0.6 × 15 cm) as above and the nonrenatured, end-labeled short DNA chains with K_d values of 0.4–0.8 were pooled. Short pieces for *l*- or *r*-strand synthesis were enriched in these fractions. To each sample from I and II were added 30 μg of λoriR20 and λoriL15, respectively, as probes (see Fig. 1), and denaturing and renaturing were effected as above (second hybridization). To isolate renatured DNA after this treatment, the samples were applied on a Sepharose CL-4B column (0.6 × 15 cm), and DNAs in the excluded fractions were pooled. The labeled materials in I and II represent short-chain DNAs of *l* and *r* strands, respectively, newly synthesized at or around the λ *ori* region.

The hybrids of the short-chain DNAs and probe DNAs were digested with S1 nuclease at 10 units/ml in 0.5 ml of 50 mM sodium acetate buffer, pH 5.2/0.28 M NaCl/5 mM $ZnSO_4$ at 50°C for 30 min to remove the single-stranded region (21), and the mixture was passed through a Sephadex G-100 column. The excluded DNAs were pooled and digested with appropriate restriction enzymes. Through this series of treatments, about 3.0% and 10% of initially incorporated ^{32}P were recovered in I and II, respectively. Considering the loss of labeled DNA chains during two cycles of hybridization, we can argue that these recoveries are reasonable. The digested DNAs were electrophoresed in denaturing gels of 10% polyacrylamide in Tris borate buffer containing 8 M urea and were autoradiographed. The lengths of the fragments banded in the electrophoresis were determined from size markers run in parallel, and the locations of 5' ends of nascent DNA were estimated by their length and the position of the restriction enzyme cleavage site used for assay. The results were confirmed each time by using at least two different restriction enzymes. The size of most DNA fragments was determined to within two nucleotides. Since short-chain DNAs of 60–160 nucleotides were the major products under the conditions employed, no attempt was made to fractionate fragments of more than 160 nucleotides.

Determination of Nucleotide at Transition Site. See ref. 22. The DNA band was extracted from the autoradiographed gel, dissolved in 30 μl of 30 mM sodium acetate buffer, pH 5.2/0.1 mM $ZnSO_4$/nuclease P1 (Yamasa Shoyu, Choshi, Japan) at 0.1 mg/ml, and completely digested by incubating at 65°C for 3 hr. The 5'-end-labeled mononucleotides were identified on polyethyleneimine-cellulose plates (Polygram Cell 300 PEI, Macherey & Nagel) by successive use of 0.25 M formic acid and 0.5 M lithium formate buffer, pH 3.0, followed by autoradiography.

RESULTS

Transition Sites are Located Outside of the λ *ori* Region. As noted in the Introduction, a 165-bp segment of the λ genome carries all the DNA signals needed for O and P protein-dependent replication initiation *in vitro*. We define this region as the "λ *ori* region." We have shown previously that, when the chain-terminator ddCTP is administered, practically all DNA synthesis occurs in or around this region (14). This observation can be taken to imply that the majority of the short DNA fragments that accumulate in the presence of ddCTP

represent the earliest phase of replication. In this study, we employed an even higher concentration of ddCTP relative to dCTP (see *Materials and Methods*). In 300 μl of the assay mixture, 1.9 pmol of short-chain DNAs were produced from 0.9 pmol of active template; i.e., about two short-chain DNAs were formed per active template.

To eliminate the possible contamination of Okazaki fragments representing chain elongation at other positions of the plasmid DNA, two cycles of hybridization with appropriate probes were used to select only those fragments carrying the sequences of the *ori* region and its vicinity. Thus, the junction between primer RNA and short-chain DNA in our sample can be taken to represent the transition site(s) in the earliest phases of replication. The purified short-chain DNA was specifically labeled with ^{32}P at the 5′ ends of the DNA at the RNA-DNA junction (transition site; see *Materials and Methods*). Approximately 10% of total short-chain DNA molecules were end-labeled in those experiments. The remaining 90% of fragments were not labeled, indicating that they had cleaved off the primer RNA during *in vitro* replication or sample preparation.

From the end-labeled DNA pieces, the "*l*-strand pieces" were selected by hybridization with λoriR20 DNA (see Fig. 1). These pieces, in hybridized form, were treated with S1 nuclease to eliminate the single-stranded region, and the remaining double-stranded DNA was digested with *Eco*RI and electrophoresed. This test was designed to detect *l*-strand DNA with a transition site(s) within the *ori* region, since such DNA would show band(s) shorter than 165 nucleotides. However, no such discrete bands were detected (data not shown). To locate the transition sites outside of the *ori* region, the S1-treated sample was digested with *Dde* I or *Ava* II. The results in Fig. 2*a* demonstrate that transition sites are located to the left of the *ori* region, in a stretch from 793 to 1000. Many bands of various intensities were observed in both samples. Since the region analyzed carries two *Dde* I sites (887 and 1000), the transition sites were determined more precisely with the *Ava* II-digested sample. Three clusters of transition sites (Z, Y, and V) were identified within the region from 895 to 980. Z is composed of one strong and two weak bands, Y of two strong bands, and V of four strong and seven weak bands. In addition, five clusters of weak bands (x, w, s, t, and u) were noted. s and x overlap in lane 1.

