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Membrane Association of Conjugally Transferred Deoxyribonucleic Acid in *Escherichia coli* Minicells

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The conjugally acquired deoxyribonucleic acid (DNA) of small, anucleate cells ("minicells") of a mutant strain of *Escherichia coli* K-12 was found to be predominantly associated with the bacterial membrane. Evidence from X-irradiation studies in vivo shows that there is no decrease in DNA-membrane association under conditions which reduce the DNA to one-sixth its original size and suggests the possibility of multiple DNA-membrane association sites. Preliminary enzymatic studies indicate the involvement of protein, DNA, and lipids in the membrane association of the DNA.

A mutant strain of *Escherichia coli* K-12 produces large numbers of small, anucleate cells ("minicells") as a result of aberrant cell divisions near the ends of the parent cells. Phase-contrast and electron microscopy show that the minicells are formed from a region of the parent cell which is normally devoid of nuclear structure, and biochemical analysis has confirmed that little if any deoxyribonucleic acid (DNA) is present (2, 6).

Minicells derived from F^- parents will participate as recipients in bacterial conjugation. The mated minicells contain, exclusively, DNA acquired during conjugation and, because of their small size, are easily separated from the much larger donor cells. Immediately after mating, conjugal DNA acquired from F^+, F', or Hfr donors is recovered in minicells in a single-stranded state. Under suitable postmating incubation conditions, the DNA transferred from F^+ donors and F' donors harboring smaller episomes is converted to the double-stranded state, whereas the DNA transferred from Hfr donors and F' donors harboring larger episomes is not (6, 7).

In view of evidence that the bacterial membrane may play a direct role in DNA replication (17, 25, 34, 35) and segregation (21, 26, 33) and because of reports (17, 34) of direct physical contact between DNA and the cell membrane, we considered it of interest to examine the extent of membrane association of DNA transferred to minicells during mating and to determine whether the failure of single-stranded DNA transferred by Hfr donors to be replicated to double-stranded DNA was correlated with a lack of membrane association.

MATERIALS AND METHODS

Bacterial strains. The strains of *E. coli* K-12 used in this study are listed in Table 1. The procedures used for maintaining the strains and for testing the stability of mating-type characteristics have been described (9). Donor-specific DNA phage (f1) were used to indicate the presence of F pili. All bacterial strains were kindly provided by Roy Curtiss III and H. I. Adler.

Media. The synthetic media used were minimal liquid and minimal mating medium (9). These were supplemented with 0.5% Casamino Acids (Difco) and 0.5% glucose as the carbon source unless otherwise indicated. Other supplements were purchased from Calbiochem (Los Angeles, Calif.) and, when required, were used at the following concentrations (μg/ml): L-leucine, 20; DL-threonine, 80; uracil, 40; thymidine, 4; and thiamine-hydrochloride, 2.

Complex media, when used, were L-broth and L-agar (27) and EMB agar (8).

Enzymes, radioisotopes, and detergents. Deoxyribonuclease (EC 3.1.4.5) was purchased from Worthington Biochemical Corp., Freehold, N.J. Lysozyme (egg white; EC 3.2.1.17) and lipase (EC 3.1.1.3) were purchased from Sigma Chemical Co., St. Louis, Mo. Pronase was purchased from Calbiochem, Los Angeles, Calif.

Radioactive labels (Schwarz BioResearch, Inc., Orangeburg, N.Y.) were used at the following specific

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TABLE 1. *Bacterial strains*[a]

Strain	Mating type	Relevant genotype and other characteristics
χ15	F+	Prototroph *str*[s] *T6*[s]
χ956	F+	*pyr*- *thy*-
χ876	Hfr OR56	O-*proC T6 purE gal* ... *lac* F prototroph
χ869	Hfr OR49	O-*argG xyl ile MetE* ... *lys* F prototroph
χ436	Hfr OR7	O-*T6 proC lac proB* ... *purE* F prototroph
χ925 (P678-54)	F-	*thr*- *leu*- *thi*-
χ796	F+	*thr*- *leu*- *thi*-
χ1009	R	*thr*- *leu*- *thi*- (*Rfi*- Tc Sm *drd*)
χ1041	Col *trp*+	*thr*- *leu*- *thi*- (F *colV colB colM trp*+)

[a] Nomenclature is essentially that of Demerec et al. (13), with exceptions noted by Curtiss (10). The markers are listed in the order in which they occur on the bacterial chromosome. Certain other mutations in these strains, such as resistance to phages and azide and ability to utilize various sugars as carbon sources, have not been listed. Plasmid nomenclature: Tc, tetracycline; Sm, streptomycin. Minicell producers are: χ925, χ796, χ1009, and χ1041.

activities and concentrations: [*methyl*-^3H]thymidine, 6 Ci/mmole, 5 μCi/ml; [2-^{14}C]thymidine, 40 to 50 mCi/mmole, 1 mCi/ml; L-[^{14}C]tryptophan, 34 mCi/mmole, 1 μCi/ml; L-[^{14}C]leucine, 171 mCi/mmole, 1 μCi/ml. In experiments utilizing thymidine label, 100 μg of adenosine per ml was added to the growth media (4).

