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# Strand-specific break near the origin of bacteriophage P2 DNA replication

(membrane-association/branch migration/P2 Hy *dis*/heteroduplex mapping/denaturation mapping)

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ABSTRACT **Membrane-associated P2 DNA isolated early after infection under conditions that block replication (*amB* in phage and *rep* in *Escherichia coli* C) was analyzed by electron microscopy. Most DNA was in the form of relaxed circles (40%) and circles with short single-stranded tails (60%). When this DNA was hybridized with separate strands of linear P2 Hy *dis* DNA (which provides suitable reference points along the heteroduplex molecules), an interruption was located near the previously mapped origin of P2 DNA replication in one specific strand. The same strand was sometimes extended in the direction consistent with the unidirectional mode of P2 DNA replication. Similar conclusions were reached when the intracellular DNA was analyzed after partial denaturation. These results are consistent with the rolling circle mode of DNA replication.**

Bacteriophage P2 provided the first unambiguous evidence for unidirectional replication from a unique origin (1) in support of the rolling circle model of DNA replication (2). A prerequisite for unidirectional replication in the model is a strand-specific break at the origin. Indeed, when P2 replication was blocked, molecules with a strand-specific break accumulated probably at the cell membrane (3). The break was in the strand proper for the direction of normal P2 replication (1). Also, P2 *cis*-acting gene A, which is essential for P2 DNA replication, was involved in the formation of the break (3) and in early attachment of P2 to the membrane (4). In the present study, I show that the break occurs near the origin of normal P2 replication. Under the same replication-blocked conditions, a small amount of synthesis sometimes was observed. Both the origin and the direction of the synthesis were as observed when free replication was allowed (1). Thus, these molecules appear to be early intermediates of P2 DNA replication.

## MATERIALS AND METHODS

**Phage Strains.** P2 *vir79 amB116 old17* (referred to here as P2 *vir79*) and a large-plaque derivative of P2 Hyl *dis vir14* (referred to as P2 Hy *dis*) were used. Mutation *vir79* is a small deletion and imparts insensitivity to immunity (6, 7) (Fig. 1). Mutation *amB116*, when not suppressed, produces a block in the synthesis of the phage DNA (8). Mutation *old17* (7) is probably irrelevant in the present context. It was expected that P2 Hy *dis* would show similar nonhomology regions in respect to P2 DNA as the previously studied P2 Hyl *dis* (*large*) (9) (Fig. 1), and that its *h* and *l* strands would correspond to the *h* and *l* strands of P2. Both these assumptions were borne out in the present experiments. High-titer stocks of both phages were kindly supplied by G. Bertani.

**Strand Separation.** Strands of P2 Hy *dis* phage DNA (30 μg) were separated with 30 μg of poly(U, G) (lot 2, Miles Laboratory) as described (10). Five-drop fractions from the Cs gradient were diluted 1:6 in 0.15 M NaCl/0.015 M Na citrate for measument of optical density. The optical density profile was bimodal. The two peak fractions (called *h* and *l*) were used without dialysis for heteroduplex formation.

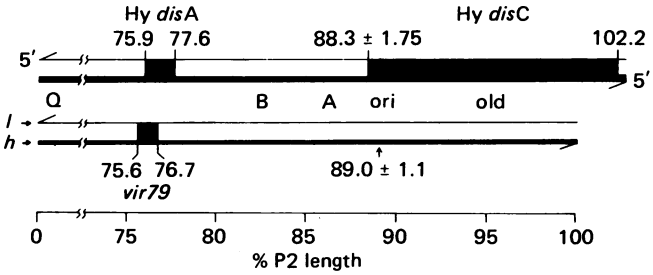


FIG. 1. Physical map of mature linear P2 Hy *dis* and P2 *vir*79 DNA. Substitutions in Hy *dis* phage, called Hy *dis*A and Hy *dis*C for convenience, are shown on the top line. The origin of P2 DNA replication (vertical arrow) and the end points of the *vir*79 deletion are shown in the next line. It is apparent that a P2 *vir*79/P2 Hy *dis* heteroduplex will show two substitution loops: one in Hy *dis*A region (2% in Hy *dis* strand and 0.9% in *vir*79 strand) and the other in Hy *dis*C region. Hy *dis*C substitution is 19% longer than its wild-type counterpart called the P2 *old* segment. Thus, P2 *old* and Hy *dis*C segments are easily recognized in heteroduplex molecules upon length measurement. There is a short region of homology beyond the right end of Hy *dis*C substitution which is exaggerated both in this and Fig. 2 for clarity. The consequence of such a homology is discussed in Fig. 2. Assignment of *h* and *l* strands (drawn in thick and thin lines, respectively) comes from transcriptional studies. Gene locations are only approximate (5). *ori*, site where DNA replication initiates.

