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Cleavage of DNA by R_I Restriction Endonuclease Generates Cohesive Ends

(SV40/restriction site/cyclization/electron microscopy/DNA joining)

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ABSTRACT **R_I restriction endonuclease cleaves duplex DNA at a specific sequence, probably 6 nucleotide pairs in length, by making two single-strand staggered cleavages, generating 5′-phosphoryl and 3′-hydroxyl termini. The single-strand ends produced at each break have identical and complementary sequences of 4 or 6 nucleotides in length. Therefore, the cleavage site possesses a 2-fold rotational axis of symmetry perpendicular to the helix axis. The ends of full-length linear SV40 DNA, generated by R_I endonuclease cleavage, can be joined by *Escherichia coli* ligase to regenerate duplex, fully infectious, covalently-closed circular molecules. It was further found that *all* R_I endonuclease-generated ends are identical and complementary. Therefore, any two DNA molecules with R_I sites can be "recombined" at their restriction sites by the sequential action of R_I endonuclease and DNA ligase to generate hybrid DNA molecules.**

Restriction endonucleases are believed to make double-strand scissions within a specific sequence of base pairs if that sequence lacks a particular modified base (see refs. 1–3 for review of restriction–modification systems). In only one instance, the restriction endonuclease of *Hemophilus influenzae*, is the nucleotide sequence specifying the restriction site and the location of the endonuclease cleavage known (4, 5). In that case, cleavage occurs in the middle of either of two sequences of six base-pairs, generating molecules that are base paired to the ends with 5′-phosphoryl, 3′-hydroxyl termini; an interesting feature of the restriction site is its two-fold rotational axis of symmetry perpendicular to the helix axis.

After the observation that R_I restriction endonuclease (6) cleaves SV40 form-I DNA [SV40 (I)] to unique linear duplex DNA molecules (7, 8), we discovered that, in contrast to the *H. influenzae* enzyme, the R_I endonuclease makes "staggered" breaks, generating "cohesive" ends. As a consequence, the linear molecules can be recyclized through intramolecular hydrogen bonds and covalently sealed by the action of *Escherichia coli* DNA ligase. Our analysis of the single-strand termini suggests that the cohesive ends generated by the R_I endonuclease are identical for all DNA substrates, are either 4 or 6 nucleotides in length, and possess the same kind of 2-fold axis of symmetry as in the *H. influenzae* site.

MATERIALS AND METHODS

DNAs and Enzymes. Monkey kidney cell lines were obtained and grown as described (7). Plaque-purified SV40 virus was obtained from J. Morrow. Purified ^{3}H-labeled SV40(I) DNA was prepared essentially as described (7). Purified, closed circular duplex λdv-120 DNA (D. Berg, J. Mertz, and D. Jackson, manuscript in preparation) was prepared and supplied by D. Jackson. The closed circular duplex DNA of the episome F_8(P17) from *E. coli* (9) was a gift of M.-T. Hsu. The RI endonuclease was the same preparation described (7). *E. coli* DNA ligase (10), 7500 units/mg (75% pure) (11), was kindly supplied by P. Modrich.

Enzyme Reactions. (*a*) R_I endonuclease reactions were performed in 0.1 M Tris·HCl (pH 7.5)–0.01 M $MgCl_2$ at 37° for 15 min. To insure that the cleavage went to completion, further incubation with additional enzyme was frequently performed. The reaction was stopped by addition of Na_3-EDTA to a final concentration of 20 mM. Where indicated, the R_I endonuclease-treated DNA was purified by neutral sucrose gradient sedimentation. (*b*) *E. coli* DNA ligase reactions were in 20 mM Tris·HCl (pH 8.0), 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.1 M KCl (10 mM $MgCl_2$ without KCl was also used), 100 μg/ml of bovine serum albumin and 100 μM DPN (chromatopure, P-L Biochemicals) at 15° for about 24 hr with an excess of enzyme (1–5 μg). The concentration of DNA varied depending upon whether intra- or intermolecular joining was to be favored. The DNA was warmed to room temperature, then kept at 0° for 5 min or longer before the ligase was added.

Equilibrium Centrifugation. Density gradient centrifugations were performed in CsCl [containing 10 mM Tris·HCl (pH 7.5)–1 mM EDTA and, where indicated, 330 μg/ml of ethidium bromide (13)]. Samples were counted and corrected for background (<10 cpm) and a channel overlap of 0.68% ^{32}P into the ^{3}H channel.

