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Strand-Specific Nick in Open Circular R-Factor Deoxyribonucleic Acid: Attachment of the Linear Strand to a Proteinaceous Cellular Component

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The fraction of covalently closed circular R-factor R12 deoxyribonucleic acid (DNA) isolated from *Proteus mirabilis* was found to depend upon the pH of the buffer used to suspend the cells at the time of lysis. Examination of the open circular DNA revealed that there is one strand-specific nick per R-factor molecule. This same DNA strand also appeared to be bound to a proteinaceous particulate fraction of the cell.

In recent years, covalently closed circular (CCC) or supercoiled deoxyribonucleic acid (DNA) has been identified in the DNA prepared from a variety of sources (4). These include the DNA from animal and bacterial viruses, the DNA from cellular organelles in eukaryotes, and extrachromosomal DNA in bacteria (colicinogenic factors. F′ factors, and R factors). The widespread occurrence of CCC DNA has prompted considerable interest in its origin and possible significance in the control of DNA replication. During the past several years, we have studied the CCC form of R-factor DNA in *Proteus mirabilis* (9; C. Hershberger, S. Mickel, and R. Rownd, Fed. Proc., 28:466, 1969; S. Mickel, C. Hershberger, and R. Rownd, Bacteriol. Proc., p. 53, 1968). Our experiments have shown that the fraction of R-factor DNA which is in the supercoiled form depends on the conditions of DNA isolation, particularly upon the pH of the buffer used to suspend the cells at the time of lysis (Hershberger et al., Fed. Proc. 28:466, 1969). Under conditions that yield little or no CCC DNA of the R factor R12, there appears to be a single break in a unique strand of the R-factor DNA duplex. Moreover, under these conditions, the linear strand of the R-factor DNA appears either to be attached to a proteinaceous particulate fraction of the cell or to be attached to a protein which facilitates the entrapment of this strand in a particulate fraction of the cell.

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MATERIALS AND METHODS

Bacterial strains. The strain of *P. mirabilis* (Pm 15) which was used has been described previously (5). The R factor R12 used in these studies is a derivative of the R factor *NR1* (10). R12 was transferred to Pm 15 essentially as described previously (10). A more detailed description of the isolation and characterization of R12 will be presented elsewhere (in preparation). Pm 15/R12 was cultured in Penassay broth (Difco) except for the experiment shown in Fig. 2 in which M9 minimal medium was used as described previously (6, 8).

Isolation of DNA. With the exception of the experiments carried out at pH 12.4, DNA was prepared from either exponential or stationary-phase cells as described previously (8) except that SSP buffer (0.15 M NaCl + 0.015 M NaH_2PO_4, pH 8.0) was used to resuspend the cells for lysis instead of saline-ethylenediaminetetraacetate (EDTA), pH 8, buffer. Cells washed in SSP were resuspended in 1 ml of SSP at a concentration of 1.5×10^{10} cells/ml. The pH of the cell suspension was adjusted to the desired value by the addition of 0.4 M K_3PO_4 (pH 13) as described in the Results section. The cells were lysed by the addition of 0.08 ml of 25% sodium dodecyl sulfate (SDS) and incubating for at least 5 min at 37 C. A 0.12-ml amount of 3 M sodium acetate (pH 8.0) was added to each lysate and, after gentle mixing, the DNA was precipitated by adding 0.64 ml of isopropanol. The DNA was collected on a small glass stirring rod and dissolved in 1.0 ml of SSC/10 (0.015 M NaCl + 0.0015 M sodium citrate, pH 7.0). DNA

samples were heated at 65 C for 30 min to inactivate any nucleases which might be present in the DNA preparation.

In the experiments carried out at pH 12.4, two procedures which are minor modifications of the methods used previously (5) were employed to lyse the cells. In the first procedure, the cells were washed several times with ice-cold SSP buffer at pH 8. The cells were lysed by adding 2×10^9 cells (in a volume of 0.1 ml of SSP buffer, pH 8) to 0.65 ml of 0.12 M K_3PO_4 which had been previously titrated to pH 13 with KOH. After complete cell lysis, 1 g of solid CsCl (Harshaw, optical grade) was added to the solution (final density, 1.750 g/cm^3). A 0.70-ml amount of this solution was centrifuged in a Spinco model E analytical ultracentrifuge. In the second procedure, 0.07 ml of cells in SSP buffer, pH 8 (cell density 2×10^{10}/ml), were added directly to an assembled 12-mm analytical ultracentrifuge cell. After the addition of 0.07 ml of 0.4 M K_3PO_4 which had been titrated to pH 13 with KOH, the centrifuge cell contents were gently mixed until complete lysis of the bacteria occurred. Next, 0.56 ml of a saturated CsCl solution in water was added and, after gentle shaking, the sample was centrifuged in an analytical ultracentrifuge. After the centrifuge run, the pH and the density of the alkaline CsCl solution were measured and found to be 12.5 ± 0.1 and 1.750 g/cm^3, respectively.

