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Genetics

Evidence of RNA in D loops of intracellular λ DNA

(electron microscopy/RNase A/RNase H/rifampin/genetic recombination)

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ABSTRACT If λ DNA replication is blocked by mutation in any one of several genes essential for replication, intracellular λ DNA often shows short three-stranded regions called D loops. In this report we show that one arm of a D loop is an RNA·DNA hybrid, whereas the remaining arm is made up of single-stranded DNA. The RNA can be partially removed by RNase A and totally removed by RNase H. Also, D loops do not appear if infections are made in cells treated with rifampin, a potent inhibitor of transcription by *Escherichia coli* RNA polymerase. Several genes associated with recombination, including the host *recA* gene, are not essential for D-loop formation.

Some DNA molecules contain regions in which the polynucleotide chain forks into two arms that subsequently reunite. One of the arms is double stranded; the other, single stranded. These regions are called D (for displacement) loops and are early intermediates of mitochondrial DNA replication (1). They are located at the origin of replication and are uniformly 0.6 kilobase long (1, 2). Replication of ColE1 DNA also starts with a D loop (3).

Similar structures have been seen in intracellular DNA of phage λ, but in this case DNA replication is blocked by mutation in genes essential for phage DNA replication (4). Unlike D loops in mitochondrial DNA, those in λ often form at more than one position along the genome and are variable in size (0.17–1.4 kilobases). In the absence of any other information, the role of these structures *in vivo* remained mysterious.

Our interest in D loops was started by the demonstration that supercoiled duplex DNA from φX174 can take up homologous pieces of single-stranded DNA *in vitro,* forming D loops (5). Upon transfection, the information from the single-stranded DNA is stably inherited *in vivo,* and this reaction is promoted by the *recA* function of the host (6). These and other more recent studies indicated that D loops may represent an early event not only in replication, but also in genetic recombination (7, 8) and encouraged us to see if λ D-loop formation *in vivo* involved *recA*. Contrary to that expectation, D loops formed normally in the absence of *recA* and several other gene functions involved in recombination. Our analysis described herein indicates that the D loops of λ contain an RNA chain paired with one of the DNA chains. Whether these D loops are related to any process *in vivo* remains to be determined.

In a recent publication, it has been independently shown that D loops of intracellular φX174 DNA also contain RNA (9).

MATERIALS AND METHODS

Isolation of Intracellular DNA. Typically, a 10-ml culture of bacteria was grown to a density of 10^8 cells per ml in tryptone broth/0.05% yeast extract/0.2% maltose. Bromo[^{3}H]uracil-labeled phage was added at a multiplicity of infection of 10. Thirty minutes after phage addition, the culture was mixed with a slush containing 8 g of crushed ice and 10 ml of 30% (vol/vol) pyridine/10 mM Tris, pH 8.0/30 mM KCN. The mixture was immediately centrifuged and the cells were resuspended in standard saline citrate (0.15 M NaCl/0.015 M Na citrate) containing 10 mM EDTA, 20 mM KCN, 10 mM Na_3N, and 0.003% tRNA (pH of the mixture was adjusted to 8.0) to a density of 10^9 cells per ml. The infected cells were subjected to two cycles of freezing and thawing; then fresh lysozyme solution was added to a final concentration of 0.5 mg/ml, followed by incubation at 0°C for 10 min. Sarkosyl was added to a final concentration of 0.5%, and the lysate was held at 40°C for 15 min. Lysis was evident by complete clearing. Proteinase K was added to a final concentration of 0.25 mg/ml, and incubation was continued for 1 hr at 37°C. The mixture was heated at 65°C for 5 min and then mixed with an 80% solution (wt/wt) of cesium formate in standard saline citrate (pH adjusted to 8.2 with HCOOH) to a final density of 1.775 ($\bar{\eta}$ = 1.403). This was centrifuged for 3 days at 10°C in a Beckman SW50.1 rotor at 38,000 rpm. Fifteen-drop fractions were collected in a sterile microtiter dish and the fractions were assayed for radioactivity by liquid scintillation spectrometry.