Electron Microscopy. DNA was mounted for electron microscopy by the formamide and aqueous techniques described by Davis, Simon, and Davidson (14). To visualize hydrogen-bonded circles, aqueous spreading was performed in a suitably temperature-controlled cold room, with all solutions equilibrated to the indicated temperature while sitting in aluminum blocks. The DNAs used in experiments on the formation of hydrogen-bonded circles remained at 5° for at least 2 days before they were used. Grids were examined and photographed with a Philips EM 300 electron microscope. Length measurements were made with a Hewlett-Packard 9864A Digitizer and 9810A Calculator with a fully smoothed length calculation program, giving an accuracy of ±0.5% and greater precision on sample figures of known length.

RESULTS

SV40(L_{RI}) DNA Molecules are Infectious. Circular, covalently-closed SV40 DNA [SV40 (I)] is cleaved by R_I endonuclease at a unique site to produce full-length linear duplex molecules [SV40(L_{RI})] (7. 8). Unexpectedly, we found that even purified SV40(L_{RI}), which contained less than 0.1% circles, had about 10% of the specific plaque-forming activity of SV40(I); the progeny virus, however, contained supercoiled

SV40(I) DNA. This finding suggests that the ends of SV40 (L_{RI}) can be joined *in vivo* to regenerate infectious circular molecules.

SV40(L_{RI}) DNA Molecules Can Be Circularized In Vitro. Only 0.14% of the purified SV40(L_{RI}) molecules are circular when mounted for electron microscopy at 25° in 50% formamide. The same preparation spread in 0.5 M ammonium acetate at temperatures below 25° gives an appreciable number of circular monomers, and some linear and circular dimers (Fig. 1). At 5.9 ± 0.3° there are equal numbers of linear and circular monomer structures. The T_m for melting a hydrogen-bonded circular dimer to a linear dimer is 2 ± 1° (Fig. 1), and is close to that predicted for the dimer (1°) from the relation K dimer = $(1/2)^{5/2} \cdot K$ monomer (15). Clearly, under some conditions, the ends of SV40(L_{RI}) can be annealed to produce hydrogen-bonded circles.

SV40(L_{RI}) can be converted to a covalently-closed circular form by incubation with *E. coli* DNA ligase at 15°. More than 98% closed molecules have been observed by electron microscopy. These closed circular DNA molecules produce a denser band in an ethidium bromide–CsCl density gradient (Fig. 2); the enzymically closed molecules appear at a higher buoyant density than SV40(I) extracted *in vivo* because the negative superhelical turns, normally present in native molecules, are lost under these conditions of closure (16). Incubation of SV40(L_{RI}) with ligase at high DNA concentrations (10 μg/ml) produces linear and circular oligomers, as well as monomer closed circles (Fig. 2). After covalent joining of the ends of SV40(L_{RI}) at low DNA concentration (to produce

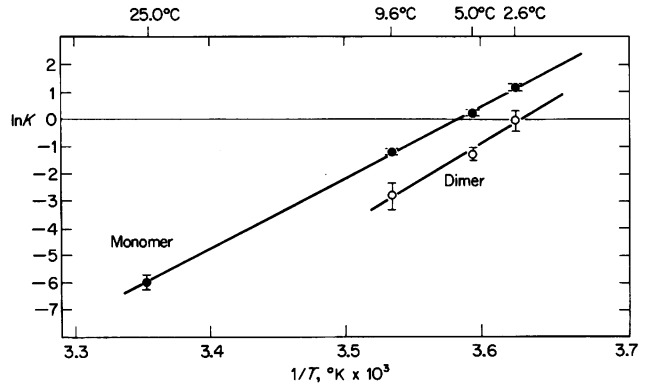

FIG. 1. The equilibrium between linear and circular forms of SV40(L_{RI}) as a function of temperature. A single sample of SV40(L_{RI}) was mounted for microscopy by the aqueous technique at the temperatures indicated. Monomers or hydrogen-bonded dimers were scored as linear (L) or circular (C) in random fields, with about 1% being unscorable because of tangling, uranyl oxide crystals from staining, or because they were residual supercoiled SV40(I). The background number of circles, obtained from a sample spread in 50% formamide–0.1 M Tris·HCl buffer (pH 8) (equivalent to 75°), was 10 of 7000 monomers scored. The data for the graph are shown below; standard error in $K = K(1/C + 1/L)^{1/2}$.