Density gradient centrifugation: The DNA samples were examined in neutral and alkaline analytical CsCl gradients as described previously (5, 6, 8). In neutral preparative gradient experiments, the DNA (400 μg) was centrifuged in a CsCl solution (1.710 g/cm^3) containing 0.01 M EDTA, pH 8. In alkaline preparative gradient experiments, the DNA (75 μg) was centrifuged in a CsCl solution (1.760 g/cm^3) containing 0.12 M K_3PO_4; the final pH was 12.4. In both neutral and alkaline runs, 5 ml of solution in polyallomer tubes was centrifuged at 35,000 rpm in a Spinco Ti 50 rotor for 60 h at 20 C. The rotor was then allowed to coast to a stop (unbraked), and 0.04- to 0.05-ml fractions were collected from the bottom of the tube. Fractions from the alkaline run were collected onto 2.4-cm glass-fiber filters.

Alkaline sucrose gradient centrifugation. An isokinetic 5 to 27% alkaline sucrose gradient, adapted to an SW40 rotor by D. Perlman in our laboratory, was prepared with the use of a Buchler Polystaltic Pump and mixing chamber. A volume of 12.2 ml of 5% sucrose, 1 M NaCl, and 0.3 M NaOH was mixed with 25.4 ml of 27% sucrose, 1 M NaCl, and 0.3 M NaOH. The height of the 5% sucrose solution in the mixing changer apparatus was adjusted to equal that of the 27% sucrose solution. The DNA sample to be analyzed was layered on the gradient and centrifuged at 20 C in an SW40 rotor at 35,000 rpm for 15 h. The rotor was then coasted (unbraked) to a stop, and 0.1-ml fractions were collected from the bottom of the tube.

RESULTS

Density profiles of DNA prepared from *P. mirabilis* harboring the R factor R12. When harbored by *P. mirabilis*, the DNA of the R factor R12 appears as a satellite band with a density of 1.712 g/cm^3 to the chromosomal DNA (1.700 g/cm^3) in a neutral CsCl gradient (Fig. 1A). The proportion of the R12 DNA band is 10% of the chromosomal DNA band in both exponential- and stationary-phase cultures. In an alkaline CsCl gradient (pH 12.5), there is an increase in density of the DNA (Figure 1B) due to the titration of guanine (G) and thymine (T) residues of the DNA strands and the binding of Cs^+ ions by these ionized bases (13). Since

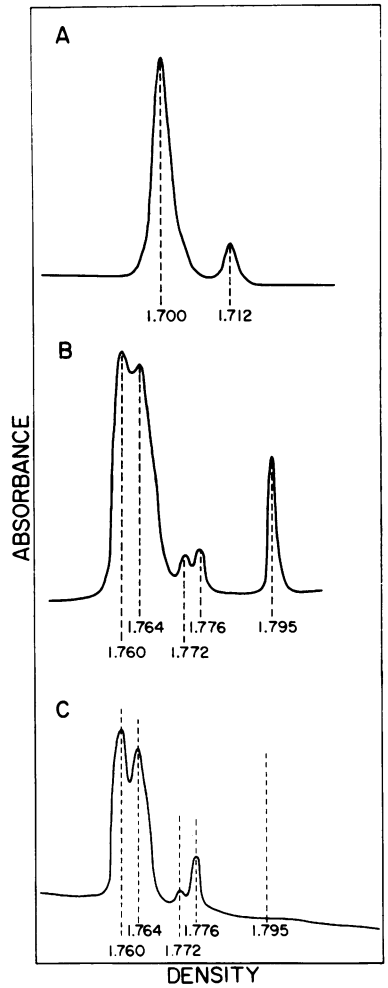

FIG. 1. *Density profiles of the DNA prepared from P. mirabilis harboring the R factor R12. (A) Native DNA in a neutral CsCl gradient (pH 8.0); (B) DNA in an alkaline CsCl gradient (pH 12.5); (C) DNA from cells lysed directly in alkaline SSP (pH 12.4); sufficient concentrated CsCl was added to the cell lysate to obtain a final density of 1.750 g/cm^3.*

there is an asymmetric distribution of G + T residues between the complementary strands of *P. mirabilis* chromosomal DNA (5), this DNA forms two bands with densities of 1.760 and 1.764 g/cm^3 in an alkaline CsCl gradient. R12 DNA also forms two bands with densities of 1.772 and 1.776 g/cm^3 in an alkaline CsCl gradient, suggesting that there is also a bias in the distribution of G + T residues between the complementary strands of the R-factor DNA. CCC R12 DNA has a density of 1.795 g/cm^3 in an alkaline CsCl gradient. This value is 0.021 g/cm^3 greater than the average density of the two R12 DNA complementary strands, which is in agreement with the density increase of CCC DNA observed previously by other investigators (11). Thus, examination of R12 DNA in an alkaline CsCl gradient provides the means of determining the percent CCC DNA as well as identifying the complementary strands of the R-factor DNA.