Electron Microscopy of DNA. Cs gradient fractions were diluted 1:3 and spread for electron microscopy as described (10). D loops were analyzed only from the fractions in which they were most frequent (usually one or two fractions on the heavy side of the peak of phage DNA; see Fig. 3).

RNase A Treatment. Ribonuclease A (phosphate-free, Worthington) solution was made in water (0.8 mg/ml) and boiled for 10 min. The activity and purity of the enzyme was checked against 16S rRNA from *Escherichia coli* (kind gift of Otto Hagenbuchle in our institute) and supercoiled ColE1 DNA. DNA from the Cs gradient was dialyzed against 20 mM NaCl/5 mM EDTA, pH 8.0. DNA and RNase A were mixed under conditions described in Table 3.

***Sac* I Endonuclease Digestion.** DNA dialyzed as above was digested under conditions specified by the manufacturer (New England Biolabs) except that triethanolamine was used instead of Tris. This permitted spreading of DNA in the presence of HCHO immediately after the enzyme digestion. To avoid possible loss of D loops by branch migration, we crosslinked the DNA with psoralen (11) and then digested it with *Sac* I at 12°C for 2 hr (12).

RNase H Treatment. DNA from a Cs gradient was dialyzed against 10 mM triethanolamine, pH 7.6/0.13 M NH_4Cl/0.1 mM EDTA/5% (wt/vol) sucrose. After dialysis, Mg acetate was added to a final concentration of 10 mM, and 1 unit of RNase H (Enzo Biochemicals, New York, NY) was added to about 1 nmol of DNA. The sample was incubated at 17°C for 2 hr and then dialyzed for electron microscopy (10).

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Table 1. Bacterial strains

Strain	Relevant properties	Source or ref.
FA77	Su⁻, *dnaBts*	13
FZ14	Su⁻, *dnaBts* $recA_{56}$*endoI*	14
RM199 (λ *tsA14 sus029 susP80*)	Su⁻	This work; ref. 15
RM202 (λ *tsA14 sus029 susP80*)	Su⁻, $recB_{21}$	This work; ref. 15
JC9937	Su⁻	A. J. Clark (unpublished)
JC11850	Su⁻, $recB_{21}recC_{22}sbcB_{15}recF_{143}$	A. J. Clark (unpublished)
ED206 (λ *red3 susgam210*)	Su⁻, $recA_{56}$	N. Dower; N. Willetts (unpublished)
NY73	Su⁻, *dnaGts3*	16

RESULTS

D Loops Form in Absence of Recombination Gene Functions. The possible dependency of λ D-loop formation on *recA*⁺ gene activity was tested with two different phage. *E. coli* strains FA77 (*rec*⁺) and FZ14 (*recA*) were infected with DKC1 phage in one experiment and with DKC8 in another experiment under identical conditions (see Tables 1 and 2 for bacterial and phage strains used). In the two experiments and in both bacteria, D loops formed in the phage DNA at comparable frequencies. Thus, the *recA*⁺ gene, which is essential for the Rec system of recombination, is not essential for D-loop formation in λ. In these experiments, genes essential for λ DNA replication (e.g., the host gene *dnaB* or the phage gene *P* or both) were inactivated. Several other genes associated with recombination (Tables 1 and 2) were also found to be dispensable. For example, when λ *bio1* phage (defective for the Int and Red recombination pathways of λ) infected JC11850 (defective for the RecBC and RecF pathways of Rec recombination), D-loop formation was still maximal (Table 3). We conclude that D loops can form in the absence of both DNA replication and recombination.

Evidence of RNA in D Loops. Because RNase A is active against single-stranded RNA and not against RNA· DNA hybrids, we tested for RNase A sensitivity only in those preparations of DNA that showed a high frequency of single-stranded "whiskers" associated with the D loops (Fig. 1). RNase A indeed destroyed the single-stranded whiskers preferentially (Fig. 2). The frequency of D loops with whiskers decreased from about 60% to 6% or less upon RNase A treatment (Table 3). The treatment was attempted at two different temperatures with the hope that, at the higher temperature, whisker formation would be facilitated by branch migration and both the size and frequency of D loops would be further reduced. The results in Table 3 support these expectations. In Exps. 1–4, the percent of circles with D loops decreased 13% (from 94% to 81%) at 23°C but 60% at 34°C upon RNase A treatment. Similarly, the size of D loops decreased 1.5% at 23°C and 3.3% at 34°C. Results were qualitatively the same in Exps. 5–8. These results were our preliminary evidence that at least the ends of D loops were made out of RNA.