Sodium dodecyl sulfate was purchased from E. I. DuPont de Nemours and Co., and Sarkosyl NL 30 (sodium lauryl sarcosinate) was purchased from Geigy Chemical Corp., Ardsley, N.Y.

Purification of minicells before mating. Minicells were purified before mating by differential centrifugation in a RC-2B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) and by rate, zone centrifugation on sucrose gradients in a Spinco L2-65B refrigerated ultracentrifuge (Beckman Instruments, Palo Alto, Calif.). Cultures of χ925 grown (for 16 to 18 hr) in 500 ml of minimal media supplemented with Casamino Acids, glucose, and thiamine were centrifuged at 2,500 × *g* for 5 min. Pellets, containing mostly cells and some minicells, were discarded, and the supernatant fluid was centrifuged at 13,200 × *g* for 10 min. The pellets were suspended in approximately 3 ml of tris-(hydroxymethyl)aminomethane (Tris)-KCl buffer [0.01 M Tris (Sigma) and 0.1 M KCl, *p*H 6.8 to 7.0], and 1-ml samples of this suspension were layered over 35 ml of 10 to 30% (w/w) sucrose or glycerol gradients buffered with the Tris-KCl. Gradients were centrifuged at 4,500 × *g* in a Spinco SW27 rotor for 15 to 20 min, and the minicell zone (Fig. 1a) was collected with a syringe. This material was diluted 1:2 in the Tris-KCl buffer and collected by centrifugation at 27,000 × *g* for 15 min. Pellets were suspended in minimal mating medium to a density of 10^{10} to 2 × 10^{10} minicells per ml. All centrifugal forces are stated at R_{max} and centrifugations were carried out at 4 C.

Mating procedure. The mating procedure was similar to that described by Cohen et al. (7), except that minicells were harvested from stationary-phase cultures and the minicells were more highly purified. Donor cells were log-phase cells grown without aeration or agitation and were usually grown in minimal medium supplemented with the growth requirements of the organism plus radioactive thymidine and 100 μg of adenosine per ml. Matings were done at 37 C, without agitation, in a 125-ml micro-Fernbach flask. A total of 10^9 to 2 × 10^9 donors and 10^{10} to 2 × 10^{10} minicell recipients were used per 10 ml of mating mixture. Excess radioactive label and adenosine were not washed from the donor cultures and were present during the mating at approximately a 1:2 dilution. Durations of matings were 30 to 40 min for F+ donors and 90 to 120 min for Hfr donors. Matings were terminated by rapid chilling to 0 to 4 C followed by vigorous agitation on a Vortex mixer.

Purification of minicells after mating. Minicells were purified after mating by two rate, zone centrifugations on 10 to 30% (w/w) linear sucrose gradients. Minicells and donors were collected from a 10-ml mating mixture by centrifugation at 12,000 × *g* for 10 min. The pellet was suspended in 1 ml of Tris-KCl buffer and layered over a sucrose gradient. After centrifugation in the Spinco SW27 rotor at 4,500 × *g* for 15 to 20 min, the minicell zone (Fig. 1a) was collected, diluted 1:2 with the Tris-KCl buffer, and pelleted by centrifugation at 27,000 × *g* for 15 min. This pellet was suspended in 1 ml of Tris-KCl buffer, incubated at 37 C for 15 min in the presence of 100 μg of deoxyribonuclease per ml and 10 mM $MgSO_4$, and then subjected to a second sucrose gradient purification. The minicell zone (Fig. 1b) was collected by fractionating the gradient into 1-ml samples while monitoring at 260 and 280 nm with an Oak Ridge flow cell in a Beckman DB spectrophotometer attached to a Brown recorder (3).

X-ray exposures. Mated minicells, purified by two cycles of rate, zone centrifugation on sucrose gradients, were X-irradiated with a GE Maxitron 250-kVp medical X-ray unit with 1-mm aluminum added filtration. Intact minicells were irradiated in Tris-KCl buffer in a 6-cm plastic petri dish in an ice bath situated on a revolving platform. The exposure rate was approximately 10 kr per min.