In an alkaline CsCl gradient, CCC DNA is converted to the open circular form at a slow rate (12). This decay follows first-order kinetics. To correct for this decrease in the proportion of supercoiled R12 DNA in our experiments, ultraviolet absorption photographs were taken at four different time intervals, and the first order plot of the logarithm of the percent supercoiled R12 DNA versus time was extrapolated to zero time of centrifugation. The average half-life for the decay of CCC DNA was typically 15 to 20 h. This procedure provides reproducible values for the percent CCC R12 DNA for all DNA samples which have been examined.

Effect of pH on the fraction of supercoiled R12 DNA. Previously, we observed that the pH of the phosphate buffer used to suspend the *P. mirabilis* cells for cell lysis and subsequent isolation of DNA could greatly affect the fraction of R12 DNA which was in the supercoiled form (Hershberger et al., Fed. Proc. 28:466, 1969). We therefore conducted a systematic study of the effect of pH on the yield of CCC DNA. A culture of stationary-phase cells was washed and resuspended in SSP buffer, and samples of this cell suspension were titrated with 0.4 M K_3PO_4 (pH 13) to the desired pH. The rest of the DNA isolation was as described in Materials and Methods. The DNA was examined in an alkaline CsCl gradient to determine the percentage of supercoiled R12 DNA in the different samples. As shown in Table 1, the percentage of CCC DNA in the different samples is very sensitive to the pH of the buffer between pH 10 and 11. Examination of the DNA prepared at pH 11 or below in a neutral CsCl gradient revealed that all of the DNA was in the native state. In the experiment at pH 12.4, the bacterial cells were lysed directly in alkaline SSP (pH 12.5), and sufficient concentrated CsCl was added so that the resulting solution had the proper density for equilibrium sedimentation. Under these conditions, no CCC DNA was observed (Fig. 1C). It should be noted that under these conditions there was an extremely large asymmetry in the areas of the two bands corresponding to the two complementary R12 DNA strands (1.772 and 1.776 g/cm^3, respectively) whose origin will be discussed at a later point. These experiments show that the fraction of R12 DNA in the supercoiled form depends markedly on the pH of the SSP buffer in which the cells are suspended at the time of DNA isolation. The addition of EDTA to the buffer greatly reduces the difference in the CCC fraction observed at the different pH values.

To examine whether a nuclease with an alkaline pH optimum might be nicking the CCC DNA, we carried out an experiment in which ^{15}N-labeled supercoiled R12 DNA was added to a suspension of ^{14}N-labeled R$^+$ cells in SSP at pH 11. SDS was added to the mixture of ^{14}N-labeled cells and ^{15}N-labeled DNA, and the DNA was isolated as described in Materials and Methods. When the DNA was examined in an alkaline CsCl gradient (Fig. 2), the two bands corresponding to the two strands of the open circular ^{14}N-labeled R12 DNA (1.772 and 1.776 g/cm^3, respectively) could not be resolved from the bands corresponding to the complementary strands of the ^{15}N-labeled chromoso-

TABLE 1. *Effect of pH on the fraction of closed circular R12 DNA in P. mirabilis*[a]

pH of SSP buffer after lysis	Fraction closed circular R12 DNA[b]
8.0	0.65
10.0	0.85
10.5	0.30
11.0	0–0.10
12.4 (direct alkaline lysis)	0

[a] Stationary-phase cells of Pm 15/R12 were washed and resuspended in SSP buffer. Samples of the cells were titrated with 0.4 M K_3PO_4 (pH 13) to the desired pH. DNA was isolated from the different samples as described in Materials and Methods. In the experiment at pH 12.4, the cells were lysed directly in alkaline SSP (pH 12.5), and sufficient concentrated CsCl solution was added to obtain the appropriate density for equilibrium sedimentation.