Two further experiments supported the hypothesis of RNA in the loops. D loops were treated with RNase H, which degrades RNA from RNA·DNA hybrids. Without RNase H treatment, in a typical sample of 78 molecules, 46 contained D loops. A similar sample after the enzyme treatment had no D-loop-containing molecules in 57 analyzed. Thus, D loops may contain a polynucleotide fragment made entirely of RNA. In order to test this idea, the cells were treated with rifampin, a potent inhibitor of initiation of transcription by *E. coli* RNA polymerase. Because λ is essentially dependent upon the host polymerase for transcription, it was expected that in the presence of the drug, no transcription would occur and, hence, no D loops would be seen. When rifampin was added 10 min prior to phage infection (final concentration 500 μg/ml), the frequency of D loops was 0/41; in the control sample without the drug, the frequency was 5/21.

D Loops Formed in *dnaGts* Mutant. The *dnaG* protein of *E. coli* has RNA polymerizing activity associated with priming

Table 2. Phage strains

Phage	Complete genotype
DKC1	*cI857* χ^{+}*C159 susP80 susS7*
DKC8	*tsA14 susred270 susgam210 cI857* χ^{+}*C158 susS7*
DKC97	*tsA14 bio1 cI26 sus029 susS7*
DKC121*	Δ*Eco*RI.B *cI857* χ^{+}*C159 susP80 susS7*
DKC136	*tsA14 susred270 susgam210 cI857* χ^{+}*C158 sus029 susS7*

* Δ*Eco*RI.B indicates deletion of the λ segment from 44.5% to 54.3% (17).

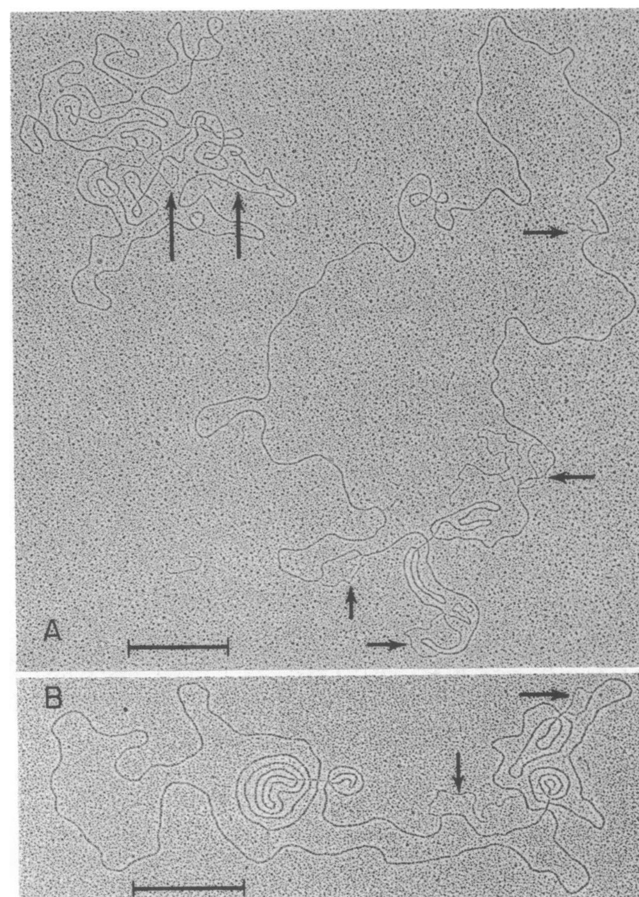

FIG. 1. Electron micrograph of λ *bio1* DNA (DKC97) showing D loops (arrows). (*A*) In this example, most of the loops have hanging single strands (whiskers) at their ends. (*B*) The same DNA sample after treatment with RNase A. Note the absence of whiskers. For other details see Exps. 1 and 2 in Table 3. Bar, 0.5 μm.

