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Release of *Escherichia coli* DNA from membrane complexes by single-strand endonucleases

(origin of replication/S1 nuclease/mung bean nuclease/M band)

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ABSTRACT **Treatment of gently prepared lysates of *Escherichia coli* with single-strand-specific endonuclease (S1 or from mung beans) results in the release of about 90% of the DNA from membranes, as determined by the M band technique. The released DNA has an average molecular weight of about 1.2×10^8.**

Data obtained with endonuclease S1 fit a mathematical model in which substrate sites are at or near membrane attachment sites. Data obtained with pancreatic deoxyribonuclease or x-rays fit a model for double-strand breaks at random sites along the DNA. Fitting data to these models, we estimate that there are 18 ± 5 membrane attachment sites.

The DNA remaining after S1 nuclease treatment is enriched for the region near the origin of chromosome replication. Therefore, attachment at this region appears to be chemically different from that at the other sites along the DNA.

The chromosome of *Escherichia coli* appears to be attached to the cytoplasmic membrane at 20–30 sites (1, 2). The chemical nature of DNA attachment to membranes is not known. In an attempt to study the chemical properties of this interaction we took as a point of departure the finding that exogenous denatured DNA sticks to membranes under conditions where native DNA does not (2, 3). This suggested to us a study of the effect of treating cell lysates with the single-strand-specific nucleases. We found that such nucleases from *Aspergillus oryzae* or mung bean remove DNA from the membrane in a mode that is consistent with action of the enzymes at or near the membrane-attachment sites. These results are different from those obtained by random scission along the DNA. DNA near the origin of replication is not released from membranes by treatment with S1 nuclease.

MATERIALS AND METHODS

Preparation of Spheroplasts. Ten-milliliter cultures of *E. coli* D-10 *met*$^-$ (obtained from R. Jayaraman) were grown at 37° in M9 salt glucose medium containing 0.2% casamino acids. At OD_{600} = 0.15, the culture was labeled with 50–100 μl of [*methyl*-^{3}H]thymidine (New England Nuclear Corp.; 1 mCi/ml, 6.7 Ci/mmol), and harvested at OD_{600} = 0.35. Cells were resuspended in 0.25 ml of 0.1 M Tris·HCl buffer, pH 8.1 at 4°, containing 0.1 M NaCl and 15% sucrose (wt/vol). To this was added 0.01 ml of 0.25 M Na_2EDTA at pH 8.1 and 0.025 ml of lysozyme (three times recrystallized, Sigma) at 4 mg/ml in 0.01 M Tris buffer. After gentle mixing for 3 min at 4°, the reaction was stopped by adding 0.01 ml of 1.0 M Mg acetate, pH 5. Spheroplasts were kept on ice until use. Experiments were done with *E. coli* PC2 *dnaC thy*$^-$ (obtained from Y. Hirota) using similar methods.

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Incubation of Lysate with Endonucleases. Spheroplasts (0.025 ml) were added to tubes containing 0.5 ml of buffer (0.1 M sodium acetate, pH 5.0/1 mM $ZnSO_4$/0.01 M Mg acetate) with heat-denatured calf thymus DNA at 5 μg/ml. After the addition of 0.01 ml of 5% Sarkosyl (Geigy Chemical Additives Co.) and 0.2–10 units of S1 or mung bean endonuclease, the tubes were gently rolled to mix and incubated at 45° for 10–20 min. Shark liver nuclease treatment was for 30 min at 37°, and samples with pancreatic deoxyribonuclease (0.01–0.1 μg/ml) were incubated at 0° for 5 min.

Isolation of Membrane-Associated DNA (M Band). Membrane-associated DNA is defined as DNA that sediments with Mg Sarkosyl crystals (M bands). M bands were prepared as follows. Samples were transferred by carefully pouring onto 15–47% sucrose gradients containing 0.01 M $MgSO_4$/0.01 M Tris, pH 7.6 at 4°/0.1 M KCl. Preformed Mg Sarkosyl crystals (0.1–0.2 ml), obtained by mixing two parts 0.1 M Mg acetate and one part 5% Sarkosyl, were added (without mixing) to the sample layer of each gradient. After 15 min, gradients were centrifuged in a Beckman SW 41 rotor at 15,000 rpm for 20–25 min at 4°. Tube contents were separated into a top fraction containing all material above the crystal band, and an M-band fraction containing the crystals themselves. These fractions were treated with an equal volume of 10% trichloroacetic acid for 30 min on ice, filtered through GF/A filters, and washed with 5% trichloroacetic acid followed by 1% acetic-acid or 95% ethanol before drying and measuring radioactivity in Liquifluor/toluene scintillation fluid.