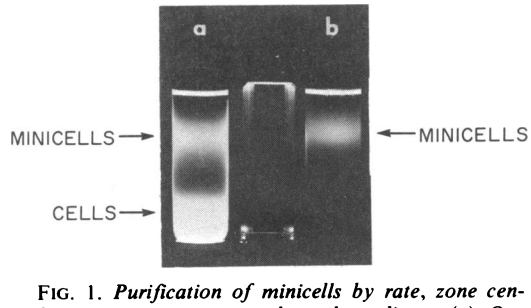

FIG. 1. *Purification of minicells by rate, zone centrifugation on sucrose or glycerol gradients. (a) One cycle of centrifugation; (b) two cycles of centrifugation.*

Assay for membrane association of DNA. The method of isolation of membrane-associated DNA was essentially that of Tremblay et al. (37). Spheroplasts of cells or minicells were prepared by incubation at 37 C with 600 to 1,000 μg of lysozyme per ml for 15 to 30 min in Tris-Mg-KCl (same as Tris-KCl with 0.01 M magnesium acetate). In some cases, the lysis mixture contained Tris-KCl buffer plus 0.001 M ethylenediaminetetraacetic acid (EDTA) and 0.25 M sucrose, and in these cases the lysozyme incubation was carried out at 4 C. Equal volumes of spheroplasts and 0.2% Sarkosyl were layered over 10 to 45% or 15 to 48% (w/w) linear sucrose gradients. Centrifugations were at 18,000 rev/min (~39,000 $\times$ *g*) for 25 to 30 min in the Spinco SW 50.1 rotor. After centrifugation, gradients were either fractionated onto 3MM Whatman filter discs (0.1 ml/disc) or collected by syringe in three large fractions—the top, M band, and bottom of the gradient. In either case, the cold trichloroacetic acid-insoluble counts (DNA labeled) were obtained by using 2,5-bis[2-(t-*tert*-butylbenzoxazolyl)]-thiophene (Packard Instrument Co., Downers Grove, Ill.) dissolved in toluene (5 g/liter) as the scintillator and a Packard Tri-Carb liquid scintillation spectrometer.

CsCl centrifugation. The use of isopycnic CsCl buoyant-density centrifugation to determine the strandedness of DNA recovered from mated minicells was previously described (7).

Alkaline sucrose sedimentation analysis. The size of the conjugally acquired DNA in minicells was determined as described by McGrath and Williams (30), except that gradients were at *p*H 13. Sedimentation rates and molecular weights were estimated as described by Burgi and Hershey (5) by interpolation from published values for λb2 and T4 phage DNA species (36), which were used as reference markers. Centrifugations were at 142,000 $\times$ *g* (at R_{avg}) for 3 to 4 hr and at 10 C.

Phage T4D infection of minicells. Phage T4D were grown on the minicell-producing parent strain, χ925, in minimal medium supplemented with Casamino Acids, glucose, and the appropriate growth requirements plus [^{3}H]leucine and [2-^{14}C]thymidine and were assayed on *E. coli* B by standard procedures (1). Minicells purified by two cycles of centrifugation on 10 to 30% (w/w) sucrose gradients were infected with the doubly labeled phage at multiplicities of 0.8 to 2.4. Ninety-eight per cent of the phage was absorbed in approximately 2 min. Phage ghosts were sheared from the minicells by rapid blending with a Waring Blendor fitted with an ice jacket. Minicells were recovered by centrifugation and incubated in the presence of 600 μg of deoxyribonuclease per ml at 37 C for 10 min. The loss of phage ghosts into the medium and the appearance of phage DNA in the minicell fraction were estimated by measuring the appropriate labels in the cold trichloroacetic acid-insoluble fraction. Infected minicells prepared in this manner were assayed for the presence of membrane-associated phage DNA.

RESULTS

Membrane association of conjugal DNA. Table 2 shows the extent of membrane association of DNA acquired by F$^-$ minicells in matings with selected F$^+$ and Hfr donor strains. The DNA of the donor strains was prelabeled by growth in [^{3}H]- or [^{14}C]thymidine, and membrane association was measured by determining the amount of the DNA that sedimented on sucrose gradients as DNA-membrane-Mg-sarcosinate complex (the M band). Although there is some variation between experiments, it is clear that the major portion of the DNA transferred to minicells was recovered in the M band; this was true for all donor types and strains tested.

Specificity of membrane association. Although Schaechter and his group (14, 37) reported a variety of studies in which they tested the specificity of the Sarkosyl method for determining membrane association of DNA in bacterial cells, we have performed some additional control experiments with the minicell system.

The possibility that the minicells were not lysed and that the DNA recovered in the M band was in intact minicells was excluded by phase-contrast microscopy of the lysate and of the material recovered from the M band. Table 3 shows that only a relatively small percentage (~15%) of exogenously added DNA is recovered in the M band. This is true for both single- and double-stranded DNA and for DNA either added directly onto the gradient, mixed on the gradient with minicells at the time of lysis, or preincubated with minicells before lysis. The degree of binding was likewise unaffected by the purity of the exogenous DNA. DNA in crude cell lysates or DNA purified by the phenol (23) or Marmur (29) isolation techniques was bound to the same extent. The amount of exogenous DNA added to the Sarkosyl gradients was determined by adding an equal number of counts to those contained in the minicell conjugal DNA. Usually, samples of either type contained 2,000 to 5,000 counts/min of [^{3}H]- or [^{14}C]thymidine-labeled DNA.