We then examined end-labeled short-chain DNAs of *r*-strand specificity that had been hybridized with λoriL15 DNA and treated with S1 nuclease. The hybridized duplex DNAs were digested with *Dde* I. A band of 152–155 nucleotides appeared (data not shown), indicating that a transition site is located at around position 1155, to the right of the *ori* region (marked A in Figs. 3 and 4). No shorter bands representing *r*-strand transition sites within the *ori* region were observed. The same sample was digested with *Eco*RI or *Hpa* II (Fig. 2*b*), to locate precisely the transition sites lying between positions 1150 to 1416 to the right of the *ori* region. Seven major clusters (A, B, E, F, G, H, and I) of transition sites were observed. Each cluster appeared in ladders of 4–8 bands. Bands of weak transition sites (c, d, j, k, and l) were also visible. The weak ladders between G and H clusters and between H and I clusters (lanes 2 and 3) are background bands that cannot be eliminated in the assay system (see legend to Fig. 2).

Next, the transition sites were examined in a wider stretch of DNA covering about 1 kb around the *ori* region, using λoriL3 and λoriR3 as probes. No further strong transition sites were detected, although weak sites were found on both strands (data not shown: the results are incorporated in Fig. 3). Weak transition sites appeared in the *l* strand, dispersed through the regions covering 750–800 and 1230–1310. Similarly, weak transition sites were found to be clustered in the *r* strand in the region from 910 to 930.

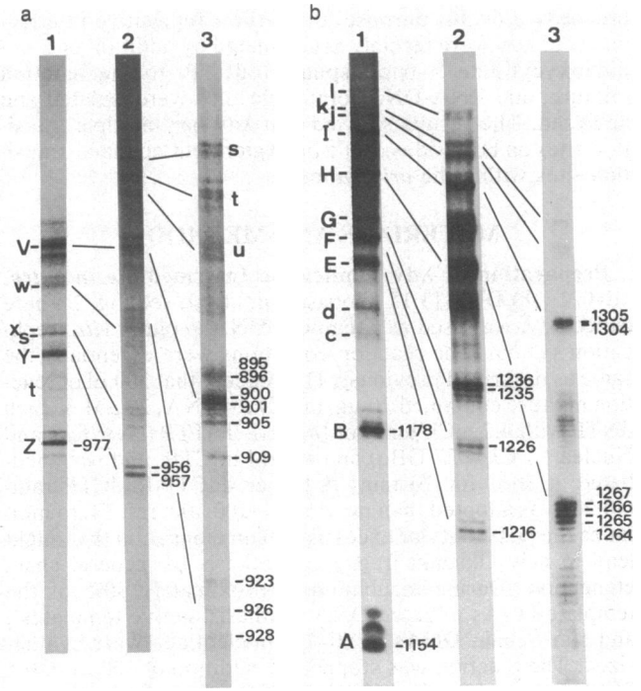

FIG. 2. Analysis of transition sites located to the left (*a*) and right (*b*) of the *ori* region. (*a*) Purified, end-labeled *l*-strand short-chain DNA, hybridized with λoriR20 and treated with S1 nuclease, was digested with *Dde* I (lane 1) and *Ava* II (lanes 2 and 3). The data are assembled from three independent electrophoreses. Lane 3 is the result of long electrophoresis of the same sample as in lane 2. Z, Y, and V are the major transition clusters that consist of strong bands and some flanking weak bands. x, w, u, t, and s are the minor transition sites. (*b*) Purified, end-labeled *r*-strand short-chain DNA, hybridized with λoriL15 and treated with S1 nuclease, was digested with *Eco*RI (lane 1) or *Hpa* II (lanes 2). Lane 3 is the result of longer electrophoresis of the same sample as in lane 2. Clusters of major transition sites are indicated as A, B, E, F, G, H, and I, and minor sites are indicated as c, d, j, k, and l. Corresponding bands are linked by lines. Numbers indicate the locations of the 5′ ends of the DNA pieces along the nucleotide sequence of λ (see Figs. 3 and 4). These locations were determined from the positions of restriction enzyme sites and the nucleotide lengths of bands measured by size markers. The *Hpa* II digest (lanes 2 and 3 in *b*) shows two ladders of background bands, which seem to have been produced by preferential chain termination of DNA synthesis by ddCTP at the cytosine cluster. Ladders between H and I or G and H may represent the DNA chains initiated at sites A and B, respectively, and terminated at the cytosine cluster at positions 1092–1096. There are three reasons for this assumption: (*i*) The chain lengths of these bands, 57–61 and 76–84 nucleotides, correspond to the distance from sites B and A to the termination, respectively. (*ii*) Nucleotides at the 5′ ends of these bands are mainly C and T, suggesting that they share the same ends with the A and B bands, respectively (see Fig. 4). (*iii*) These bands did not appear in the sample digested with *Eco*RI, which cuts just downstream of the sites A and B. But they appeared at the same positions upon digestion with other enzymes.