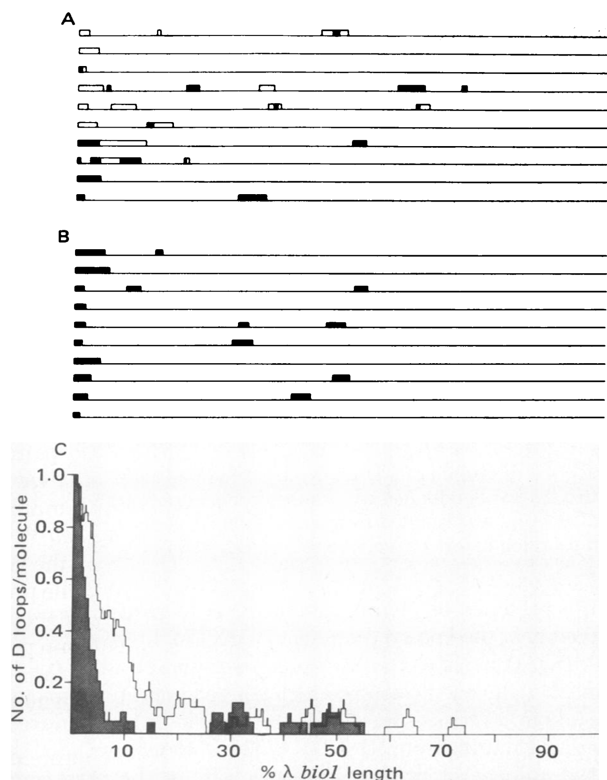

FIG. 2. (*A* and *B*) Diagrams of further examples of the molecules of the type shown in Fig. 1 *A* and *B*, respectively. Empty rectangles in *A* represent the whisker part of the loops; solid rectangles represent the three-stranded regions (*A* and *B*). When only open rectangles are shown (e.g., the first two rectangles of the top line drawing), single-stranded branches were seen attached to the circle with no discernible three-stranded region. The horizontal lines represent the entire circle broken artificially so as to end in one of the loops. In molecules containing more than one loop, the break point was chosen so that the majority of the loops belong to one half (left) of the molecules. (*C*) Average size and position of the loops (including whiskers) are shown in the form of a histogram. The empty profile represents 29 molecules of the type shown in *A*; the shaded profile represents 25 molecules of the type shown in *B*.

of DNA synthesis (18). However, this activity is insensitive to rifampin. The apparent ability of rifampin to block D-loop formation indicates that *dnaG* is not involved. To challenge this conclusion, we checked a *dnaGts* host (NY73, Table 1) at permissive (26°C) and nonpermissive (42°C) temperatures by using two different phage, DKC1 and DKC121. As expected, D loops were present at both temperatures. At 42°C, the mutant was as effective as a *dnaBts* mutant (FA77, Table 1) in blocking λ DNA replication (M. Stahl, personal communication). It thus appears that D loops are made exclusively by *E. coli* RNA polymerase.

Does D-Loop Formation Occur Outside the Cell? It is conceivable that D-loop structures as such do not exist inside the cell, but form from free RNA and covalently closed DNA after cell lysis, analogous to the process of R-loop formation *in vitro* involving superhelical circular DNA and homologous linear RNA (19). The following experiment argues against this possibility. In this experiment we took advantage of the fact that rifampin stops D-loop formation. Prior to phage infection, one of the cultures was treated with rifampin (Fig. 3*A*) and the other was used as a control (Fig. 3*B*). Just before cell lysis (50 min after addition of phage), part of the cultures from both the tubes were mixed into a third tube (Fig. 3*C*) and the three tubes were lysed in parallel. Two different phage were used to infect the first two tubes so that they could be physically distinguished upon mixing in the third tube. One phage (DKC121) was labeled with a mixture of [^{14}C]thymidine and bromouracil and infected the drug-treated cells (Fig. 3*A*); the other phage (DKC1) was labeled with bromo[^{3}H]uracil alone and infected the control cells (Fig. 3*B*). As can be seen in Fig. 3, the two phage DNAs can be separated in the cesium gradient by virtue of their different bromouracil content. In order to reduce cross contamination, the DNA in fractions 11 and 12 from the third tube was treated with *Sac* I. This enzyme cuts DKC1 DNA twice but leaves DKC121 DNA intact (the region containing the *Sac* I sites is deleted in DKC121, indicated by Δ*Eco*RI.B in Table 2). Because the DKC121-infected cells were treated with rifampin, no D loops were expected among circular molecules unless they are formed *in vitro*. However, circles containing D loops were seen. Twenty such circles were photographed and, upon length measurement, all were found to be DKC1 DNA that apparently escaped enzyme digestion. It thus appears that D loops do not form outside the cell under our conditions. It remains possible that the D loops form at the onset of cell lysis though not outside the cell.