**Heteroduplex Analysis.** To 50 μl of intracellular DNA (Fig. 3), 5 μl of 0.2 M EDTA (pH 8.0) and 15 μl of 1 M NaOH were added. After 20 min at room temperature, 15 μl of 2 M Tris·HCl (pH 7.0) was added followed by 15 μl of *h* or *l* strands of P2 Hy *dis* DNA. Formamide (0.1 ml) was added and the mixture was allowed to anneal at room temperature and finally was dialyzed against 0.02 M NaCl/5 mM EDTA (pH 8.0) for examination in the electron microscope (13).

## RESULTS

**Description of Intracellular DNA.** Earlier studies (3) have shown that circular P2 DNA with an *l* strand-specific break and associated with a fast-sedimenting "membrane" complex accumulates when mutations in the host gene *rep* and the phage gene B are present. P2 *vir79* DNA was isolated under similar conditions in the present experiments, and after purification it showed two kinds of structures: simple circles (40%) and circles with short single-stranded branches (60%); 61% of the branched circles showed a second tail from the same branch

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Abbreviations: OC, one-strand circle heteroduplex; TC, two-strand circle heteroduplex; L, linear heteroduplex; RL, replicating linear heteroduplex; RC, replicating circular heteroduplex.

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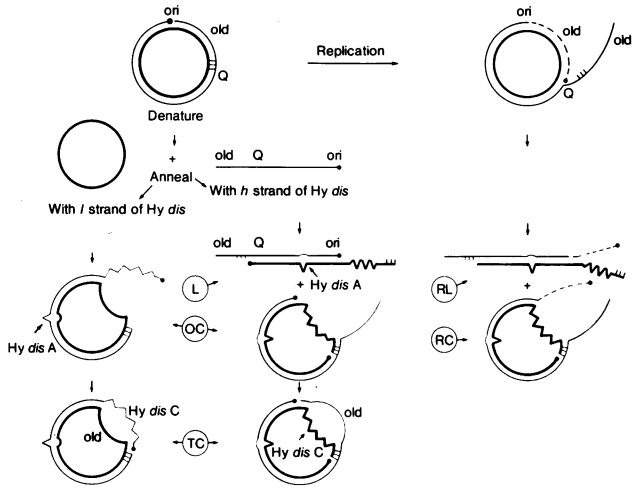


FIG. 2. Predicted structure (schematic) of the heteroduplexes between intracellular P2 *vir*79 DNA and the separated strands of P2 Hy *dis* DNA. Heavy strands (*h*) are drawn thicker than the light strands (*l*), 3′ ends are knobbed, and nonhomologous Hy *dis* DNA strands are made zigzag for convenience. A common feature of the molecules is the Hy *dis*A substitution loop. Different types of heteroduplex molecules are referred to as: L for linear; OC for one-strand circles when one of the two strands in the Hy *dis*C/P2 *old* region has visible ends; and TC for two-strand circles when no ends are seen. Linear and circular heteroduplexes in which the intracellular DNA is replicating with only the *l*-strand extended are called RL and RC. New synthesis is shown by broken lines. Cohesive ends and joints are indicated by three short lines at right angles to the strands. Both types of OC can give rise to TC. When the *l*-Hy *dis* strand is used, the short homology beyond the right end of Hy *dis*C can hybridize as shown. When the *h*-Hy *dis* strand is used, if the *ori* site is not covered by the *dis*C substitution, the free end of the P2 *old* strand can hybridize.

point (Fig. 3*A*) and were thought to result from branch migration of the growing point (12, 14, 15). The combined length of the two strands was used in the plot of branch lengths (Fig. 4 *lower*). Of 41 branched circles analyzed, 1 had a short double-stranded tail with a single-stranded connection to the circle.