Temp, °C	No. of molecules: Monomers C	Monomers L	Dimers C	Dimers L	(Ratio of circles to linears): K(monomers)	K(dimers)
2.6	394	119	16	16	3.30 ± 0.35	1.00 ± 0.35
5.0	533	415	21	72	1.28 ± 0.08	0.29 ± 0.07
9.6	271	875	5	75	0.307 ± 0.02	0.068 ± 0.03
25.0	24	5973	0	3	0.0026 ± 0.0007	

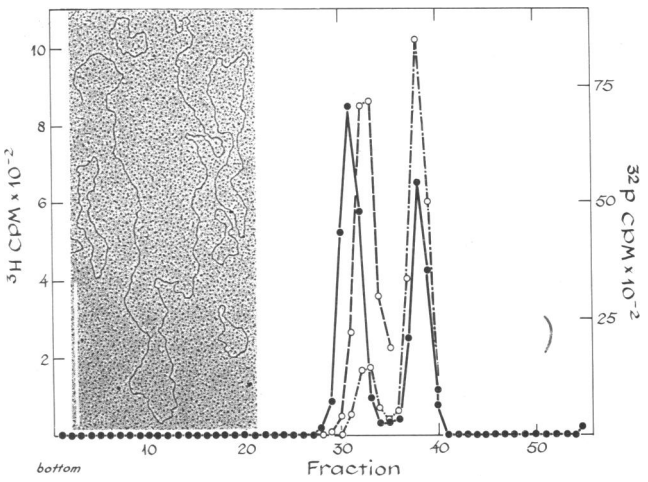

FIG. 2. Equilibrium centrifugation of ligase-treated SV40(L_{RI}) in a CsCl–ethidium bromide gradient. SV40(L_{RI}) [³H]DNA (1 μg/ml) was incubated with *E. coli* ligase for 24 hr at 18° and, after addition of EDTA, ethidium bromide (330 μg/ml), cesium chloride (final density 1.5656), and a marker of SV40 [³²P]DNA [about 17% SV40(I)–83% SV40(II)] were added. The mixture (3.0 ml) was contrifuged at 38,000 rpm and 4° for 47 hr in an SW50.1 rotor. ●——●, ³H-SV40(L_{RI}) DNA after ligase treatment; O–·–·–O, ³²P-SV40 marker DNA; O– – –O, the dense peak of SV40 [³²P]DNA plotted at five times the scale shown. The micrograph is a similar sample treated with ligase at 10 μg of DNA per ml.

largely monomer circles), the infectivity and R_I sensitivity is restored to that of the original SV40(I) DNA. Thus, the original cleavage site is probably regenerated during joining.

We conclude from these findings that R_I endonuclease cleavage of SV40(I) DNA generates short "cohesive" ends, and that these "cohesive" ends, under suitable conditions, can be paired through hydrogen-bonds to produce circular structures; treatment of these molecules with DNA ligase restores the covalently-closed structure and full infectivity.

An Estimate of the Number of Nucleotides in the Cohesive End. The equilibrium constant, K, for joining the cohesive ends of SV40(L_{RI}) is defined by the ratio of circular to linear monomers (Fig. 1). By Eq. 1, the slope of the plot of ln K against $1/T$ for the monomer yields an enthalpy change, ΔH, of −50 ± 3 Cal/mol. The number of bases involved in the cy-

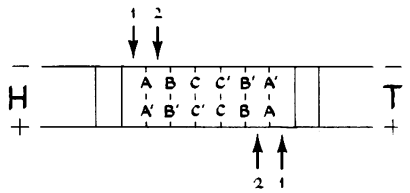

FIG. 3. Model for R_I endonuclease recognition site and possible modes of cleavage. The letters *A*, *B*, and *C* signify DNA bases, and the *prime symbol* indicates the complement of that base. The *numbered arrows* indicate two alternate sites of cleavage that would generate either 6- or 4-base single-strand ends. *Plus* and *minus* distinguishes the two complementary strands of the duplex. *H* and *T* are arbitrary designation for head and tail.

clization, n, can be estimated from Eqs. 1 and 2 (17):

$$\frac{d \ln K}{d(1/T)} = -\Delta H/R \quad [1]; \qquad n\Delta h = \Delta H + 7 \quad [2]$$

where Δh is the enthalpy change per mol base-pair [-8 to -9 Cal/mol (18)] and -7 Cal/mol is assumed to be the contribution from an additional stacking interaction accompanying pairing of the ends (17). Substituting, one obtains n, the length of the single-strand ends = 5 ± 1 nucleotides.