Stability of D Loops. To see if D loops are stable, we allowed them to form for 20 and 30 min after infection in two experiments and then blocked the formation of new D loops by adding rifampin. DNA extracted 20 and 12 min later, respectively, had D loops with frequencies of 0/109 and 3/174, respectively. In

Table 3. Effect of RNase A on D loops

Exp.	Bacterial strain	Reaction condition	% of circles with D loops	% of D loops with whiskers	Average size of a D loop (% λ length)*	Total length of D loops per circle (% λ length)*
1	JC11850	23°C, 30 min	94 (29/31)	68 (41/61)	4.7 ± 3.8 (61)	9.9 ± 5.8 (29)
2	JC11850	23°C, 30 min, RNase A	81 (25/31)	2.5 (1/40)	3.2 ± 1.7 (40)	5.1 ± 1.9 (25)
3	JC11850	34°C, 30 min	90 (9/10)	56 (9/16)	5.7 ± 3.5 (16)	10.1 ± 2.9 (9)
4	JC11850	34°C, 30 min, RNase A	30 (11/37)	6 (1/16)	2.4 ± 1.3 (16)	3.5 ± 1.7 (11)
5	JC9937	23°C, 30 min	84 (27/32)	57 (21/37)	4.1 ± 3.2 (37)	10.1 ± 5.3 (15)
6	JC9937	23°C, 30 min, RNase A	85 (22/26)	0 (0/19)	3.5 ± 1.6 (19)	5.6 ± 1.9 (12)
7	JC9937	34°C, 45 min	86 (12/14)	60 (15/25)	4.4 ± 4.0 (25)	8.5 ± 4.2 (13)
8	JC9937	34°C, 45 min, RNase A	72 (13/18)	0 (0/18)	3.1 ± 1.7 (18)	4.6 ± 1.5 (12)

Bacteria were infected with λ *bio1* phage. RNase A, when added, was at 5 μg/ml. Numbers within parentheses represent number of molecules measured or counted.

* ± SD.

the control samples the frequencies were 16/72 and 23/85, respectively. It appears that in the absence of new initiation of transcription, D loops are rarely seen if analyzed as little as 12 min after addition of the drug.

DISCUSSION

The new findings in this report are: (*i*) the D loops of intracellular λ DNA contain an RNA fragment and (*ii*) several genes involved in DNA replication and recombination do not seem to be essential for their formation. These include the DNA replication genes *O* and *P* of the phage and *dnaB* and *dnaG* of the host as well as the DNA recombination genes *int* and *red* of the phage and genes *recA*, *recBC*, *sbcB*, and *recF* of the host. The frequency of molecules with D loops varied widely in different experiments (from a few percent to more than 90%); thus we were unable to determine whether these genes, though not essential for the formation of D loops, in any way influence their structure. D loops were only rarely recorded under conditions of free replication of phage λ (10). However, D loops were seen when phage 186 DNA replication was studied under similar conditions (20) in the host used for λ DNA replication (10). D loops were restricted to simple circles only and were absent from branched replicating molecules. It is conceivable that the RNA strand (branch) migrates out due to unwinding of parental DNA during replication.

The presence of RNA has also been demonstrated in D loops of ϕX174 replicative form I (RF I) DNA (9). Baas *et al.* (9) further showed that the position of the loop depends upon whether chloramphenicol was present during the experiment.