**Expectation and Results of Heteroduplex Analysis.** To determine if the simple circles have an *l* strand-specific break, as reported earlier (3), and if the break is at the origin of replication as expected from the rolling circle model, duplexing of the intracellular DNA with separated strands of a P2 mutant, Hy *dis*, was performed, which provided two suitable nonhomology regions called Hy *dis*A and Hy *dis*C (Fig. 1). Expectations from the heteroduplex experiment, assuming an *l* strand-specific break at the origin, are described in Fig. 2.

When the *l* strand of Hy *dis* is used, only circular heteroduplexes should be seen. With respect to the Hy *dis*C nonhomology region, these can be of two types: one-strand circles (OC), in which case one of the strands will show free ends; and two-strand circles (TC), in which case no free ends can be seen under the experimental condition used. TCs are derived from OCs because the free end of the hanging Hy *dis* strand is homologous to P2. Examples are shown in Fig. 3 *B* and *C*).

In contrast, when the *h* strand of Hy *dis* is used, linear heteroduplexes (L) can be seen. The left end of the L should be single stranded and contain the P2 *old* segment; its length should determine the position of the break from the right end of mature P2 DNA. The length distribution of such single strands should be unimodal in the case of a unique break but will be broad in the case of random breaks. The right end of the L molecules should also be single stranded for a length equal to the Hy *dis*C substitution. It should be noted that P2 Hy *dis* phage was chosen primarily to provide the nonhomologous Hy