Intact T4D bacteriophage added directly onto the Sarkosyl gradients, without minicells, were not lysed by the detergent and were recovered as intact particles in a zone of the gradient between the M band and the bottom of the tube. Only a small percentage of purified T4 DNA added to a lysate is recovered in the M band. However, assay for membrane association in minicells infected with T4D before lysis on the gradients shows that 65% of the phage DNA is membrane associated; the remainder of the phage DNA is recovered at the top of the gradient.

Donor cell contamination. Two types of experiments were done to assess the contribution of contaminating donor cells to the observed membrane association of DNA in isolated minicells. (i) The number of contaminating donor cells remaining in the mated minicell preparations

TABLE 2. *Typical studies on membrane association of conjugally acquired DNA in minicells*

Donor strain[a]	Mating type	Duration of mating (min)	Per cent DNA recovered in	
			M band[b]	Top of gradient[b]
χ15	F+	0[c]	98	2
χ956	F+	30	58	42
χ15	F+	40	81	19
χ15	F+	40	75	25
χ15	F+	40	80	20
χ869	Hfr	0[c]	91	9
χ869	Hfr	90	79	21
χ869	Hfr	90	91	9
χ876	Hfr	90	97	3
χ876	Hfr	90	95	5
χ436	Hfr	120	79	21

[a] χ925 minicells are the recipient strain in all cases.

[b] Calculated as M/(M + T) or T/(M + T). The sum of counts recovered from M band and top of gradient (M + T) = 100%, based on microscopic observation that counts at bottom of tube below M band are intact cells. Purified cell or minicell samples containing approximately 2,000 to 5,000 counts/min of labeled DNA were used for assay of membrane association.

[c] Unmated donor cells.

after two cycles of sucrose gradient centrifugation was estimated by viable cell counts (minicells do not divide or form colonies) and by direct counts in a Petroff-Hauser counting chamber. Both types of enumeration revealed about one contaminating donor cell per 10^5 to 10^7 minicells. We normally obtain 1 to 2% transfer of donor DNA to minicells. On the basis of this percentage, one contaminating donor cell per 100 to 50 minicells could account for the conjugal DNA recovered in minicells after mating and purification. Our data show an average of one contaminating donor cell per 10^6 minicells, which would account for no more than 0.01 to 0.005% of the DNA transferred. (ii) As an additional check for donor cell contamination, DNA from conjugated, purified minicells was routinely subjected to CsCl density-gradient centrifugation. Our results (Fig. 2) agree with previous results (6, 7) showing that, immediately after conjugation, conjugally acquired DNA in minicells is single stranded. Any DNA present from contaminating donor cells would be double stranded.

Effect of X-irradiation on membrane attachment of DNA. X-ray exposures of up to 1,000 kR to mated minicells did not alter the degree of

TABLE 3. *Studies on the specificity of the Sarkosyl technique*[a]

Material studied	How added to gradient	"Real" recovery (%)[b]	DNA recovered in[c]		Special notes
			M band (%)	Top of gradient (%)	
χ15 DS DNA	Alone	58	23	77	Purified by two phenol extractions
T4 phage labeled with [^{3}H]thymidine and [^{14}C]leucine	Alone	100	0	0	Added intact; recovered as a zone; no apparent lysis
T4 [^{3}H]DNA	Minicells infected with phage	—	65	35	Infected before lysis on gradient
T4 [^{14}C]DNA	Alone	—	16	84	Marmur isolation of DNA
DS [^{14}C]DNA	Alone	—	30	70	Crude lysate[d]
DS [^{3}H]DNA	Alone	71	15	85	Crude lysate
DS [^{3}H]DNA	With minicells on gradient	70	16	84	Crude lysate
DS [^{3}H]DNA	With minicells on gradient preincubated 10 min, 37 C	65	26	74	Crude lysate
DS [^{3}H]DNA	With minicells on gradient preincubated 10 min, 37 C	80	10	90	Layered over SDS in- instead of Sarkosyl
DS [^{14}C]DNA	With minicells on gradient	72	2	98	Crude lysate
SS [^{14}C]DNA	With minicells on gradient	81	0	100	χ15 DS DNA melted and quickly cooled

[a] Symbols and abbreviations: dashes indicate that data are not available; DS, double-stranded DNA; SS, single-stranded DNA.

[b] (Counts added to gradient)/counts recovered from gradient. Loss was due to handling, degradation, adsorbtion to tube, etc.

[c] Calculated as M/(M + T) or T/(M + T). The sum of the counts recovered from the M band and top of the gradient (M + T) = 100%.

[d] Treated with lysozyme, Pronase, and sodium dodecylsulfate, and then stored frozen. Lysate was added to the gradient without further purification.