Sizing of DNA. Samples (0.3 ml) of the treated lysate were adjusted to 0.2 M NaCl and 1% sodium dodecyl sulfate. [^{14}C]Thymidine-labeled T4 phage were added and the mixture was incubated at 63° for 20 min. The samples were poured gently onto 5–20% linear sucrose gradients containing 0.05 M sodium phosphate at pH 6.8, 0.1 M NaCl, and 0.05 M Na_2EDTA, and spun at 8000 rpm for 16 hr at 20° in an SW 50.1 rotor. Gradients were dripped onto filters (Whatman 3MM, presoaked with 10% trichloroacetic acid), dried, and assayed for radioactivity in Liquifluor/toluene scintillation fluid.

Molecular weights (M_r) were calculated by the method of Freifelder (4) using $S_1/S_2 = (M_{r1}/M_{r2})^{0.38}$. The T4 standard was assumed to have a sedimentation coefficient of 60 S and a molecular weight of 1.2×10^8 (4). The molecular weight of each fraction of the gradient was determined by its relative distance from the marker. The average number of breaks was calculated from the equation:

$$m = \sum_1^n \frac{M_r \text{ chromosome}}{M_r \text{ fraction}} \times (\text{proportion cpm in fraction}).$$

Enzymes. S1 was obtained from Miles Laboratories or pu-

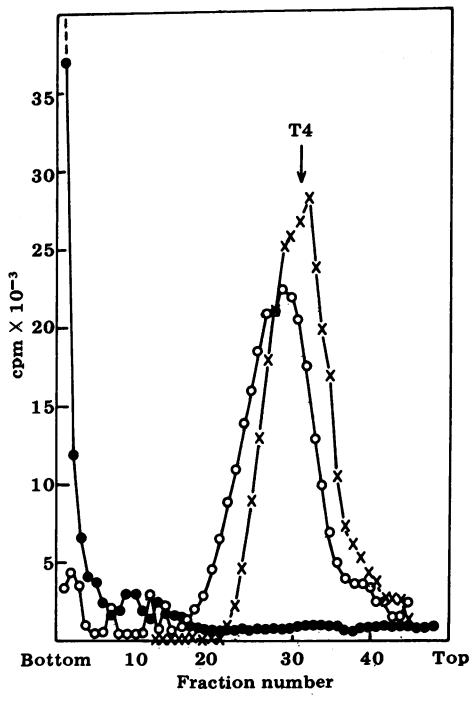

FIG. 1. Size of DNA after nuclease treatment. Lysates were incubated and sized as described in *Materials and Methods*. A portion of each sample was also fractionated on an M band gradient. Results are similar with different DNA concentrations and when T4 phage DNA marker is eliminated. The extrapolated molecular weight for the untreated sample is $\geq 1 \times 10^9$. Similar curves were obtained using mung bean nuclease. ●, Sample incubated without nuclease, 5% of the DNA is in the top fraction of the M band gradient; ○, 2 units S1, 63% of the DNA is in the top fraction; ×, 5 units S1, 85% of the DNA is in the top fraction. Arrow indicates position of T4 phage DNA marker.