Another method of estimating the number of nucleotides in the single-strand ends created by R_I endonuclease is that used for determining the length of the cohesive ends of coliphage lambda DNA from the melting temperature of the hydrogen-bonded circles (19). This approach introduces an empirical term, which accounts for additional effects on the stability of the cohesive joint; for example, the steric and electrostatic effect of two phosphodiester breaks (end effect). By use of a value for that term, that proved applicable to the cohesive ends of lambda and 186 DNA (17, 20), and the T_m for hydrogen-bonded SV40 monomeric circles ($5.9 \pm 0.3°$), the estimated length of the SV40 cohesive ends ranges between 6 AT pairs and 4 GC pairs. However, since the R_I endonuclease-generated ends may differ significantly in their base composition, sequence, and length from those of lambda DNA, and since K for SV40(L_{RI}) DNA may be affected by electron microscopic mounting, the estimate of the length of the R_I endonuclease cohesive ends is approximate.

The R_I Endonuclease Cleavage Site Possesses a 2-Fold Axis of Symmetry. It is possible that the R_I endonuclease restriction site has a 2-fold rotational axis of symmetry perpendicular to the helix axis and that the cleavages occur in a staggered

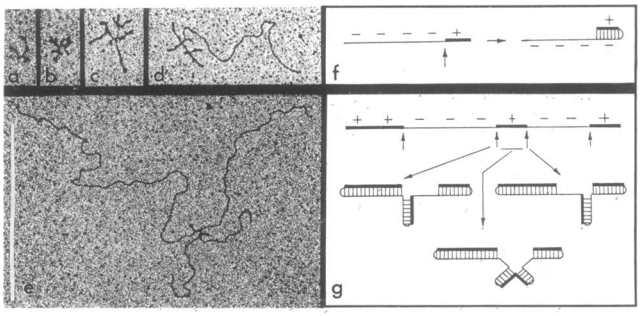

FIG. 4. Covalent joining of plus and minus strands to produce "snap-back" molecules. The covalently joined linear (and circular) oligomers of SV40(L_{RI}) were prepared by treatment of the DNA sample at high concentration (15 μg/ml) with DNA ligase. About $^1/_3$ of the mass of this sample was linear oligomers before denaturation, with the longest oligomer observed being 33 monomer units. The DNA (0.01 μg) was denatured with NaOH (pH 13) and quickly neutralized with Tris·HCl (total volume = 50 μl). The sample, containing "snap-back" linear oligomers and undenatured closed circles, was then quickly mounted for electron microscopy by aqueous spreading and stained with uranyl acetate. Panels *a*, *b*, and *c* are electron micrographs of single-stranded monomer, dimer, and trimer SV40 DNA molecules, respectively. The molecular weight of the "bushes" that result from aqueous spreading of single-stranded DNA can be estimated from the periphery length of a "bush" (25). The interpretation of the molecules in panels *d* and *e* is diagrammed in panels *f* and *g*, respectively. The diagram in panel *g* depicts several possible structures that the 9-mer containing joined plus and minus strands could assume after "branch migration" (26); the middle figure most closely resembles the photograph in panel *e*. Our estimate of the ratio of single-strand to double-strand mass in the 5-mer (panel *d*) and 9-mer (panel *e*) (characteristic of those tabulated in Table 2) are 1.5 and 0.125, respectively. The *white bar* is the length of one SV40 duplex.