[b] Value obtained by extrapolating fraction of CCC R12 DNA to zero time of centrifugation.

mal DNA (1.773 and 1.777 g/cm³, respectively). However, the fraction of supercoiled ^{14}N-labeled R12 DNA (1.795 g/cm³) could be computed from the ratio of this band to the ^{14}N-labeled chromosomal DNA band and the ratio of the total ^{14}N-labeled R12 DNA to the ^{14}N-labeled chromosomal DNA (determined by examining the DNA from ^{14}N-labeled cells in a neutral CsCl gradient). The fraction of supercoiled ^{15}N-labeled R12 DNA (1.808 g/cm³) could be determined either by this method or, more directly, from the relative amounts of the DNA in the two bands corresponding to the complementary strands of open circular ^{15}N-labeled R12 DNA (1.785 and 1.789 g/cm³, respectively) and the CCC ^{15}N-labeled R12 DNA. After extrapolation to zero time of centrifugation, these calculations showed that less than 10% of the ^{14}N-labeled R12 was in the supercoiled form, whereas over 90% of the ^{15}N-labeled R12 DNA was CCC. Thus, exogenously added supercoiled R12 DNA is not nicked in a pH 11 lysate; only the R12 DNA from cells which are lysed at this pH is found to be in the open circular form. In other experiments, it was found that essentially all of the R12 DNA was in the open circular form when the cells were lysed directly at alkaline pH (12.5) at either 0 or 37 C. It seems unlikely that a nuclease would be active at 0 C. Taken together, these findings suggest that a simple endonucleolytic attack could not account for the low percentage of supercoiled DNA at pH 11 or above.

Selective entrapment of an R12 DNA strand in a proteinaceous particulate fraction of the cells. In the experiments shown in Table 1, a characteristic variation in the nature of the density profile of R12 DNA was observed, depending on the condition used for the isolation of the DNA. Under conditions which yielded a high fraction of supercoiled DNA, the areas of the two bands corresponding to the complementary R12 DNA strands (Fig. 1B, 3A, 3B) were essentially the same. Under conditions that produced a lower fraction of CCC DNA, a marked asymmetry was observed which was due to a reduction in the relative amount of the less dense (1.772 g/cm³) R12 DNA strand. The degree of this asymmetry was greater as the fraction of supercoiled DNA in the samples decreased (Fig. 1C, 3C, 3D, 3E). Very little asymmetry was observed in a sample of supercoiled R12 DNA which was isolated by ethidium bromide preparative density gradient centrifugation and then allowed to decay with time in an alkaline CsCl gradient in the analytical ultracentrifuge (Fig. 3F). This suggests that the asymmetry observed in samples having a low percentage of supercoiled R12 DNA is due to the selective loss of the less dense R12 DNA strand.

In experiments in which the cells were lysed directly in alkaline SSP, examination of the DNA in an alkaline CsCl gradient shows that there is no supercoiled R12 DNA and almost a complete loss of the less dense R12 DNA strand (Fig. 1C and 3E). Examination of the contents of the analytical ultracentrifuge cell after the

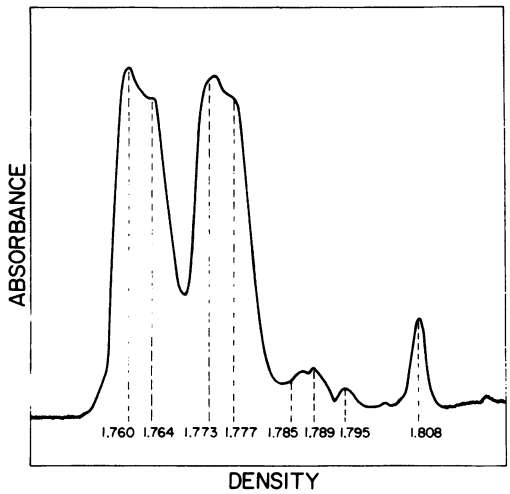

FIG. 2. *Stability of exogenous CCC R12 DNA in a P. mirabilis cell lysate in SSP buffer at pH 11. Pm 15/R12 cells were cultured in media containing $^{15}NH_4Cl$ to density-label the DNA. The cells were lysed in saline-EDTA buffer (0.1 M EDTA, pH 10.2, and 0.15 M NaCl) and isolated as described in Materials and Methods except that the DNA (60 to 75 μg) was dissolved in 0.2 ml of SSP/10. This DNA was then added to ^{14}N-labeled Pm 15/R12 cells which would contain an equivalent amount of DNA. The cells had previously been washed twice and resuspended in 0.5 ml of SSP, pH 8. This mixture of ^{14}N-labeled cells and ^{15}N-labeled DNA was titrated to pH 11.0 with 0.4 M K_3PO_4, pH 13. SDS was added to a final concentration of 2% to lyse the cells, and the DNA was isolated as described in Materials and Methods. The DNA was analyzed in an alkaline CsCl gradient, and the fraction of ^{14}N- and ^{15}N-labeled CCC R12 DNA was calculated as described in the text. In the density profile shown in this figure, the ^{15}N-labeled CCC R12 DNA band was 14% of the ^{15}N-labeled chromosomal DNA band after extrapolation to zero time of centrifugation. This corresponds to a ^{15}N-labeled CCC R12 DNA fraction of between 90 and 100%. The ^{14}N-labeled CCC R12 DNA band extrapolated to only about 1% of the area of the ^{14}N-labeled chromosomal DNA. This would correspond to less than 10% of the total ^{14}N-labeled R12 DNA being CCC after cell lysis in SSP buffer at pH 11.*