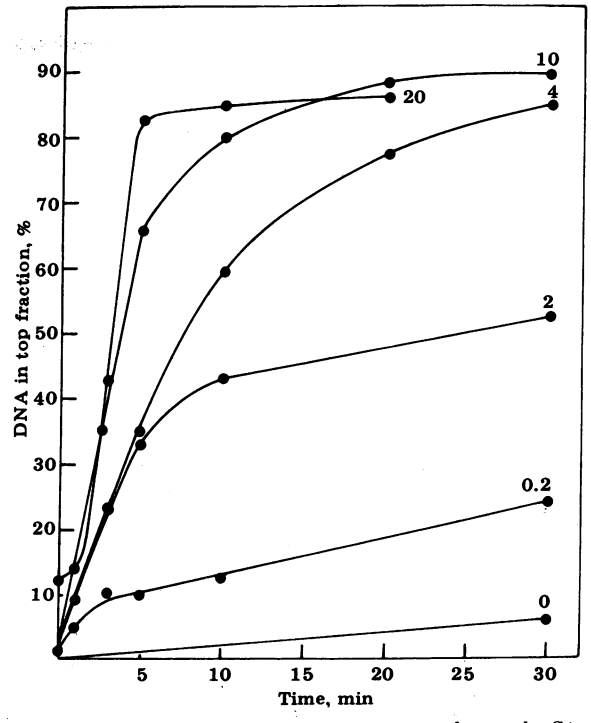

FIG. 2. Time course of DNA release from membranes by S1 nuclease. Incubation with nuclease and M band fractionation are described in *Materials and Methods*. Numbers next to curves indicate units of enzyme used.

rified from α-amylase (see below). Mung bean and shark liver endonucleases were kindly provided by R. Wells and E. Goldberg, respectively. Electrophoretically purified pancreatic deoxyribonuclease was obtained from Worthington.

Purification of S1 Endonuclease. A modification of the procedure of Vogt (5) was used to purify S1 endonuclease from α-amylase (crude, type IV-A from *Aspergillus oryzae*) obtained from Sigma. Purification steps included heat treatment, two $(NH_4)_2SO_4$ precipitations, dialysis, DEAE-cellulose chromatography, and Sephadex G-100 gel filtration. A_{260} and nuclease activity (using either native or denatured DNA as substrate) were monitored across the column fractions.

Assay of Single-Strand Endonuclease Activity. One or two microliters of each fraction was assayed for solubilization of single- or double-stranded calf thymus DNA by the procedure of Vogt (5). One unit of nuclease activity is the amount of enzyme that solubilizes 10 μg of nucleic acid in 10 min at 45°.

RESULTS

Single-Strand Nucleases Remove DNA from the Membrane. Incubation of *E. coli* lysates with the single-strand-specific endonuclease S1 from *Aspergillus oryzae* results in extensive release of DNA from membranes as determined by the M band technique. In controls incubated without nuclease, over 90% of the DNA is found in the M band. The average size of DNA fragments in the treated lysate is quite large, 1.2×10^8 daltons or approximately 1/20 of the chromosome (Fig. 1). The time course of release of DNA from the membrane is shown in Fig. 2. In samples treated with large amounts of enzyme, a plateau is reached when 85–90% of the DNA is released from the membrane. The size of the DNA fragments released from the membrane is relatively constant throughout the time course, whereas the DNA that remains in the M band decreases to this size limit with increasing enzyme activity (Fig. 3, which shows two of a series of analogous experiments).

Similar results are obtained with other single-strand-specific enzymes such as mung bean and shark liver endonucleases. The ability of these enzymes to hydrolyze RNA is apparently not responsible for release of DNA from the membrane because pretreatment with pancreatic ribonuclease has no effect on the amount of DNA in the M band either with or without S1 treatment. In a double-label experiment (not shown) it was found that when the proportion of DNA released from the membrane has reached a plateau, the enzyme is fully active against newly added lysate and that when a second plateau is reached, both the original and the newly added DNA approach the same size limit. The incubation conditions do not reduce the activity of S1 on single-stranded calf thymus DNA. Release of DNA from the membrane by S1 is inhibited strongly by the addition of single-stranded DNA to the lysate but only slightly by the addition of double-stranded DNA (Fig. 4).

On this basis we conclude that the release action is complete in the plateau region, that the DNA fragments are a limit digest of the chromosome by single-strand-specific nucleases under the conditions employed, and that the remaining membrane-associated DNA is significantly more resistant to single-strand nuclease than the DNA that has been released.

Location of the Sites of Single-Strand Endonuclease Activity. We have compared in greater detail the release of DNA from the membrane by single-strand- and double-strand-specific nucleases. We assume that our measurements of breaks in the DNA result from double-stranded scission of the DNA molecule by the nucleases. We expect that breaks of DNA at the membrane attachment sites would give results different from those expected from random breaks along the chromo-