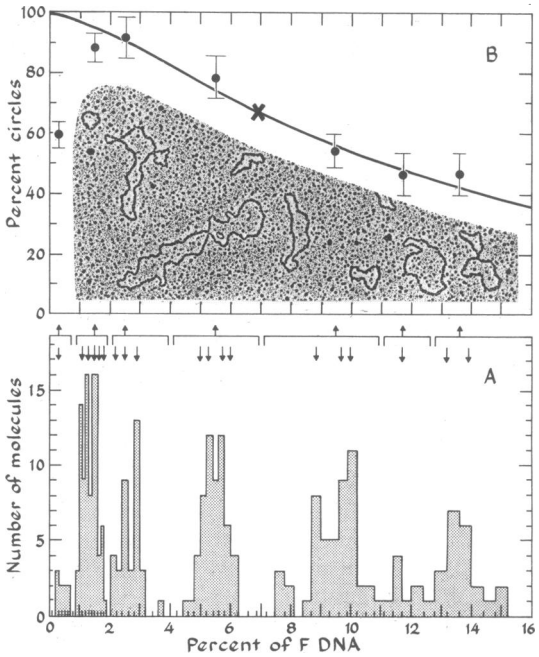

FIG. 5. (*A*) Fragments produced by R_I endonuclease treatment of F_s(P17) DNA. Circular F_s(P17) DNA was reacted to completion with R_I endonuclease. After aqueous spreading at 25° of the cleaved DNA (0.5 μg/ml), random fields were photographed, and the lengths of 286 fragments were determined relative to circular lambda DNA present on the same grid. 10 Molecules that were greater than 16% of the length of F_s(P17) were discarded, on the assumption that they resulted from incomplete cleavage by R_I. The small divisions on the abscissa are lengths plotted in standard deviation intervals calculated from the equation $\sigma = 0.1$ [% length of F_s(P17)]$^{1/2}$ (14). 19 Size classes (denoted in their approximate locations by *downward arrows*) containing about 15 molecules each were obtained. The length of the smallest molecule (about 200 nucleotides) has a larger standard deviation than expected, probably due to measurement error. Whole F_s(P17) DNA is 1.584 the length of lambda DNA and contains about 74,000 base pairs. (*B*) Cyclization of the different size fragments in panel *A*: theoretical curve and experimental points. The R_I endonuclease cleavage fragments of F_s(P17) DNA (panel *A*) were mounted for electron microscopy by incubation of a 50-μl drop of the DNA (in 0.45 M ammonium acetate containing 0.5 μg of DNA/ml and 70 μg/ml of cytochrome *c*) on a Teflon block in a 3.0° cold room; a parlodion-coated grid was touched to it. The above conditions are equivalent to 0.5 M ammonium acetate at 3.7° (27). The lengths of 300 random molecules were measured, put into one of seven distinguishable size classes (indicated by *upward arrows*), scored as circular or linear, and plotted at the position corresponding to the mean length of the molecules within each size class. The *error bars* are calculated as given in Fig. 1. An additional 86 molecules were scored for the smallest size class. Very little polymerization occurred at this low temperatue and concentration, since the number average molecular lengths of the fragments at 25° and 3.7° were 5.1 and 5.8% of the F_s(P17) DNA, respectively. The micrograph shows a field of hydrogen-bonded fragments at a magnification as shown on the *abscissa* in panel *A*.

TABLE 1. *Ratios of single- to double-strand mass in denatured (snap-back) linear oligomers of SV40(L_{RI})*

Oligomer size (n, $n+1$)	Total number of monomer units scored	Ratio of single- to double-strand mass	
		Experimental*	Predicted†
2, 3	612	1.00	1.00
4, 5	315	0.57	0.60
6, 7	175	0.57	0.46
8, 9	91	0.14	0.38
10, 11	30	0.25	0.25
12, 13	12	0.20	0.22
14, 15	14	0.17	0.21
16, 17	16	0.00	0.20
	Weight average	0.723	0.736

* See legend to Fig. 4.

† Calculated from the binomial coefficients on the assumption of equal probability of joining "head-to-head" and "head-to-tail." For example, a tetramer can join as (4+); (3+, 1−); (2+, 2−); (1+, 3−); (4−), in the respective ratio 1:4:6:4:1. After denaturation, the average single- to double-strand mass ratio is 0.60. The calculated ratio is the same for n and $n+1$, where n is any even integer.

fashion about the site, as shown by the numbered arrows in Fig. 3. The base sequence of the two cohesive ends generated from such a symmetrical site must be identical, as well as complementary.

If they are identical, heads and tails can join at random, and, if two heads or two tails are covalently joined, then a plus strand will be joined to its complementary minus strand. After denaturation a single strand of such a molecule would instantly renature (snap-back) to form a "hairpin" duplex.