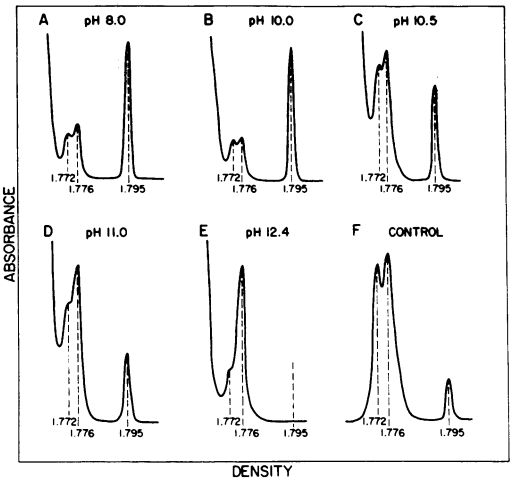

FIG. 3. *Asymmetry in the areas of the complementary strands of R12 DNA as a function of the pH of the buffer used to suspend the cells for DNA isolation. The P. mirabilis chromosomal DNA bands have not been included in this figure to emphasize the DNA distribution of the R12 DNA bands in the gradient. After cell lysis at the indicated pH, the DNA was isolated as described in Materials and Methods and then examined in an alkaline CsCl gradient (profiles A-D). In profile E, the cells were lysed directly in alkaline SSP. In profile F, CCC R12 DNA isolated by ethidium bromide-preparative CsCl density gradient centrifugation was allowed to decay for 25 h during centrifugation in an analytical alkaline CsCl gradient.*

completion of a centrifuge run revealed a thin pad of material at the meniscus which presumably consists of cellular debris resulting from direct lysis of the cells in alkaline SSP. To examine whether the missing strand might be trapped in this material, we lysed bacterial cells in alkline SSP in an analytical ultracentrifuge cell as in the previous experiments; sufficient concentrated CsCl was added to attain the proper solution density (1.750 g/cm^3), and the solution was centrifuged at 44,000 rpm for 18 h. As in the previous experiments, the density profile of the DNA showed no CCC R12 DNA and a large asymmetry between the R12 complementary strands (Fig. 4A). After the centrifuge run, the CsCl solution was removed from the analytical cell, and the pad was washed with SSC while still in the analytical ultracentrifuge cell. A solution of Pronase (final concentration of 2 mg/ml) was added, and the cell contents were incubated at 37 C for 30 min. Alkaline CsCl solution was then added to obtain the correct density, and the Pronase-treated material was centrifuged to equilibrium. As shown in Fig. 4B, Pronase treatment of the insoluble pad resulted in the release of both chromosomal and R12 DNA. This solubilized DNA had a lower molecular weight than in our previous experiments as indicated by the broader DNA bands, presumably as a result of some degradation which occurred during resolubilization of the DNA. This prevented the resolution of the DNA bands corresponding to the complementary strands of the chromosomal DNA and of the R12 DNA. However, it can be seen that the asymmetry in the R12 complementary DNA strands is essentially reversed. Thus, during direct cell lysis in alkaline SSP, it appears that the less dense R12 strand is entrained in the particulate material which forms the pad during centrifugation. Since this strand can be released by Pronase treatment, it appears that the entrainment of this strand is mediated by a protein.

The DNA isolation procedure which we have used in our experiments is an abbreviated version of the Marmur procedure (7; see Materials and Methods) which results in an almost quantitative recovery of bacterial DNA. Exten-

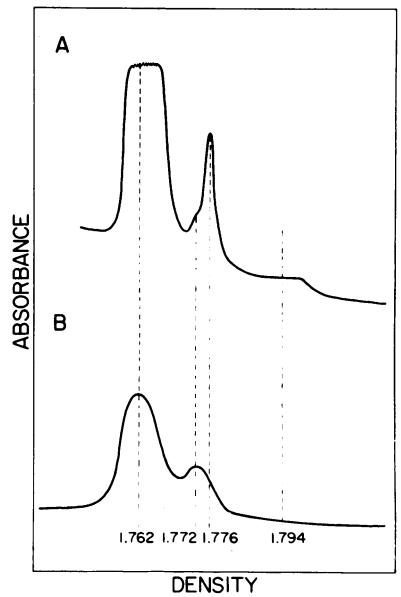

FIG. 4. *Release of the less dense strand of R12 DNA from the proteinaceous material at the meniscus in an alkaline CsCl gradient by treatment with Pronase. The details of these experiments are described in the text. The densities of the DNA bands were calculated from the position of a Micrococcus lysodeikticus marker DNA (not shown) which was included in the alkaline CsCl gradients. The P. mirabilis chromosomal DNA in profile A is not on the linear range of the photographic film and cannot be used for quantitation of the areas of the bands.*