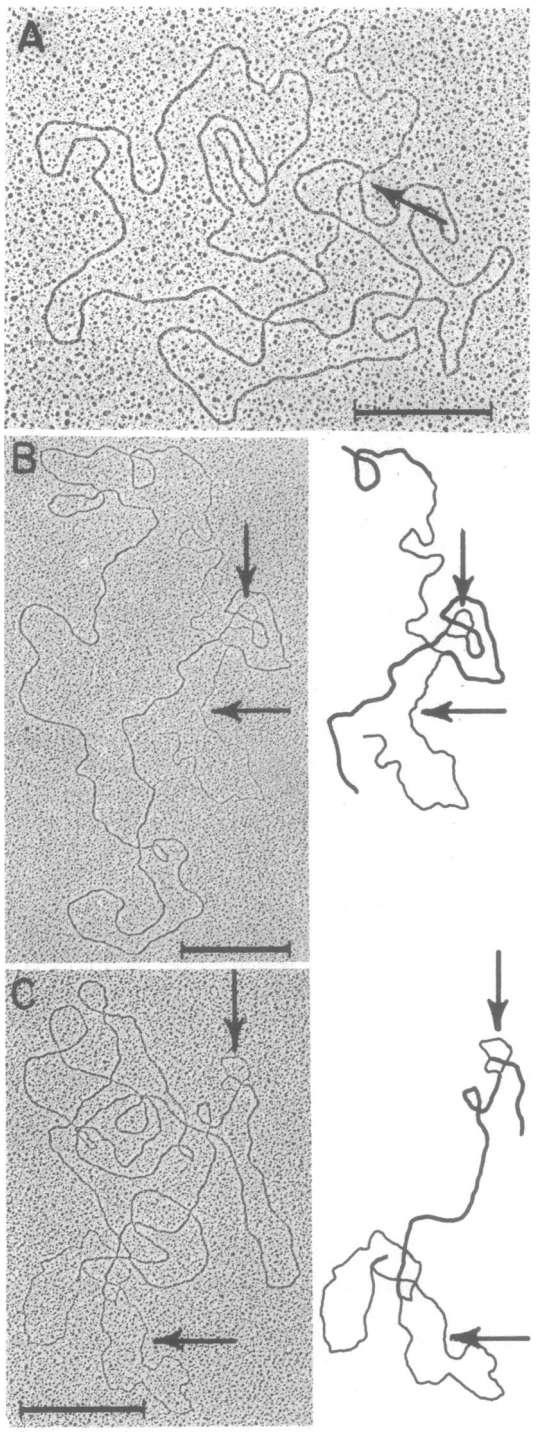


FIG. 3. (*A*) Example of an intracellular branched circle, showing two single-stranded tails attached to the branch point (arrow). A short gap between the origins of the two single-stranded tails indicates that the circle is continuous through one strand only. For isolation of intercellular DNA, an overnight culture of C1704 ($Su^-$, *thy rep3 uvrA*) was grown in the presence of thymine [50 μg/ml in TPG/CAA medium (8)], resuspended without thymine, diluted 1:50 into fresh TPG/CAA medium containing thymine (1 μg/ml) and 5-bromodeoxyuridine (20 μg/ml) and grown to $OD_{600\ nm}$ = 0.20. Extra $CaCl_2$ was added (final concentration, 3 mM) followed by phage (P2 *vir*79) at a multiplicity of infection of 20. After 20 min the culture was cooled in ice and then lysed as described (11). Lysate (final volume, 1 ml) from a 10-ml culture was layered onto a 27-ml 5–30% sucrose gradient (over a 3-ml cushion) and run for 150 min at 22,000 rpm at 5° in a Spinco SW 25 rotor. The opalescent band from the cushion was removed with a pasteur pipette into a dialysis bag and dialyzed against 0.15 M NaCl/0.015 M Na citrate/0.01 M EDTA, pH 8.5. Sarkosyl was added to a final concentration of 0.25% and the solution was heated at 70°

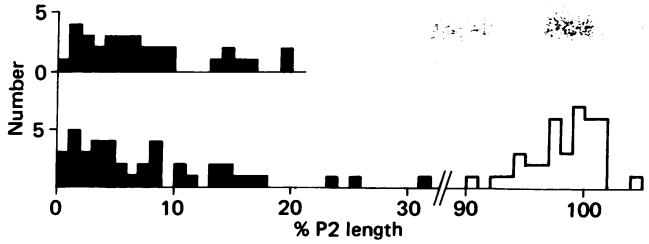


FIG. 4. (*Lower*) Length distribution of P2 *vir*79 circles (open bars) and of their single-strand branches (solid bars) in undenatured intracellular DNA. (*Upper*) Length distribution of the single strands thought to represent new synthesis in heteroduplex molecules designated RL and RC in Fig. 2 and Table 1. Most of the replication is restricted to <10% of the P2 length in both cases. Mean (±SD) lengths, of the branches were 8.6 ± 7.2 (*Lower*) and 7.3 ± 5.4 (*Upper*) from 32 and 41 measurements, respectively.

*dis*C region; otherwise the single-stranded segments at the ends of L would have hybridized to form a circular molecule and the break would not have been visible. Some circle formation from L is possible because the right end of the Hy *dis* strand is homologous to P2 and also carries the cohesive end. These circles (also called OC) are easily distinguished from the OCs described for the Hy *dis* *l*-strand sample because in the two cases the hanging strands are different in length (being *old* and Hy *dis*C, respectively) and connected to the circle at different positions (near *cos* and *ori* sites, respectively). If *ori* is slightly to the left of Hy *dis*C substitution, as assumed in Fig. 2, then the free end of the hanging P2 *old* strand will hybridize to form TC.

If the branched circles represent new synthesis by covalent extension of the *l* strand, then their presence will be inconsequential to the Hy *dis* *l*-strand sample but will give rise to RL and RC in the *h*-strand hybridized sample as shown. Thus, by comparing the structures seen in the *h*- and *l*-strand hybridized samples, it should be possible to check both the strand specificity and the site specificity of the break.

Heteroduplexes were recorded whenever a Hy *dis*A nonhomology loop was observed (Fig. 3 *B* and *C*) and were classified on the basis of the Hy *dis*C nonhomology region (Table 1).

When the *l* strand of Hy *dis* was used, only two kinds of structures (OC and TC) were observed (Fig. 3 *B* and *C*), as predicted in Fig. 2. The two single strands in the Hy *dis*C loop of TC molecules were different in length. The short and long strands were plotted separately (Fig. 5 *top*). Although the distributions were broad, the two species could be resolved, and their mean (±SD) lengths (11.30 ± 1.31 and 13.86 ± 0.98) corresponded well with the expected lengths of P2 and P2 Hy *dis*C segments (11.6 ± 0.29 and 13.89 ± 0.32, respectively) (9). With these distributions as internal standards, the distribution of the single strands in the same region of OC molecules was plotted (Fig. 5 *middle*). Clearly, the continuous strand was shorter, on the average, than the hanging strand, as predicted.