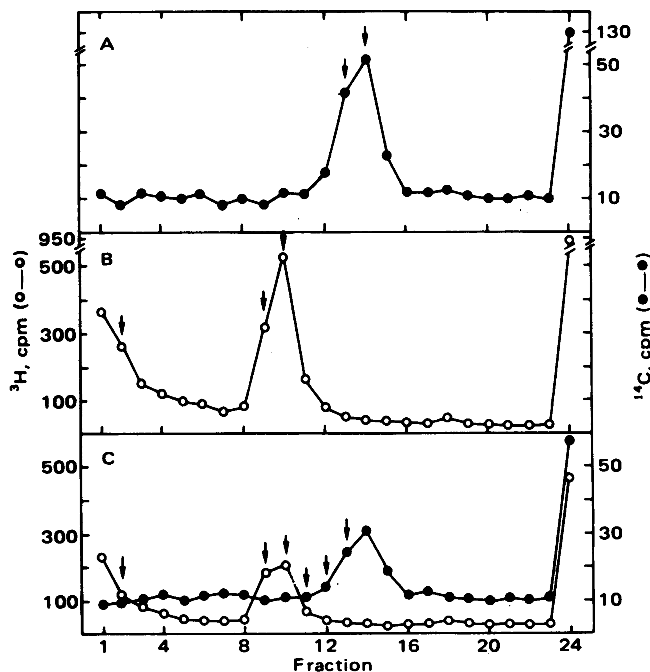

FIG. 3. Cesium formate density-gradient fractionation of DNA from JC9937 cells infected with DKC121 phage labeled with [^{14}C]-thymidine and bromouracil (*A*) and DKC1 phage labeled with bromo[^{3}H]uracil (*B*). In *A*, cells were treated with rifampin before phage infection. (*C*) Just before cell lysis, part of the cultures from *A* and *B* was mixed together. Arrows indicate fractions that were further characterized. Fraction 2 consisted entirely of short single-stranded material (less than 5% of λ length) and was not characterized further. D loops were maximal in fractions 9 of *B* and *C* and absent in fractions 13 of *A–C*. Fractions 11 and 12 were treated with *Sac* I (see *Results*). Fractions 18–22 consisted of *E. coli* DNA. Radioactivity in the top of the tube (fraction 24) was present in aggregated material that could not be characterized.

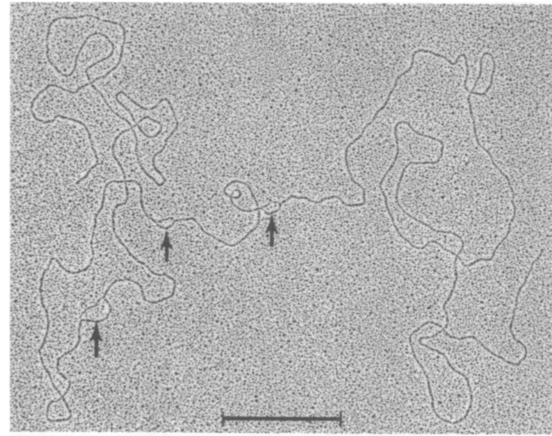

FIG. 4. Example of a D-loop-containing molecule (arrows) linearized by *Sac* I endonuclease. The molecule represents λ *bio1* DNA (DKC97) and was isolated from an RM199 lysogen and crosslinked with psoralen before enzyme digestion. Bar, 0.5 μm.