sive deproteinization is not involved in this procedure, and generally a thin pad is observed at the meniscus after centrifugation in a CsCl gradient. Since R-factor DNA strand asymmetry was also observed for DNA isolated at a higher pH under conditions that do not result in denaturation of the DNA (Fig. 3D), we examined whether a protein attached to a unique strand of the native DNA duplex could also be responsible for this observed asymmetry. A DNA sample which was isolated from cells lysed in SSP buffer at pH 11 was divided into two equal samples. Pronase was added to one of them to a final concentration of 2 mg/ml. Both samples were then incubated at 37 C for 30 min and their DNA was then examined in an alkaline CsCl gradient. A CCC R12 DNA band (1.795 g/cm^3) was not observed for either sample in these experiments. As shown in Fig. 5, the untreated sample (A) was found to have an asymmetry in the areas of the bands corresponding to the R12 DNA complementary strands, whereas this asymmetry was found to be greatly reduced in the sample which had been treated with Pronase (B). This suggests that a unique strand of the native R12 DNA duplex is attached to a protein and that this association facilitates the selective entrapment of this strand in the pad material during centrifugation in an alkaline CsCl gradient.

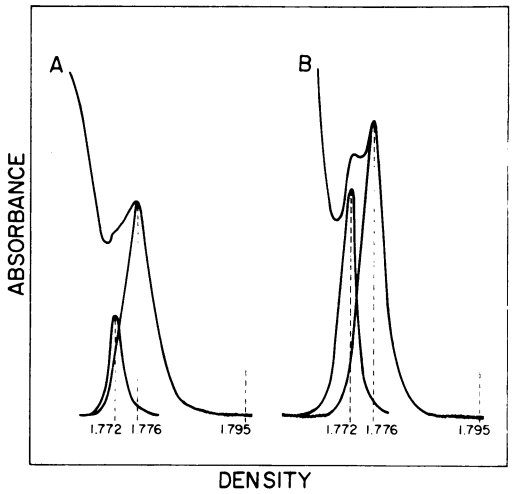

FIG. 5. *Effect of Pronase on R12 DNA strand asymmetry. A Penassay broth culture of Pm 15/R12 cells was washed and resuspended in SSP buffer, pH 8. The cells were then titrated to pH 11 with 0.4 M K_3PO_4, pH 13, and lysed with SDS. After DNA isolation and heat treatment at 65 C (see Materials and Methods), the DNA sample was divided into two portions. Pronase was added to one of them to a final concentration of 2 mg/ml, and both samples were incubated for 30 min at 37 C. A CCC band (1.795 g/cm^3) was not observed for either sample when the DNA was examined in an alkaline CsCl gradient. The P. mirabilis chromosomal DNA bands have not been included in this figure to emphasize the distribution of the R12 DNA bands in the gradient. (A) DNA isolated from cells lysed in SSP buffer at pH 11; (B) same DNA sample as in A after treatment with Pronase. The 1.772 and 1.776 g/cm^3 bands shown in A and B were resolved in the following way. The less dense half of the 1.776 g/cm^3 band was drawn as the mirror image of the more dense half of this band about the mean peak density of 1.776 g/cm^3. The less dense half of the 1.776 g/cm^3 band was then subtracted from the actual density profile in this region to construct the more dense half of the 1.772 g/cm^3 band. The less dense half of the 1.772 g/cm^3 band was then drawn as the mirror image of the more dense half about the mean peak density of 1.772 g/cm^3 band.*

Strand specificity of the nick in open circular R12 DNA. After determining that a specific R12 strand was entrained in a proteinaceous pad in an alkaline CsCl gradient under DNA isolation conditions yielding a low fraction of supercoiled DNA, we then examined the structure of the open circular DNA. ^{3}H-thymine-labeled DNA was prepared from cells lysed in SSP at pH 11 where the CCC DNA fraction would be greatly reduced and yet the R12 DNA could still be isolated in its native state. The DNA was then treated with Pronase (final concentration of 2 mg/ml), and the R12 DNA was fractionated in a neutral CsCl gradient in a preparative ultracentrifuge. After dialysis against SSC/10, the ^{3}H-thymine-labeled R12 DNA was centrifuged in an isokinetic 5 to 27% alkaline sucrose gradient. Two DNA peaks were observed with essentially no skewing toward the lower molecular weight side (Fig. 6A). This indicates that the open circular molecules contained only a single break in one of the two strands of the R12 DNA circular duplex. The more rapidly sedimenting species in Fig. 6A (peak 1) should correspond to the circular R12 DNA strand of the same molecular weight as the linear R12 DNA strand (peak 2). The circular strand sediments more rapidly because it has a lower frictional coefficient (11).