for 10 min and then banded in a Cs gradient (12). [$^{14}$C]Thymidine-labeled T7 DNA was used as a marker. T7 DNA was kindly provided by G. Bertani. (*B* and *C*) Examples of the two types of circular heteroduplexes seen when *l*-Hy *dis* strand was hybridized in intracellular P2 *vir*79 DNA. In both (and in the accompanying tracings), the vertical arrow points to the marker Hy *dis*A loop and the horizontal arrow to Hy *dis*C strand in the P2 *old*/P2 Hy *dis*C nonhomology region. (*B*) OC type molecule. The hanging strand is recognized as Hy *dis*C (horizontal arrow) because it is longer than the other single strand (*old*) and because its point of attachment to the circle is rather close to the marker Hy *dis* A loop (vertical arrow) as predicted in Fig. 2. (*C*) TC type molecule (no free ends can be seen). The bigger single-stranded loop is the P2 *old*/P2 Hy *dis*C region, showing that the substitution is unequal [the longer strand is designated as Hy *dis*C (horizontal arrow)]. On all panels, bar represents 0.5 μm.

Table 1. Distribution of different types of heteroduplex molecules* formed between intracellular P2 *vir*79 DNA and separated strands of P2 Hy *dis* DNA

| Hy *dis* strand used | Time of renaturation, hr | L | RL | OC | RC | TC | Amb.† | Total heteroduplex molecules analyzed |
|---|---|---|---|---|---|---|---|---|
| *l* | 3 | 0 | 0 | 9 | 0 | 13 | 0 | 22 |
| *l* | 1 | 0 | 0 | 16 | 0 | 12 | 8 | 36 |
| *h* | 3 | 6 | 2 | 1 | 4 | 10 | 8 | 31 |
| *h* | 1 | 8 | 21 | 4 | 5 | 9 | 14 | 61 |

* L, linear; RL, replicating linear; OC, one-strand circular; RC, replicating circular; TC, two-strand circular.

† Ambiguous structures, defined as having a clear Hy *dis*A/*vir*79 heteroduplex loop but not assignable to the structures in Fig. 2 and in other columns of this table, are either incomplete and multistranded molecules or structures that could not be completely followed.

Eight other molecules, recorded as ambiguous, were either incomplete Ls (indicative of broken strands) or were difficult to follow (Table 1, top two lines).

When the *h* strand of Hy *dis* was used, the structures were also as predicted in Fig. 2; their distribution is shown in Table 1 (bottom two lines). The predominance of replicating molecules (RL and RC) is perhaps to be expected in view of the high frequency of branched circles in the intracellular DNA preparation. The length distribution of single strands (assumed to contain the P2 *old* segment) at the left ends of L and RL and those hanging from the cohesive end side of OC and RC were compared with the Hy *dis*C strands at the right end of the L and RL and the continuous strand of OC and RC (Fig. 5 *bot-*

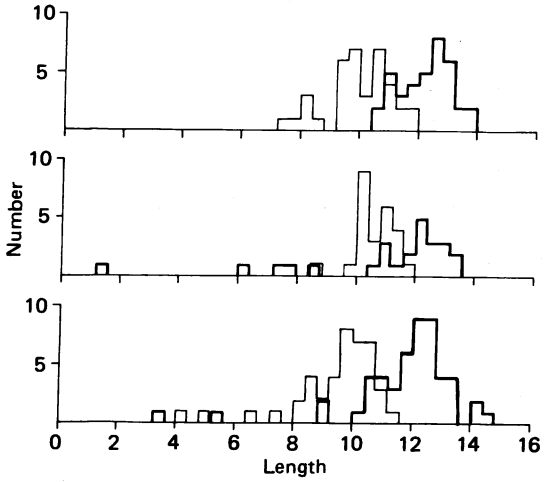


FIG. 5. Comparison of length of nonhomologous P2 *old* (thin lines) and P2 Hy *dis*C (thick lines) strands in heteroduplex molecules (from Table 1 with the exception of the ambiguous heteroduplexes). (*Top*) TC molecules of Fig. 2. These provide internal length reference for P2 *old* and P2 Hy *dis*C strands. The histogram contains 37 of 44 molecules of Table 1; the 7 others were only characterized visually. (*Middle*) All 25 OC molecules of Table 1 when the *l* strand of Hy *dis* was used (Fig. 2). The hanging strand (thick line) was longer, as predicted, except for five cases. These presumably result from broken Hy *dis* *l*-strands and are not included in the mean (see below). (*Bottom*) L, RL, OC, and RC molecules (total = 51) when the *h* strand of Hy *dis* was used (Fig. 2). The main point to note is the similarity of distribution of P2 *old* strands (thin lines) in (*Top* and *Bottom*). Mean (±SD) lengths, *Top* to *Bottom*, are 12.2 ± 0.09 ($n$ = 37), 12.2 ± 0.8 ($n$ = 20), and 12.1 ± 0.8 ($n$ = 43) for Hy *dis*C and 10.0 ± 1.2 ($n$ = 37), 10.6 ± 0.7 ($n$ = 25), and 9.7 ± 0.9 ($n$ = 39) for P2 *old* strands. $n$ = the number of molecules included in the mean. This does not include the strands outside the range in *Top*. Length units are arbitrary.