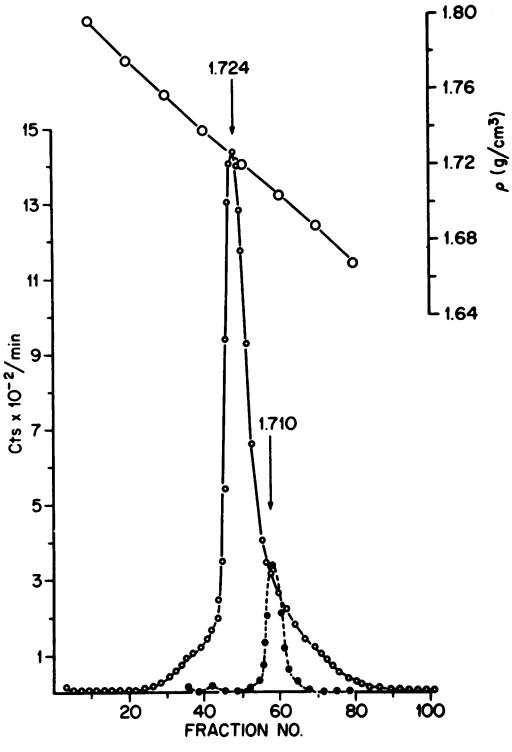

FIG. 2. *Cesium chloride density-gradient centrifugation of minicells mated with χ436 labeled with [methyl-³H]thymidine. Minicells were purified by two cycles of rate, zone centrifugation on sucrose gradients and then lysed by using lysozyme, Pronase, and sodium dodecyl sulfate. Equilibrium runs of approximately 72 hr.*

membrane association of conjugally acquired Hfr DNA. However, the same exposures caused a marked reduction in the size of the DNA. The average molecular weight of transferred DNA in unirradiated minicells (Fig. 3) was estimated by interpolation from the known values for the λb2 and T4 phage reference DNA species used (36). The relative change in size of the DNA in minicells after irradiation was obtained by assigning the *S* value calculated above to the unirradiated control and observing the shift in the alkaline sucrose sedimentation profiles. Figure 4a shows the sedimentation profiles of Hfr DNA from irradiated minicells lysed directly on alkaline sucrose gradients. The calculated molecular weights are 25×10^6 daltons for the unirradiated control and 7×10^6 and 4×10^6 daltons for exposures of 500 kR and 1,000 kR, respectively. Although the molecular weight of the membrane-associated DNA was markedly reduced by X irradiation, the degree of association was not affected (Fig. 4b).

Sensitivity of the DNA-membrane complex to enzymatic digestion. Preliminary data (Table 4) show that the addition of Pronase, lipase, or deoxyribonuclease to a lysate of minicells mated to Hfr (χ436) cells decreased the degree of membrane association of the DNA. Less than 30% of the DNA in the lysate was recovered in the M band after 10 min of incubation at 37 C at enzyme concentrations of 100 μg/ml; without the addition of exogenous enzymes, 68% of the DNA was membrane associated.

Membrane association of plasmid DNA. Recently, several reports appeared showing that DNA can be introduced into minicells by the segregation of extrachromosomal elements from

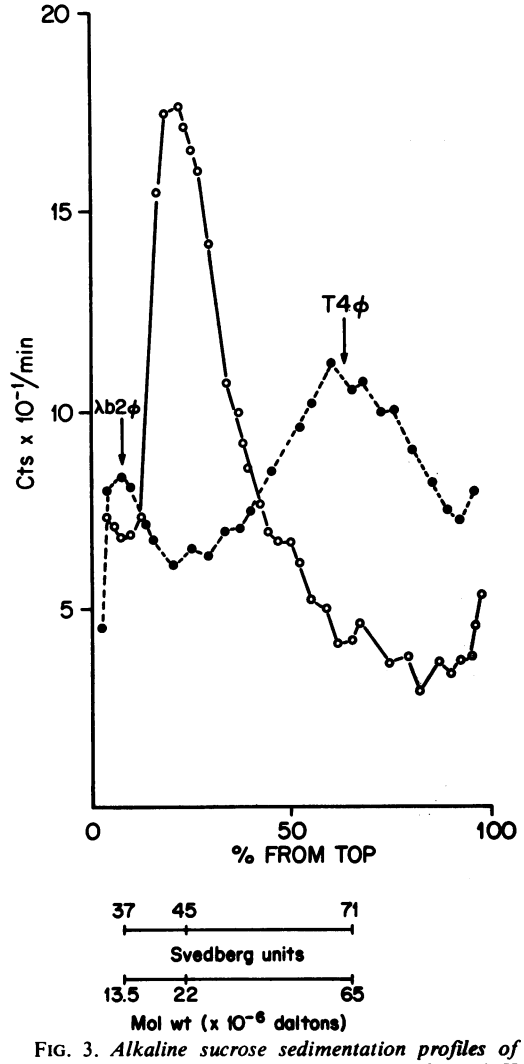

FIG. 3. *Alkaline sucrose sedimentation profiles of DNA on 5 to 20% (w/w) linear sucrose gradients (pH 13). Symbols: (○) Minicells containing [methyl-³H] thymidine DNA transferred from χ436 Hfr, cells lysed on gradient; (●) λb2 and T4 phage reference [2-¹⁴C] thymidine DNA species, phage lysed on gradient.*