To examine whether the nick was in a unique strand of the duplex, fractions were pooled from peak 1 (circular strand) and from peak 2 (linear strand). These pooled fractions of ^{3}H-thymine-labeled R12 DNA strands were then mixed with ^{14}C-thymine-labeled DNA prepared from *P. mirabilis* harboring R12 and centrifuged in an alkaline CsCl preparative gradient. In Fig. 6, it is shown that most of the circular R12 strand from peak 1 has the same

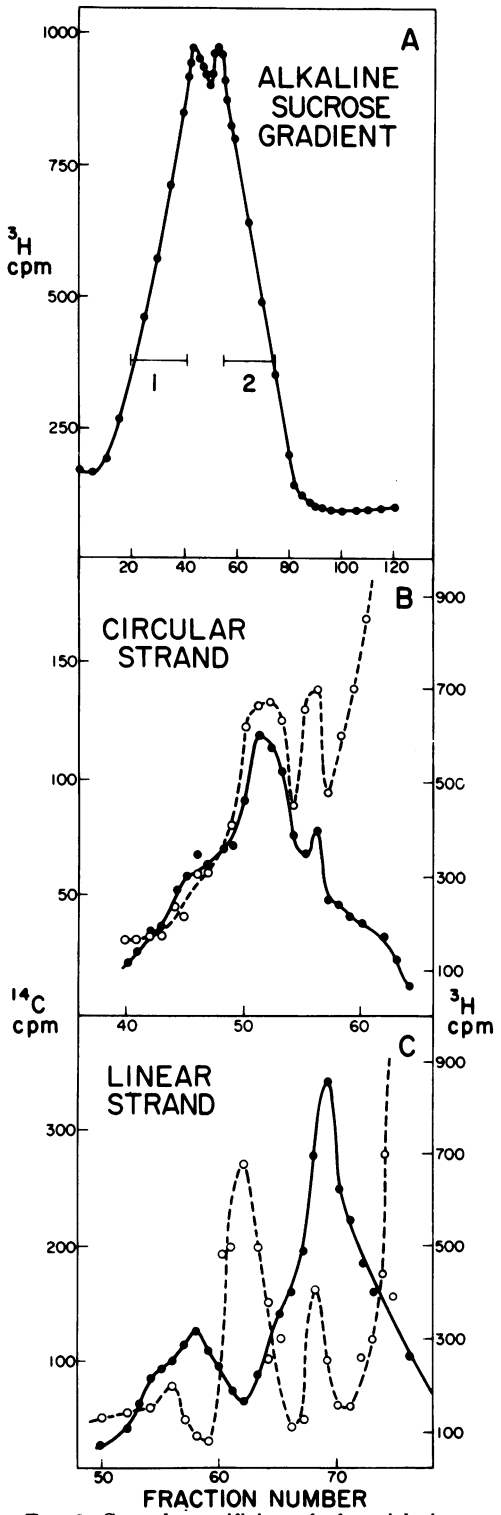

FIG. 6. *Strand specificity of the nick in open circular R12 DNA. ^{3}H-thymine labeled circular and linear strands of R12 were separated in an isokinetic alkaline sucrose gradient (A) as described in the text. Pooled fractions containing the circular (peak 1) and linear (peak 2) strands were then mixed with ^{14}C-thymine labeled DNA from P. mirabilis harboring R12 and centrifuged in an alkaline CsCl preparative gradient. (B) circular R12 strand; (C) linear R12 strand. Symbols: ●, ^{3}H-labeled strands of purified R12 DNA; ○, ^{14}C-labeled Pm 15/R12 DNA. In B and C, the solution density increases from right to left.*

density as the more dense R12 complementary strand in an alkaline CsCl gradient (Fig. 6B) and that the linear R12 strand corresponds to the less dense R12 complementary strand (Fig. 6C). Therefore, the single strand interruption must occur in a specific strand of the R12 duplex. It follows that the linear strand is the one which is almost totally entrained in the proteinaceous particulate fraction which forms the pad when the cells are lysed directly in alkaline SSP and then examined in an alkaline CsCl gradient.

DISCUSSION

Our results indicate that the open circular form of R12 DNA resulting from lysis of *P. mirabilis* at pH 11 contains only one single-strand break in a unique strand of the R-factor DNA duplex. In an alkaline CsCl gradient, this same DNA strand appears to be entrained in a pad which forms at the meniscus. Since this strand can be released by treatment with Pronase, it appears that entrainment of the linear strand in the particulate fraction which forms the pad is mediated by a protein.