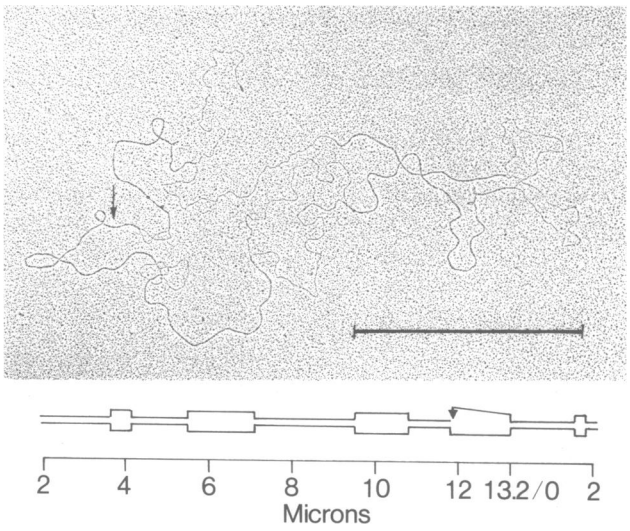


FIG. 6. Electron micrograph of a partially denatured intracellular P2 *vir*79 DNA, showing the presence of a single-strand break in simple circles. Seven microliters of DNA in 0.02 M NaCl/5 mM EDTA, pH 8.0 was added to 3 μl of 0.068 M $Na_2CO_3$/0.017 M EDTA/34% HCHO, pH 9.0. To this was added 10 μl of twice-crystallized Merck (pro analysi) formamide, and the mixture was incubated for 10 min at room temperature and then spread with 0.01% cytochrome *c* on double-distilled water. The denatured regions are easily seen as closed loops of thinner strands except that one of the loops is broken. (Bar represents 1.2 μm.) A schematic drawing of the molecule (drawn to scale) is shown below the micrograph. For convenience, the circular molecule is shown as linear. The break point (arrow in the micrograph) is chosen at 2 μm from the mature ends of the molecule. The molecule is 13.2 μm long and its mature ends are at 0/13.2 μm on the scale. The mature ends are located from the knowledge of the denaturation map of mature linear molecules (see Fig. 7*E*). The closely spaced lines are duplex regions and the rectangles represent the denatured regions. Arrow shows denatured region containing the break (at 12 μm).

*tom*). Distinguishing between the Hy *dis*C strand and the newly synthesized strand of RL was straightforward in most cases, because the amount of replication was small. The close agreement in length distribution between the putative P2 *old* and P2 Hy *dis*C strands and the controls (Fig. 5 *top*) conforms with a break in the P2 *l* strand near the origin (Fig. 2). The broad distribution precludes precise mapping of the break.

The length distribution of the single strands thought to represent new synthesis (discontinuous lines of Fig. 2) was plotted separately (Fig. 4 *upper*) for comparison with the branch length of intracellular circles (Fig. 4 *lower*). A broad distribution is seen in both cases. The majority of the molecules terminate before 10% replication has taken place.

**Mapping the Single-Strand Break by Partial Denaturation Mapping.** With the hope of directly visualizing and mapping the single-strand breaks, intracellular P2 *vir79* DNA was partially denatured (13). Molecules were photographed if they had at least one visible single-strand break in any one of the denatured regions (Fig. 6). Fifty such molecules were analyzed to map the position of the break (Fig. 7).