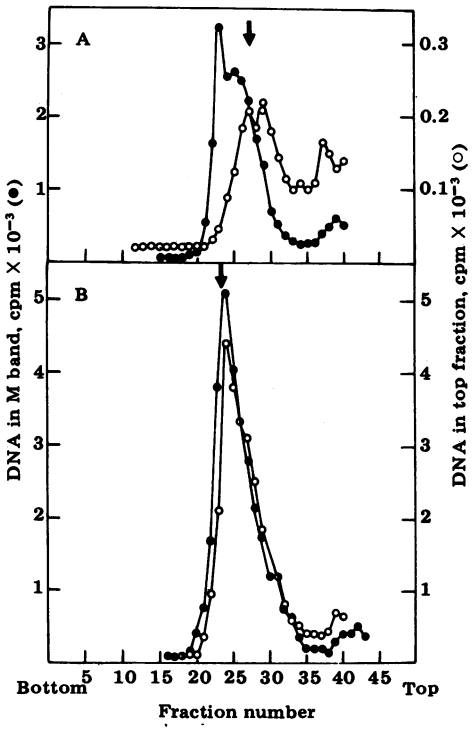

FIG. 3. Size of released and M band-associated DNA. After M band fractionation, the top fractions and M bands were carefully removed using 3-mm diameter tubes to avoid shearing. M bands were washed three times with buffer and samples were then extracted and analyzed as described in *Materials and Methods*. (*A*) 63% of the DNA was released by S1. (*B*) 88% of the DNA was released by S1. The DNA in fractions 35–41 of *A* for released DNA is also found in control samples. Arrow indicates position of T4 marker DNA.

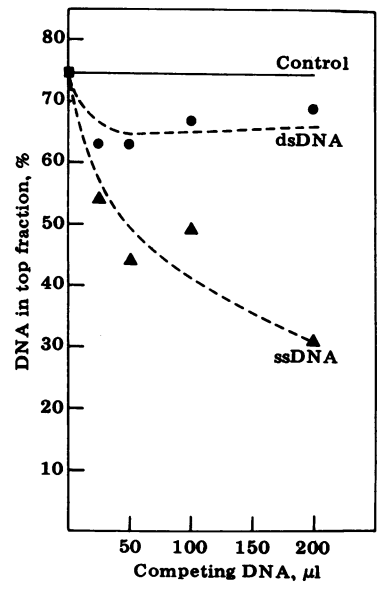

FIG. 4. Inhibition of S1 activity by exogenous DNA. Exogenous DNA: 25 to 200 μl of a stock solution containing 2.25 mg of vortexed *E. coli* DNA per ml was added to the lysate prior to the addition of S1 endonuclease. Single-stranded DNA was produced by heating to 95° for 20 min. ■, Amount of sample in top fraction after incubation with 4 units of enzyme for 10 min; ●, incubation in the presence of double-stranded DNA; ▲, incubation in the presence of single-stranded DNA.

some. The first case, breaks at the attachment points, results in the release of more DNA for a given number of breaks per chromosome than the second case. Similarly, for any given number of attachment sites, DNA released by breaks at these attachment sites will be larger than DNA released by random breaks.

The fact that the size of fragments released is so large and remains constant throughout the time course is consistent with the hypothesis that the enzyme acts at or near the sites of attachment and is itself responsible for releasing the DNA from the membrane.

In constructing mathematical models, we assume that the number of double-strand breaks per chromosome follows a Poisson distribution. The mean number of breaks per chromosome is denoted by m, and this is calculated from the sizing data. If all breaks occur at the attachment sites, then from the Poisson distribution the probability that the DNA is broken at a given attachment site is $1 - e^{-m/N}$ when N equals the number of attachment sites per chromosome. Consider a loop of DNA where the length is a_i (measured as a proportion of the total length of the chromosome). Because two breaks are required to release a loop from the membrane, the average amount released is $a_i(1 - e^{-m/N})^2$. Summing over all loops the proportion remaining after sites have been broken an average of m times is: $P_1(m) = 1 - (1 - e^{-m/N})^2$.

Equations for random breaks have been previously derived by Dworsky and Schaechter (2). Using the same arguments, the equations here have been derived in more useful form. The mean number of breaks within a loop is $a_i m$, and the amount remaining attached is $(2/m)(1 - e^{-a_i m}) - a_i e^{-a_i m}$. Summing over all loops, $P_2(m) = \Sigma\,[(2/m)(1 - e^{-a_i m}) - a_i e^{-a_i m}]$. If attachment sites are approximately equidistant, the amount in the M band will be: $P_2^a(m) = (2N/m) - e^{-m/N}(2N/m + 1)$. If attachment sites are randomly spaced, the amount remaining will be: $P_2^b(m) = e^{-m} + \Sigma_{r=1}^{\infty}(e^{-m} m^r/r!)\,[N(N - 1 + 2r)/(N + r)(N - 1 + r)] \simeq N(N - 1 + 2m)/(N + m)(N - 1 + m)$, in which the number of breaks in a particular chromosome is r. It should be noted that these models assume multiple breaks may occur at an attachment site. Also, if sites were closely bunched together, these would be calculated as much fewer in number.