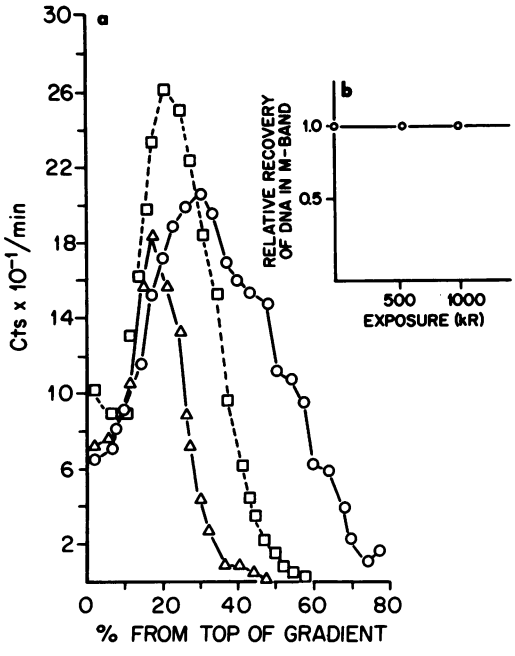

FIG. 4. *(a) Alkaline sucrose sedimentation profile of conjugally acquired minicell DNA. Minicells mated to χ436 labeled with [methyl-³H]thymidine were purified by two rate, zone centrifugations on 10 to 30% (w/w) linear sucrose gradients. Purified minicells were irradiated with 0, 500, and 1,000 kR of X rays and then lysed on 5 to 20% (w/w) alkaline sucrose gradients (pH 13). Centrifugation was at 142,000 × g for 3 to 4 hr at 10 C. Symbols: O (control), 0 kR; □, 500 kR; Δ, 1,000 kR. (b) Sample of the irradiated minicells described in part a treated with lysozyme and lysed on the Sarkosyl gradients. Relative recovery was calculated as (recovery of DNA in M band at 500 or 1,000 kR)/(recovery of DNA in M band at 0 kR).*

TABLE 4. *Sensitivity of the DNA-membrane complex to enzymatic digestion*

Treatment of lysate before M band analysis[a]	M band (%)	Per cent of control
None, held at 4 C	98	100
Incubated 10 min at 37 C (control)	68	100
Deoxyribonuclease, 10 min at 37 C[b]	12	18
Pronase, 10 min at 37 C[b]	27	39
Lipase, 10 min at 37 C[b]	29	43

[a] Minicells mated with χ436 labeled with [*methyl-³H*]thymidine were converted to spheroplasts by incubation in the Tris-KCl buffer in the presence of 10^{-3} M EDTA, 0.25 M sucrose, and 600 μg of lysozyme per ml for 30 min at 4 C. Spheroplasts were lysed by the addition of Sarkosyl to a final concentration of 0.1%. Lysates were variously treated, as above, layered over 15 to 48% (w/w) linear sucrose gradients, and centrifuged at 39,000 × *g* for 30 min at 4 C. The effect of the various treatments was determined by locating the cold trichloroacetic acid-insoluble counts in the gradient.

[b] Concentration, 100 μg/ml.

TABLE 5. *Membrane association of segregated plasmid DNA in minicells*[a]

Strain	Plasmid type	M band (%)
χ1009	Rfi⁻	93
χ1041	Col *trp*	78
χ796	F	79

[a] Cultures of χ1009, χ1041, and χ796 were grown to stationary phase in the presence of [*methyl-³H*]thymidine and 100 μg of adenosine per ml. Minicells were isolated by two cycles of sucrose gradient centrifugation on 10 to 30% (w/w) linear gradients. Assay of membrane association (M band) as in text.

parental cells carrying sex factors, colicin factors, or drug resistance factors (18, 19, 22, 28, 31). The data in Table 5 show that such DNA is also largely membrane associated.

DISCUSSION

The DNA acquired by minicells during mating with F⁺ or Hfr donor strains is largely membrane associated, and the extent (80 to 100%) of association of the DNA present agrees well with published values (14, 32, 37) obtained by the same assay technique in other bacterial systems. Arguments that the DNA in minicells is conjugally acquired (6, 7) were confirmed in the present study by showing that the degree of donor cell contamination of minicell preparations is low (1 per 10^5 to 10^7 minicells) and therefore inadequate to account for the observed transfer of up to 1 to 2% of the labeled donor DNA.