Of the 50 molecules, 24 were characterized as branched because the broken strands were longer than the complementary continuous single strands. In most cases, such molecules had two hanging single strands (Fig. 7*B*). The distribution of the breaks in Fig. 7*A* and *B* is shown in Fig. 7*D*. The breaks were most numerous at the position of the previously mapped origin of replication [11.75 ± 0.15 μm (1)]. The degree of replication was mostly confined to within 10% of P2 length although the maximum extent could be about 25% of P2 length. These observations were in agreement with those obtained earlier. (Fig. 4). The denaturation mapping data confirm that the branched

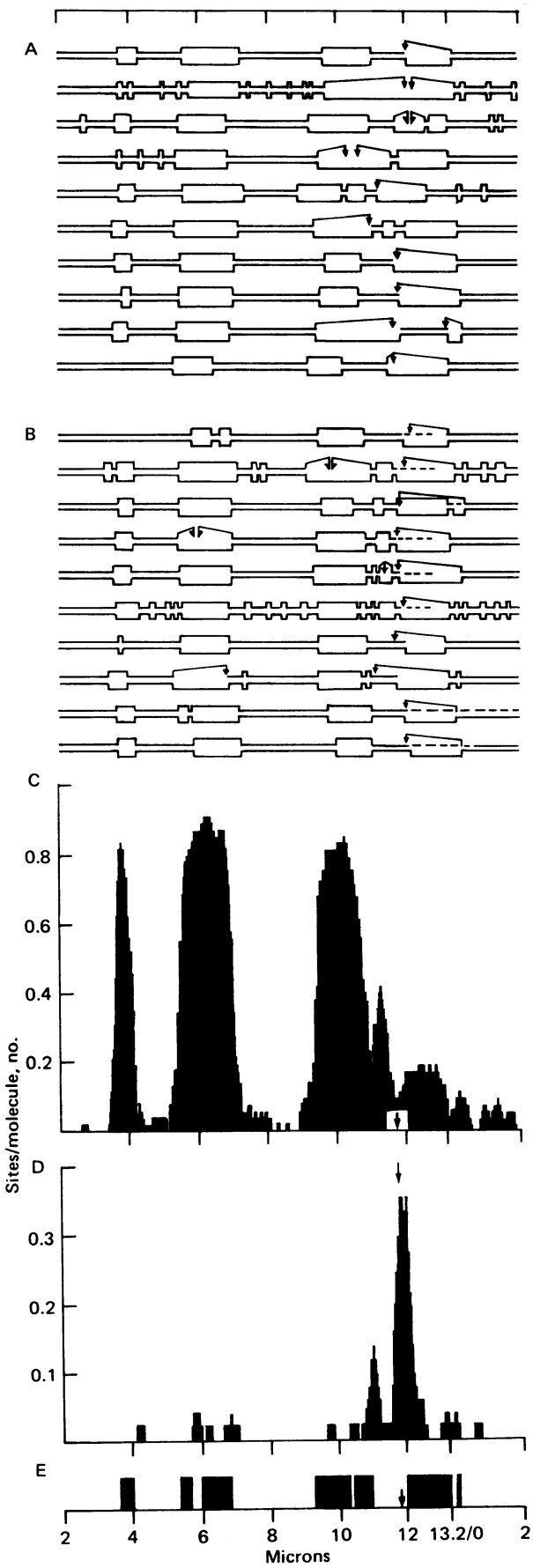


FIG. 7 Location of single-strand breaks by partial denaturation mapping. (*A*) Further examples of simple circles containing single strand breaks as shown in Fig. 6; 26 such molecules were analyzed.

molecules represent partially replicated molecules from the origin of the first round of normal P2 replication.

## DISCUSSION

The present study was attempted to see if P2 replication is initiated by a strand-specific break at its origin of replication as predicted in the rolling circle model. In the hope of stopping replication immediately after the production of this break, two mutational blocks were used: one in gene B of the phage and the other in gene *rep* of the host, *Escherichia coli* C. The presence of branched circles indicated that some synthesis still occurred when two genes essential for P2 DNA replication were mutated. However, the synthesis was highly specific in that it occurred by extension of the *l* strand only, and for about 10% of P2 length only. The origin and direction of this synthesis were consistent with that observed when free replication was allowed (1). Thus, the molecules observed in the present study could be early intermediates in normal P2 replication. If this is true, then P2 seems to follow several predictions of the rolling circle model: strand-specific break at the origin (ref. 3 and present study), early attachment to cellular membrane (refs. 3 and 4 and present study), and double-stranded branches with a single-stranded connection to the circle (M. Schnös and R. B. Inman, unpublished, cited in ref. 12).