This possibility was tested by denaturing, in alkali, covalently-joined linear oligomers of SV40(L_{RI}) (produced by treatment with DNA ligase at high DNA concentration), quickly neutralizing the pH, and mounting the DNA for electron microscopy by the aqueous technique. The unrenatured single strands, appearing as bushes (Fig. 4*a–c*), are readily distinguished from the renatured (snap-back) double strands (*d* and *e*). Since the DNA before denaturation is entirely duplex and a DNA sample not treated with ligase yields only single strands after denaturation, it is clear from Fig. 4 that the ligase has joined plus and minus strands. As shown in Table 1 the observed ratio of single- to double-strand mass in all size classes of all "snap-back" molecules corresponds closely to the predicted ratios for random covalent joining of heads and tails of SV40(L_{RI}) molecules. Therefore, the two cohesive ends of SV40(L_{RI}) have identical sequences, and it follows that the R_I endonuclease cleavage site must have a 2-fold axis of symmetry. Also, the single-strand ends must contain an even number of nucleotides; with an odd number of nucleotides there would be at least a one base-pair mismatch in the head-to-head or tail-to-tail joined molecules. This one mismatch would lower the T_m by 15–20° (21) (assuming ligase treatment has no effect on T_m) and would thus lower the probability of this type of joining at least 200-fold.

All R_I Endonuclease-Generated Ends Are Probably Identical in Their Nucleotide Sequence. Although the cohesive ends produced by R_I endonuclease cleavage of SV40 DNA are the same, one may ask whether identical ends are generated by

TABLE 2. *Number of R_I endonuclease cleavages in various DNAs*

DNA (Molecular weight × 10^{-6})		Number of cleavages	Average number of base-pairs/cleavage
SV40	3.4	1	5,100
Polyoma*	3	1	5,000
PM2	6.6	0	>10,000
Mouse mitochondrial*	10.3	2	7,800
Adenovirus 2†	24.8	5	7,500
F_8(P17)	48.7	19 ± 1	3,900
		Weighted average	5,260

* J. F. Morrow and D. L. Robberson, (unpublished).

† C. Mulder, U. Petterson, H. Delius, and P. A. Sharp, (unpublished).

cleavages in other DNAs. To answer this question, fragments generated by R_I endonuclease cleavage of the large episomal DNA, F_8(P17), were tested for their ability to cyclize under

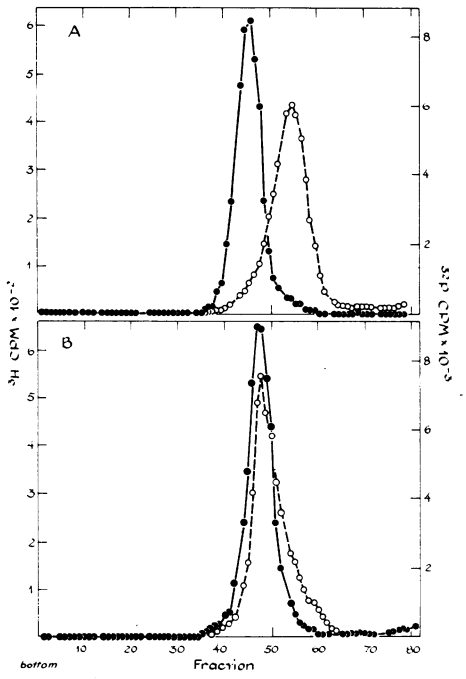

FIG. 6. Demonstration of the covalent joining of λdv-120 and SV40 DNA by CsCl density gradient centrifugation. 0.58 μg of λdv-120(L_{RI}) [³H]DNA (12,000 cpm/μg) and 0.25 μg of SV40(L_{RI}) [³²P]DNA (about 70,000 cpm/μg) were incubated with *E. coli* DNA ligase at a final DNA concentration of 83 μg/ml. In one mixture, each of the DNAs was treated with the ligase in separate reactions; the two were mixed together after the reactions were stopped with EDTA (panel *A*). In the other reaction (panel *B*) both DNAs together were incubated with the ligase. Electron microscopy of each of the mixtures showed that 65% of the mass was contained in molecules averaging 7.7 SV40 DNA equivalents. Each reaction was added to 4.4 ml of CsCl in 10 mM Tris·HCl–1 mM EDTA (pH 7.5) (final ρ was 1.698), then centrifuged at 15°, 35,000 rpm for 46 hr in a Spinco no. 50 rotor. ●——●, λdv-120 DNA; ○——○, SV40 DNA.