In the presence of chloramphenicol, D loops preferentially formed near the known promoters of ϕX174. In the absence of the drug, D loops were distributed everywhere along the genome. Usually one D loop was seen per genome, and its average size (about 300 bases) was of just the right length to remove all superhelical turns from RF I DNA. The authors concluded that in the absence of protein synthesis (i.e., in the presence of chloramphenicol), transcription proceeded by RNA·DNA hybrid formation and stopped when all superhelical turns were removed from RF I DNA. However, when protein synthesis was allowed, the 5′ end of RNA could come out of the hybrid region so that transcription could proceed away from promoters without changing the length of the hybrid region. No single-stranded RNA whiskers were recorded, presumably because they were digested during the isolation. It appears that in λ also, D loops form at random locations in the absence of chloramphenicol (4). In the present study, D loops were mapped in two cases with similar results. In one case λ *bio1* phage infected homoimmune λ lysogens of RM199 (Rec$^+$) and RM202 (RecB$^-$). The primary intent of this experiment was to see if D loops formed in the absence of the RecB function. Though infrequent (2–5% of the circles showed them), D loops were in fact seen in both cases with no apparent site specificity (Figs. 4 and 5*A*). Because the infections were made in lysogens, it was expected that most transcription would be in the immunity region of λ and in the *bio* substitution. However, it is possible that at the multiplicity of infection used (at least 20), there was breakdown of immunity (21). [In an independent experiment with DKC121 phage in a RecA$^-$ lysogen (ED206), D loops were again seen in about 3% of the circles. In this case, the multiplicity of infection was 30.] In the second experiment, cells (JC11850) were treated with 200 μg of chloramphicol per ml prior to phage infection and DNA was analyzed by partial denaturation mapping. In contrast to the results with ϕX174, D loops were present again at random locations (Fig. 5*B*). Thus, we lack evidence that λ D loops result from transcription starting from the known promoters of λ.

In addition to location, D loops in λ and ϕX174 were also different in other respects. In ϕX174, D loops occurred once per circle and seldom showed whiskers. On the other hand, multiple D loops occurred frequently in λ (Fig. 1) and could have whiskers (Table 3). These apparent differences can be rationalized as follows. If the length of D loops is determined by the degree of superhelicity as proposed for ϕX174, then in λ we expect the D loops to be longer by a factor of about 9, the

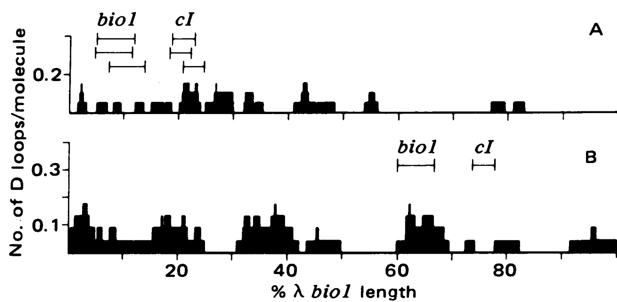

FIG. 5. (*A*) Histogram showing the average position of the D loops from 20 different molecules of the type shown in Fig. 4. The molecules were oriented so as to maximize concentration of the loops on the left half. Because *Sac* I has two closely spaced sites in λ (50.8% and 53.1% from the left mature end), there are three expected positions for the *cI* and *bio* transcripts (bars). (We assume that in some of the molecules one of the sites might escape enzyme digestion.) (*B*) Same as *A* but in this case the free ends of the molecules correspond to the ends of mature λ DNA (zero map coordinate corresponds to the left mature end). The position of mature ends on circular molecules was determined by partial denaturation mapping (10). The DNA in this experiment is the same as in Exp. 1 of Table 3.

length ratio of the two DNAs [the degree of superhelicity being independent of length of DNA (22)]. The longer possible hybrid region in λ could thus have encouraged multiple initiations of transcription. Whisker formation might also be related to longer lengths of transcripts in λ, and one might expect longer whiskers to survive the isolation procedure better and to be visible. Unknown differences in the isolation procedures could also determine the stability of whiskers. We also cannot be sure that we analyzed populations of covalently closed molecules only. Considering these complications, the mechanism proposed for the formation of D loops in ϕX174 remains an attractive one and essentially is not contradicted in the present study.

The roles that D loops could play in DNA replication and transcription regulation have already been proposed (1, 9). *In vitro* studies have shown that D-loop formation may be an early step in genetic recombination (5, 7, 8). *In vitro,* D loops form as a result of uptake of homologous DNA. In view of the finding that transcription can also give rise to D loops, an alternative role of D loops could be in facilitating strand displacement (see ref. 23 for description of strand uptake and displacement). It is conceivable that the opening of the double helix by transcription makes the single-stranded region of the D loops available for uptake by other DNA molecules. In other words, both the strand displacement and strand uptake steps during recombination could go through a D-loop structure. In fact, it has been observed that the actively transcribed regions of λ recombine preferentially (via a low-level, *recA*-independent pathway) (24).

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