Breaks other than at the origin were present both in partially denatured and in heteroduplex molecules. The extent of such breaks could not be quantitated because of technical difficulties. In partial denaturation mapping, a random break in a (G + C)-rich region is unlikely to show up. In heteroduplex mapping, random breaks in most cases will lead to long, linear, single-stranded regions at the end of Ls. These structures, less likely to remain extended and able to form multistranded structures (two such structures were seen) or circularize to form OC and TC, can go undetected.

The duplex replicative form of several single-strand lytic phage also showed single unique discontinuities (16). The results suggest that the unique site could be the origin of replication. Recent *in vitro* results in $\phi$X174 also support the above contention (17). Thus, in this respect, these phages seem to replicate identically to a double-strand temperate phage P2. Another similarity was noted from the present study. Under rep$^-$ conditions, a small amount of synthesis was observed both in $\phi$X174 replicative form (14, 18) and in P2. However, no Okazaki synthesis took place on the displaced strand in either case. *In vitro* studies in $\phi$X174 indicate that the Rep protein is involved in strand displacement (19). The limited extent of replication in the present study and in $\phi$X174 (14, 18) could then be understood as due to the failure of displacing the parental strands under rep$^-$ condition. The requirements for the synthesis of Okazaki fragments were found to be more numerous than extension of parental strands from 3′-OH ends in $\phi$X174 replication *in vitro* (19). P2 gene B might be essential for the initiation of Okazaki fragments.

One of the objectives of using Hy *dis* DNA was to see if Hy *dis* has its own origin of replication in the Hy *dis*C substitution. From independent mapping, the left end point of the Hy *dis*C substitution and the P2 origin of replication coincided within experimental error (Fig. 1). The results of the present study of heteroduplexes suggest that they are separate. It was argued that, when the *h* strand of Hy *dis* was used, TCs would form only if P2 *ori* was to the left of the Hy *dis*C substitution (Fig. 2). The high frequency of TCs indicates that P2 Hy *dis* retains P2 *ori* (Table 1). The evidence is not conclusive because there are possibilities of formation of TC from random breaks other than at the origin. But for this ambiguity due to random breaks, the heteroduplex method provides a powerful way of characterizing rolling circle replication.

Elisabeth Ljungquist most generously provided the expertise needed to isolate membrane complex. B. Forslind and G. Bjursell kindly provided the electron microscope facilities. Computer analysis of the denaturation mapping data was done in R. Inman's laboratory. Jeff Engler helped in the analysis of the data. Janet Geisselsoder provided thoughtful comments on the manuscript. Finally, this work could not have been possible without the hospitality and help of G. Bertani and my other colleagues in his laboratory. The Swedish Medical Research Council provided a Visiting Scientist Fellowship (B76-16V-4713). Funds were also used from National Institutes of Health Grant GM 20373-03 to F. W. Stahl.

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(*B*) Same as *A* except that these molecules have some new synthesis (branched circles) shown by dashed lines in the denatured region around 12 $\mu$m. The new synthesis is indicated by the fact that, often from the same denatured region, two hanging strands can be seen whose total length is clearly longer than the complementary continuous single strand; 24 such molecules were analyzed. Note that, in the last two molecules, the dashed lines continued beyond the branch point of the parental strand (containing the arrow)—i.e., the newly synthesized strand was longer than the observed length of the parental strand. Such structures are easily understood in terms of branch migration. (*C*) Distribution of the position of denatured regions in all 50 molecules analyzed in *A* and *B*. Denatured regions containing single-strand breaks were excluded. The peaks of the histogram were in places expected from the denaturation map of mature linear molecules (see *E*). This shows that, at the degree of denaturation used (38.8 ± 8.4%), in addition to revealing the single-strand breaks the denatured regions can provide suitable reference points for physical mapping in circular molecules. The arrow marks the position of the previously determined origin of replication at 11.75 ± 0.15 $\mu$m (1). (*D*) Weight-average histogram showing the distribution of the position of single-strand breaks (arrows in *A* and *B*) after they are given a fixed width of 0.2 $\mu$m to account for the experimental error involved in the present mapping. The arrow denotes the origin of replication as in *C*. (*E*) Denaturation map of mature P2 DNA at an average degree of denaturation of 32.0% (kindly provided by R. Inman). The arrow is the same as in *C* and *D*.