Summary Map Showing RNA-DNA Transition Sites in Nascent Short DNA Chains. The foregoing analyses by electrophoreses enabled us to locate the transition sites within two nucleotides. The relative intensities of the major bands were estimated from the results of two or three independent experiments. These results are assembled in Fig. 3. Most of the transitions for the rightward DNA synthesis occur to the left of the *ori* region, while minor transition sites are found to the right of *ori*. Conversely, most of the transition sites for the leftward DNA synthesis are located to the right of the *ori* region. Therefore, most of the DNA chain initiations occur on both sides of the *ori* region and proceed covergently. The

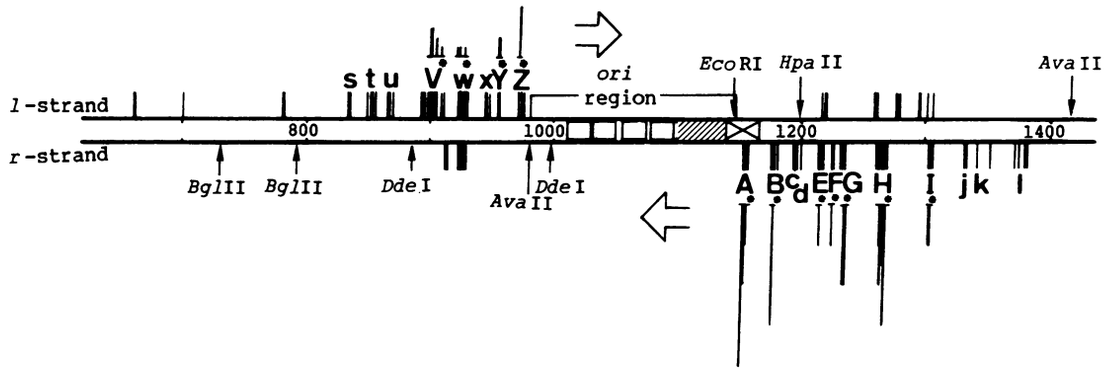

FIG. 3. Map of the transition sites on *l* and *r* strands of the λ genome. Vertical bars on each strand indicate locations of the transition sites observed in this work. The letters correspond to those in Fig. 2. Additional bars above and below the asterisked letters represent relative frequency of occurrence of the transition. These were obtained from densitometric tracing of the autoradiographed bands. Results for minor bands that could not be measured precisely are not shown. The results for *l* and *r* strands were comparable, since they were taken from the same autoradiography of ^{32}P-labeled DNAs that were prepared from two equally divided samples for each strand (not shown). Arrows show the direction of DNA synthesis. The *ori* region carries *ori* repeats (four boxes) and an A+T-rich stretch (▨), along with a part of a palindromic 28-bp sequence (⊠). The cutting sites of restriction enzymes used in the analyses are also shown. It is possible that some weaker transition sites have been missed in these analyses.

results indicate bidirectional replication, with the leftward DNA synthesis several times more frequent than the rightward. This asymmetry is reflected also in the higher recovery of *r* strand than *l* strand in the sample after the S1 nuclease treatment.

To locate the transition sites precisely, the ^{32}P-labeled mononucleotides obtained from the 5′ ends of the short DNA chains (V, Y, and Z and A, B, E, F, G, H, and I) were analyzed. The results (data not shown) agreed well with those obtained from the estimation of chain length. The transition sites are summarized on the nucleotide sequence of the λ *ori* region (Fig. 4). So far, we have not been able to detect a consensus sequence for the transition sites, except for the preferential use of thymidine residues. Thus, 12 out of 19 strong transition sites were T, and only 4, 2, and 1 were A, C, and G, respectively.