Since the linear strand of R12 DNA has a molecular weight of approximately 35×10^6 (in preparation), it seems unlikely that the attached protein would be large enough to lower the buoyant density of the linear strand-protein complex so that it would float at the meniscus. Assuming the protein to have a density of 1.3 g/cm^3, it would have to have a molecular weight greater than 6×10^6 to produce this effect. Our present data do not distinguish whether the linear strand is attached directly to a particulate fraction of the cell, perhaps the cell membrane, or is attached to a protein which facilitates the entrainment of the linear strand in the pad at the meniscus. In any case, the explanation is unlikely to be trivial, since a unique strand of the R factor duplex is involved.

Since the replication of bacterial episomes and plasmids occupies only a small fraction of the cell division cycle (1, 8, 9), our findings raise the question of the structure of the nonreplicating form of R-factor DNA in situ. Depending on the pH of the buffer used to

suspend the cells for lysis, R12 DNA can be isolated in either the supercoiled or open circular form. R12 DNA could exist as CCC molecules which are nicked during cell lysis or as a nicked circular molecule which is closed during lysis. If R12 DNA is CCC in situ and the single strand scission is due to an endonuclease in the cell lysate, this enzyme must have some very unique properties. It must have maximal activity above pH 11 and have essentially no activity at pH 10. Moreover, the enzyme must introduce only one single strand scission per R-factor molecule which is in a unique strand of the duplex. Finally, it would only attack the DNA released from cells at the time of cell lysis and not exogenous CCC DNA which has been added to the cell suspension. The DNA in vivo might also be present as a nicked circle having a break in a unique strand which is closed by a ligase enzyme upon lysis. This enzyme would have maximal activity at pH 10 but have little or no activity above pH 11. Although it is very difficult to distinguish between these two possibilities in any rigorous way at the present time, the latter explanation seems to be the simpler alternative. However, our findings are also consistent with a nonenzymatic mechanism if one assumes that a specific strand of CCC R12 DNA is attached to a particulate fraction of the cell, such as the cell membrane, in such a way that certain conditions of cell lysis would cause a preferential DNA strand break to occur in the strand to which the protein is attached.

Our data indicate that the linear strand of R12 DNA is covalently attached to a protein when the R factor DNA is isolated in the open circular form. On the other hand, when isolated in the supercoiled form, there is no indication that a protein is attached to the R factor DNA. It seems most reasonable to assume that the protein is normally attached to the R-factor DNA in situ, since it seems extremely unlikely that a unique strand of R12 DNA would acquire a covalently bound protein as an artifact during cell lysis. If R12 DNA exists in the supercoiled form in situ, then it would appear that the protein is released from the DNA under cell lysis conditions in which the R-factor DNA remains CCC. Under conditions yielding a strand-specific nick in R12 DNA, the linear strand-protein complex would appear to be stabilized. If R12 DNA exists in the open circular form in situ, then our findings are simply explained by assuming that the protein would remain attached to the linear strand until conversion of the R12 DNA to the supercoiled form by ligase action results in its release. There is presently no evidence to distinguish between these possibilities.

While our work was in progress (9; Hershberger et al., Fed. Proc. 28:466, 1969; Mickel et al., Bacteriol. Proc., p. 53, 1968), Helinski and his co-workers reported a DNA-protein complex for the DNA of colicin factors E_1 and E_2 in *Escherichia coli* which has many of the properties of CCC DNA (2, 3). Various treatments can relax this complex to form open circular DNA having a nick in a unique strand of the DNA duplex. In the case of *Col* E_2, prior heat treatment alters the complex in such a way that it is converted to authentic supercoiled DNA by agents which were capable of converting it to the open circular form (2). The open circular DNA-protein complex of R12 may be the R-factor counterpart of the DNA-protein complex observed by Helinski and co-workers. In *E. coli*, about 10% of R12 DNA exists as a DNA-protein relaxation complex (in preparation). We have not been able to isolate this type of complex in *P. mirabilis* since this host is much more difficult to lyse than *E. coli* and isolation requires gentle lysis of the cells. Since essentially all of the linear strands of open circular R12 DNA can be entrained in the pad at the meniscus in an alkaline CsCl gradient, all of the R12 DNA in *P. mirabilis* could be considered to be complexed with protein. A relationship of this complex in *P. mirabilis* to the DNA-protein complex observed in *E. coli* is an interesting possibility.

It should be mentioned that variation of the pH of the buffer used to suspend the cells for lysis does not affect the fraction of CCC R12 DNA isolated from either *E. coli* or *Salmonella typhimurium*. These differences may reflect differences in the chemical composition of the cytoplasmic membranes or R-factor attachment sites in *P. mirabilis*, *E. coli*, and *S. typhimurium*.

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