The conclusion that DNA transferred to minicells during conjugation becomes membrane associated is based on results obtained from the Sarkosyl technique, in which portions of the cell membrane adhere to the hydrophobic surfaces of crystals formed by the addition of Mg^{2+} to the detergent Sarkosyl (14). The Mg^{2+}-Sarkosyl-membrane complex, along with any DNA attached to the membrane, is recovered as an isopycnic zone after centrifugation on sucrose gradients. Tests of the specificity of the technique for membrane associated DNA in other bacterial cells were previously reported (14, 37). Similar types of control experiments were repeated with the minicell system, where the DNA is largely in a single-stranded state. These experiments showed that exogenously added DNA, from a variety of sources, and in either a single- or double-stranded state, is not entrapped in the M band. The sensitivity of the membrane-detergent

complex to enzymes argues against spurious extracellular aggregation and suggests that lipid, protein, and DNA are involved in the attachment. However, as with most isolation procedures, the controls do not rule out all possible artifacts, and this reservation should be noted in interpreting the biological significance of the data.

Data obtained by X irradiation of intact minicells containing conjugally acquired DNA suggest multiple attachment sites between the DNA and the minicell membrane. Doses of irradiation which were adequate to produce several breaks in the DNA and reduce its calculated molecular weight from 25×10^6 daltons to 4×10^6 daltons did not alter the degree of membrane association. This argues against a single and specific membrane attachment site for DNA, although it is possible that reattachment of the DNA occurred after irradiation. Our conclusion suggesting that there are multiple attachment sites agrees with the results obtained in some phage systems (24) as well as the results in a bacterial system (32). In the latter study, the DNA recovered from the M band was of relatively low molecular weight (5×10^6 daltons), and calculations for the percentage of the DNA remaining membrane associated suggested a minimum of 20 DNA-membrane attachment sites and probably many more.

Our evidence that F^+ and Hfr DNA transferred to minicells is largely membrane associated indicates that the reported failure of Hfr DNA and large F′ episomal DNA to synthesize a complementary strand on postmating incubation (6) was not due to either the absence of membrane association sites or lack of attachment of transferred DNA to these sites. Instead, as suggested previously (6, 10), conversion of single-stranded DNA to double-stranded DNA in vivo may depend on the availability of a distally transferred region of the DNA molecule.

F^- minicells have very limited synthetic capacities (15, 16), and the presence of membrane attachment sites for DNA suggests that such sites are already present in the polar or end regions of the parental cells. However, the ends of the parent cells are devoid of nuclear structure and give rise to DNA-less minicells, which raises the interesting question of why there is no attachment of chromosomal DNA at the polar ends of the parental cells. There are several possible explanations. In spite of their limited demonstrated synthetic capacity, minicells may still be able to synthesize new attachment sites or at least activate preexisting sites in the presence of conjugally transferred DNA. A more intriguing possibility is that minicell DNA sites are specific, possibly for some region(s) of the nonchromosomal DNA. This notion is consistent with our data, since the F^+ and F′ DNA species are episomal, and with some current hypotheses (11, 12, 20) which assume that the lead region of the Hfr chromosome contains some portion of the F DNA. However, it is inconsistent with our interpretation of the X-ray data presented in this report, which suggest multiple, nonspecific membrane sites for DNA association. Finally, the observed membrane association could be artifactual and not reflect the state of attachment of DNA in the functioning cell.

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LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc. New York.
2. Adler, H. I., W. D. Fisher, A. Cohen, and A. A. Hardigree. 1967. Miniature *Escherichia coli* cells deficient in DNA. Proc. Nat. Acad. Sci. U.S.A. **57**:321-326.
3. Anderson, N. G. 1962. Analytical techniques for cell fractions. II. A spectrophotometric column monitoring system. Anal. Biochem. **4**:269-283.
4. Boyce, R. P., and R. B. Setlow. 1962. A simple method of increasing the incorporation of thymidine into the deoxyribonucleic acid of *Escherichia coli*. Biochim. Biophys. Acta **61**:618-620.
5. Burgi, E., and A. D. Hershey. 1963. Sedimentation rate as a measure of molecular weight of DNA. Biophys. J. **3**: 309-321.
6. Cohen, A., W. D. Fisher, R. Curtiss III, and H. I. Adler. 1968. The properties of DNA transferred to minicells during conjugation. Cold Spring Harbor Symp. Quant. Biol. **33**:635-641.
7. Cohen, A., W. D. Fisher, R. Curtiss III, and H. I. Adler. 1968. DNA isolated from *Escherichia coli* minicells mated with F^+ cells. Proc. Nat. Acad. Sci. U.S.A. **61**:61-68.
8. Curtiss, R., III. 1968. Ultraviolet-induced genetic recombination in a partially diploid strain of *Escherichia coli*. Genetics **58**:9-54.
9. Curtiss, R., III, L. J. Charamella, D. R. Stallions, and J. A. Mays. 1968. Parental functions during conjugation in *Escherichia coli* K-12. Bacteriol. Rev. **32**:320-348.
10. Curtiss, R., III. 1969. Bacterial conjugation. Annu. Rev. Microbiol. **23**:69-136.
11. Cuzin, F., and F. Jacob. 1964. Deletions chromosomiques et intergration d'un episome sexuel F-lac$^+$ chez *Escherichia coli* K-12. Compt. Rend. **258**:1350-1352.
12. Cuzin, F., G. Buttin, and F. Jacob. 1967. On the mechanism of genetic transfer during conjugation of *Escherichia coli*. J. Cell Physiol. (Suppl. 1) **70**:77-88.
13. Demerec, M., E. A. Adelberg, A. J. Clark, and P. E. Hartman. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics **54**:61-76.
14. Earhart, C. F., G. Y. Tremblay, M. J. Daniels, and M. Schaechter. 1969. DNA replication studied by a new