the conditions described for forming hydrogen-bonded SV40 DNA circles. F_8(P17) DNA is cleaved to 19 ± 1 fragments (Fig. 5A), ranging from 200 to 10,000 nucleotide pairs in length. If R_I endonuclease produces only one type of single-stranded end; all fragments will form hydrogen-bonded circles. On the other hand, if a fragment has a terminal sequence that differs by even one base, <1% hydrogen-bonded circles will be formed under these conditions. Accordingly, the limit digest of R_I endonuclease on F_8 (P17) was incubated and prepared for electron microscopy at 3°; the % circles in various size classes (Fig. 5A) was then scored (Fig. 5B). If all R_I-produced single-strand ends are identical, the expected % circles in any size class would be as indicated by the curve in Fig. 5B. The % circles as a function of length is obtained from the relation K for any fragment = K for SV40(L_{RI}) × (length SV40/length F_8(P17) fragment)$^{3/2}$ (22). K for SV40, under these conditions, is 2.0 (from Fig. 1).

Except for the two smallest size classes, the experimental points are within one standard error of the theoretical curve. If a given fragment within a size class could not be cyclized, the deviation of the experimental point would be more than two standard errors. Conceivably, one fragment, occurring in the second-smallest size class, could have had nonidentical ends. But, if one fragment had nonidentical ends, a second noncyclizable fragment also should have occurred, but none are evident. The deviation in the frequency of cyclization seen for the smallest fragment is probably due to the stiffness of a DNA helix, since the smallest fragment is considerably smaller than one statistical segment length (23). Therefore, we conclude that all the R_I-generated ends in F_8(P17) DNA are very likely identical.

How Many Nucleotides Are Needed to Specify the R_I Endonuclease Restriction Site? Assume, as a first approximation, that the nucleotide order in DNA is random and that the number and sequence of nucleotides specifying the R_I restriction site is unique; then, the probability that a given sequence of length n will occur in a long random sequence composed of 4 bases is $1/4^n$. For n equal to 4, 5, 6, and 7, the statistical frequency of the number of restriction sites is of the order of one in 256, 1024, 4100, and 16,400 base-pairs, respectively. Although the sample is limited, the number of R_I endonuclease cleavages produced in several different sizes of DNAs (Table 2) [coliphage DNAs have been omitted because of a possible selection against sequences that sensitize them to restriction (1)] gives values that fit closest to $n = 6$.

R_I Endonuclease-Generated Fragments from Different DNAs Can Be Covalently Joined. If R_I endonuclease generates identical cohesive ends at the cleavage sites of all DNAs, it should be possible to covalently join DNA segments generated in this way. This has been accomplished with SV40(L_{RI}) DNA and the linear monomer duplexes [λdv-120(L_{RI})] formed from λdv-120 supercoiled dimer circles with R_I endonuclease. When the two DNAs were incubated with *E. coli* DNA ligase separately, then mixed and centrifuged to equilibrium in a CsCl gradient, SV40 DNA formed a band at a lower density than the λdv-120 DNA (Fig. 6A). When the two DNAs were mixed together, incubated with ligase, then centrifuged in the same way, the SV40 and λdv-120 DNAs banded together at an intermediate density. This experiment proves that a cohesive end of SV40(L_{RI}) can be covalently joined to a cohesive end of λdv-120(L_{RI}). Quite likely, any two R_I endonuclease fragments can be covalently joined.

DISCUSSION

R_I endonuclease, in conjunction with DNA ligase, provides a means for *in vitro*, site-specific recombination: Any two DNAs with R_I endonuclease cleavage sites can be "recombined" at their restriction sites by the sequential action of R_I endonuclease and DNA ligase. These hybrid DNAs can be cleaved by R_I endonuclease to regenerate the original DNAs. It is more difficult to limit the mode of joining of two different DNA duplexes by this approach than can be done with the protocol developed by Jackson and Berg (24) and by Lobban and Kaiser (manuscript in preparation). Nevertheless, what the joining of R_I endonuclease-cut fragments lacks in specificity is compensated for by the ease and efficiency of the reaction; quite possibly, with appropriately chosen concentrations and molecular species of DNA one may, in this simple way, be able to generate specifically oriented recombinant DNA molecules *in vitro*.

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