DISCUSSION

Short-chain DNAs joined to primer RNA were purified from the earliest replicative intermediates of λdv, which had been prepared in an O,P-dependent *in vitro* replication system. The 5′ ends of these short DNA chains (namely, the transition sites from primer RNA to DNA synthesis) were freed from RNA, subjected to specific labeling with ^{32}P, then mapped along the known λ nucleotide sequence. The results shown in Figs. 3 and 4 clearly demonstrate that transition sites are multiple. They are located on both strands flanking the λ *ori* region and are oriented such that the chain elongations converge. This reflects the bidirectional nature of λ DNA synthesis. The major sites of transition on *l* and *r* strands are located within stretches of about 150 bp each to the left and right of the *ori* region. No transition sites were discovered within the *ori* region, which includes the *ori* repeats, the A+T-rich stretch, and a part of a palindromic sequence, all of which are essential for initiation of λ replication (14).

These observations imply that the *ori* region essential for replication initiation is not the site(s) of initiation of primer RNA synthesis. Two possibilities may be considered concerning the synthesis of primer RNA. First, the primer RNAs might be made as individual short molecules, initiated at multiple sites that may somehow be associated with the transition sites we observed. Alternatively, the primer

FIG. 4. Transition sites from primer RNA to DNA syntheses in the nucleotide sequence of λ around the *ori* region. The λ DNA sequence from 891 to 1330 is shown. The *ori* region is the region from the *Ava* II site (983) to the *Eco*RI site (1147). *ori* repeats (boxed), the A+T-rich stretch (hatched), and a palindromic sequence (thick arrows) are shown. Three clusters of strong transition sites on the *l* strand (V, Y, and Z) and seven clusters of strong sites on the *r* strand (A, B, E, F, G, H, and I) were defined from electrophoretic data (Fig. 2). Palindromic sequences outside the *ori* region that are related to sites B and I are shown by thin arrows (see text). Mononucleotides at the 5′ end of the DNA joined to primer RNA were analyzed as described in *Materials and Methods*. ▲ and △ show the sites of strong and weak transitions, respectively. In cluster A, weak sites located close to *Eco*RI cleavage site might have been missed due to technical limitations.

RNAs might be made as a large transcript starting far upstream of the transition sites. Rightward RNA synthesis from p_R is one candidate for the long rightward primer. However, there is no known long leftward RNA synthesis that goes across the *ori* region. Therefore, the leftward long primer must be made by a new mechanism; and this, of course, might also operate for rightward primer synthesis. These problems can be solved only by analysis of the 5′ ends of the primer RNAs.

The frequency of use of transition sites for leftward DNA synthesis is several times higher than that for rightward DNA synthesis. This fact agrees well with the previous observation that DNA replication of λdv *in vitro* proceeds bidirectionally, and that leftward DNA synthesis occurs more strongly than rightward DNA synthesis. The implications of this asymmetry are unknown, but it could reflect a difference in the initiation mechanisms for leftward and rightward DNA syntheses.

Our observations also demonstrate that the *ori* region is not the site(s) of transition from primer RNA to DNA. At present, too little information is available to link these observations with the fact that O protein binds to the *ori* repeating sequences (13). Several lines of evidence suggest that the O protein interacts with λ-encoded protein P (23), which in turn interacts with such host-encoded proteins as dnaB (24) and dnaK (25). The *ori* sequence may thus be used as a signal that attracts the replication machinery. This complex could alter the structure of neighboring DNA regions to facilitate initiation of primer RNA synthesis, modify or process the incoming primer RNA molecules, or regulate their synthesis.

The multiplicity of transition sites from primer RNA to DNA synthesis in λdv replication is in sharp contrast with the situation in ColE1 (2, 3). This replicon synthesizes a long primer RNA starting at a unique site, and the transition occurs at a unique site. This difference means that the "multiple-site" mechanism must be quite different from that of the "unique-site" mechanism observed with ColE1. It would be of interest to see how the multiple-site and unique-site mechanisms are distributed among different replicons in, at least, prokaryotic cells.

Although multiple, the λ transition sites seem not to be distributed randomly; in fact, the replicon seems to have favored sites that appear reproducibly in independent experiments, although how these sites are selected is unclear. (Major transition sites A, B, and I on the *r* strand lie within palindromic sequences that can assume hairpin structures. In this case, they all are located on the loop or at the border of the loop and the stem. The significance of these selections is unknown, as substitution experiments demonstrate that the palindromic sequence is not mandatory for replication.)

At this stage, we can only infer that combinations of local nucleotide sequences and higher-ordered structures of DNA are responsible for selection of the transition sites and that the thymidine residue plays an important role. The possibility remains that different transition sites might be selected when the experimental system is modified.

Since the *in vitro* system used exhibits a mode of DNA synthesis similar to that observed in *in vivo* replication, in the requirements for the reaction, the bidirectional nature of the replication, and the *ori*-dependent initiation, the initiation process of λ replication *in vivo* may also show transition at multiple sites. Alternatively, the possibility cannot be ruled out that the multiple transition sites observed reflects a lack of a factor(s) necessary for selection of a unique site(s). In this connection, it will be of interest to analyze the *in vivo* transition sites of λ DNA replication.

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