Fitting Data to the Theoretical Curves. Fig. 5 shows the theoretical lines and data points for pancreatic DNase, S1, mung bean nuclease, and x-ray treatment [the latter from previously published experiments (2)]. Data points for S1 nuclease, although considerably scattered, fit quite well the curve for breaks at attachment sites. The pancreatic DNase clearly fits a model for random breaks, with a somewhat higher estimated number of attachment sites. The x-ray data fit the curve for 19 randomly spaced or 13 equidistant attachment sites. It is not possible to differentiate between the submodels of random breaks, i.e., attachment spaced randomly or at equal distances. However, the kinetics of release by cleavage at attachment sites does not depend on the kind of spacing. Therefore, the S1 data provide a better estimate of the number of attachment sites than that obtained using x-rays.

A linear transformation of the S1 data is shown in Fig. 6. The best-fit line (least squares) has a correlation coefficient of −0.92 and extrapolates to 100% at zero breaks, as expected. From the best-fit line the number of attachment sites was estimated as 18 ± 5. Thus, Fig. 5 was drawn assuming $N = 18$. This reduces the previous range of uncertainty of 6.5 to 33 in the estimated number of sites. For pancreatic DNase the estimated number of attachment points is 26 ± 11 (linear transformation not shown). The data for breaks with x-rays give averages of 12 and 19 attachment sites for models of equidistant and random spacing, respectively. Combining the x-ray and S1 results, the

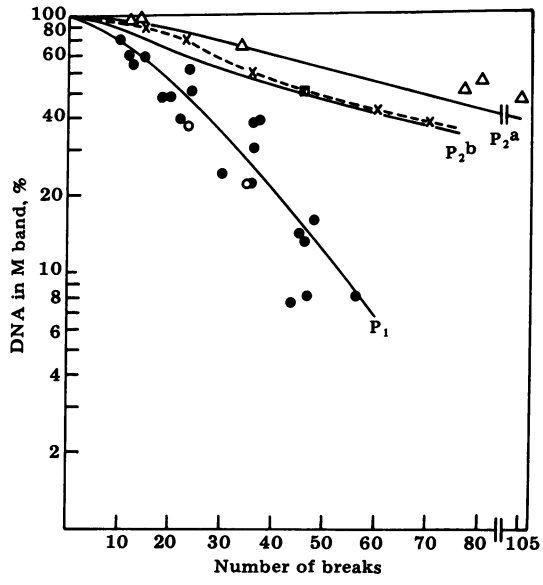

FIG. 5. Correlation of DNA released from membranes and average number of breaks per chromosome. Breaks were calculated from molecular weight determinations as described in *Materials and Methods*. ●, S1 endonuclease; ○, mung bean nuclease; △, pancreatic deoxyribonuclease; ×, best fit line for breaks by x-rays (from figure 4 of ref. 2); ⊠, x-ray data, average for several experiments. Theoretical curves: P_1, breaks at attachment sites; $P_2{}^a$, random breaks, equidistant spacing of attachment sites; $P_2{}^b$, random breaks, random spacing of attachment sites.

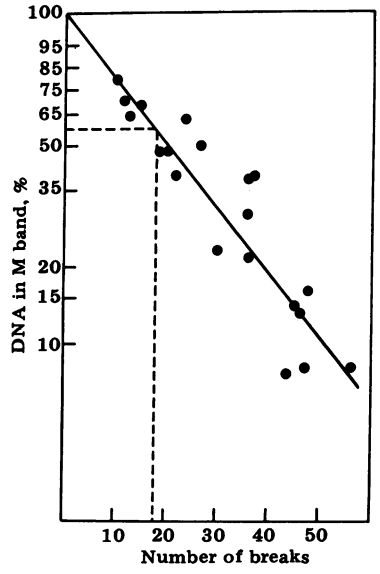

FIG. 6. Linear regression analysis of S1 data. Data for S1 nuclease shown in Fig. 5 are replotted. The ordinate is transformed to linearize theoretical curve P_1 by the equation $Y = \log [1 - \sqrt{1 - P_1(m)}]$. The slope is $[-\log(e)]/N$, and N can be determined as the value of m at which the line takes the value $-\log(e)$, or 56% on this scale. The best-fit line was calculated by least squares analysis and was found to extrapolate to 99.5% in the M band at zero breaks.

data also suggest that attachment sites are not regularly spaced.