method for the isolation of cell membrane-DNA complexes. Cold Spring Harbor Symp. Quant. Biol. **33**:707-710.
15. Fenwick, R. G., Jr., K. J. Roozen, and R. Curtiss III. 1970. RNA and protein synthesis in plasmid-containing minicells of *Escherichia coli* K-12. Genetics **64**:s20.
16. Fralick, J. A., W. D. Fisher, and H. I. Adler. 1969. Polyuridylic acid-directed phenylalanine incorporation in minicell extracts. J. Bacteriol. **99**:621-622.
17. Ganeson, A. T., and J. Lederberg. 1965. A cell-membrane bound fraction of bacterial DNA. Biochem. Biophys. Res. Commun. **18**:824-835.
18. Inselburg, J. 1970. Segregation into and replication of plasmid DNA in chromosomeless segregants of *Escherichia coli*. J. Bacteriol. **102**:642-647.
19. Inselburg, J. 1970. Replicating DNA: structure of colicin factor E1. Science **169**:590-592.
20. Jacob, F., and E. L. Wollman. 1961. Sexuality and the genetics of bacteria, p. 374. Academic Press Inc., New York.
21. Jacob, F., S. Brenner, and F. Cuzin. 1963. On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. **28**:329-348.
22. Kass, L. R., and M. Yarmolinsky. 1970. Segregation of functional sex factor into minicells. Proc. Nat. Acad. Sci. U.S.A. **66**:815-822.
23. Kirby, K. S. 1957. A new method for the isolation of deoxyribonucleic acids: evidence on the nature of bonds between deoxyribonucleic acid and protein. Biochem. J. **66**: 495-504.
24. Knippers, R., and R. L. Sinsheimer. 1968. Attachment of the parental DNA of bacteriophage ϕX174 to a fast-sedimenting cell component. J. Mol. Biol. **34**:17-29.
25. Knippers, R., and W. Sträling. 1970. The DNA replicating capacity of isolated *E. coli* cell wall-membrane complexes. Nature (London) **226**:713-717.
26. Lark, K. G. 1966. Regulation of chromosome replication and segregation in bacteria. Bacteriol. Rev. **30**:3-32.
27. Lennox, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190-206.
28. Levy, S. B., and P. Norman. 1970. Segregation of transferable R factors into *Escherichia coli*. Nature (London) **227**:606-607.
29. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **5**: 109-118.
30. McGrath, R. A., and R. W. Williams. 1966. Reconstruction *in vivo* of irradiated *Escherichia coli* deoxyribonucleic acid; rejoining of broken pieces. Nature (London) **212**:534-535.
31. Roozen, K. J., R. G. Fenwick, Jr., S. Levy, and R. Curtiss III. 1970. Characterization of DNA isolated from minicells of plasmid-harboring minicell-producing strains of *Escherichia coli* K-12. Genetics **64**:s54.
32. Rosenberg, B. H., and L. F. Calvalieri. 1968. Shear sensitivity of the *E. coli* genome: multiple membrane attachment points of the *E. coli* DNA. Cold Spring Harbor Symp. Quant. Biol. **33**:65-72.
33. Ryter, A. 1968. Association of the nucleus and the membrane of bacteria: a morphological study. Bacteriol. Rev. **32**:39-54.
34. Smith, D. W., and P. C. Hanawalt. 1967. Properties of the growing point region in the bacterial chromosome. Biochim. Biophys. Acat **149**:519-531.
35. Smith, D. W., H. E. Schaller, and F. J. Bonhoeffer. 1970. DNA synthesis *in vitro*. Nature (London) **226**:711-713.
36. Studier, F. W. 1965. Sedimentation studies of the size and shape of DNA. J. Mol. Biol. **11**:373-390.
37. Tremblay, G. Y., M. J. Daniels, and M. Schaechter. 1969. Isolation of a cell membrane-DNA-nascent RNA complex from bacteria. J. Mol. Biol. **40**:65-76.