Origin of Replication Is Not Released by S1. We have asked whether the DNA remaining membrane associated after S1 treatment represents the region of the origin of replication. To label this region preferentially, a steady-state [^{14}C]thymidine-labeled, temperature-sensitive *dnaC* mutant was allowed to finish replication at 42°. The culture was pulse labeled with [^{3}H]thymidine immediately on restart of growth at 30°. The culture was then analyzed by S1 treatment and M band fractionation with or without a chase period. Table 1 shows that the replicative origin, which is specifically labeled by the procedure, is retained preferentially on the membrane after extensive S1 treatment. The enrichment varied from 2- to 7-fold. The maximum theoretical enrichment by our method is 9-fold. The observed enrichment is a minimum estimate because the treated samples contained over 10% of the total DNA in the M band. We conclude that the attachment site near the replication origin is more resistant to S1 nuclease than the other attachment sites.

CONCLUSIONS

The key point of this work is the finding that the chromosome of *E. coli* has some twenty sites that are different from the rest of the molecule. These sites are sensitive to scission by single-strand-specific endonucleases and appear to be located at the place where the DNA is attached to the membrane. Interpretation of these results is complicated by the fact that the nature of the endonuclease-sensitive sites is not well defined (6–9) and the relevance of the *M* band technique to the *in vivo* state of the DNA is not well understood (10).

Table 1. S1 enrichment of origin label

Experiment	Treatment	% label in M band: Pulse ^{14}C	Pulse ^{3}H	Pulse–chase ^{14}C	Pulse–chase ^{3}H	Origin enrichment: Pulse	Origin enrichment: Pulse–chase
Unsynchronized	None	97.4	92.6	97.0	95.6		
control	S1	22.2	23.9	23.6	22.5	1.0	1.0
1	None	92.9	91.9	92.0	84.4		
	S1	18.5	90.4	7.0	48.1	4.9	6.9
2	None	75.5	69.0	90.8	85.0		
	S1	15.2	32.8	22.2	55.4	2.2	2.5
3	None	76.1	55.5	91.2	81.7		
	S1	15.6	50.5	22.0	65.3	3.2	3.0

The *dnaC thy*$^-$ mutant PC2 was steady-state labeled with [^{14}C]thymidine in M9 glucose thymidine medium at 30° to an OD_{600} of 0.1–0.15. The culture was shifted to 42° for 60 min, by which time DNA synthesis had ceased. [^{3}H]Thymidine was added as the culture was shifted down to 30°. After 60 sec, either incorporation was stopped by swirling in dry ice/acetone, or excess unlabeled thymidine was added for a 10-min chase. A 3-ml sample was centrifuged and resuspended in 0.1 ml of M band buffer. Treatment with S1 and M band fractionation was described in the *text*. ^{3}H and ^{14}C cpm were corrected for overlap. Enrichment was calculated as the ratio of ^{3}H cpm to ^{14}C cpm in the M band after S1 treatment.

These results correlate well with those of Dworsky and Schaechter (2). The number of attachment sites is the same, despite differences in the methods employed. In addition, two classes of attachment sites have been detected in both cases. Dworsky and Schaechter found that rifampin reduces the number of attachment sites to four or five. It is not yet clear whether the rifampin-resistant attachment sites are the same as the endonuclease-resistant sites found here. Because the *E. coli* chromosome is divided into 20 to 80 supercoiled domains (11), it is attractive to speculate that the similarity of this number and that of membrane attachment sites is more than a numerical coincidence.

At present it is still difficult to say anything about the chemical nature of the attachment of bacterial DNA to the membrane. The minimal conclusion is that the attachment site at or near the origin of replication that is resistant to S1 endonuclease is different from the other attachment sites. So far we have no evidence to indicate whether the attachment sites represent fixed chromosomal loci or can change their position on the DNA molecule. The present results indicate a convenient method for the isolation and purification of DNA at or near the replicative origin. This may be useful for studies of the origin–membrane complex.

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