JOURNAL OF VIROLOGY, Apr. 1981, p. 249-255
0022-538X/81/040249-07$02.00/0

Vol. 38, No. 1

Cellular Location of Mu DNA Replicas

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Received 19 September 1980/Accepted 17 December 1980

To ascertain the form and cellular location of the copies of bacteriophage Mu DNA synthesized during lytic development, DNA from an *Escherichia coli* lysogen was isolated at intervals after induction of the Mu prophage. Host chromosomes were isolated as intact, folded nucleoids, which could be digested with ribonuclease or heated in the presence of sodium dodecyl sulfate to yield intact, unfolded nucleoid DNA. Almost all of the Mu DNA in induced cells was associated with the nucleoids until shortly before cell lysis, even after unfolding of the nucleoid structure. We suggest that the replicas of Mu DNA are integrated into the host chromosomes, possibly by concerted replication-integration events, and are accumulated there until packaged shortly before cell lysis. Nucleoids also were isolated from induced λ lysogens and from cells containing plasmid DNA. Most of the plasmid DNA sedimented independently of the unfolded nucleoid DNA, whereas 50% or more of the λ DNA from induced lysogens cosedimented with unfolded nucleoid DNA. Possible explanations for the association of extrachromosomal DNA with nucleoid DNA are discussed.

The prophage form of bacteriophage Mu DNA can be randomly inserted into the *Escherichia coli* chromosome during the process of lysogenization. Upon induction of such a Mu lysogen, extensive Mu-specific DNA synthesis begins within 6 to 8 min (28–30). Synthesis is initiated in situ (13, 28) from the left terminus of the prophage (31) and is constrained within the boundaries of the prophage (26, 28). The form and cellular location of the replicas produced by this and the subsequent replication events is the subject of the present communication.

Numerous studies have failed to detect any form of Mu DNA devoid of host DNA present during the lytic development of the phage (3, 13, 14). One form of Mu-containing DNA that has been described in induced lysogens is Hc DNA (covalently closed circular DNA molecules containing both Mu and host DNA) (21, 27, 29). Hc DNA was first detected several minutes later than the onset of Mu DNA synthesis (at a level of about one molecule per 30 cells), and the maximum level reached shortly before commencement of lysis corresponds to only two molecules per cell (29). The location and form of the vast majority of Mu DNA molecules during lytic development remain to be determined.

Hc DNA was detected by sedimentation analysis in CsCl-ethidium bromide gradients. Other topological forms containing both Mu and host DNA, e.g., relaxed circular or linear molecules, would not be separated from chromosomal DNA in such gradients. Therefore, we have used techniques which can separate such DNA forms from host chromosomal DNA and which can be used to detect Mu copies which are associated with the host chromosome. At least some DNA is expected to be found in the host chromosome as genetic studies have demonstrated that Mu DNA integrates repeatedly throughout the lytic cycle (20, 22). The results presented here show that almost all of the replicas of Mu DNA are associated with the host chromosome, probably in an integrated form, and accumulate there until late in the lytic cycle.

MATERIALS AND METHODS

Bacteria and bacteriophage. The experimental strains were derivatives of *E. coli* K-12 AT4518 (F⁻ *thy thi sup*⁺) and were lysogenic for Mu*cts*62 (MP300) or for λ*c*I857 (AT4511) or carried an RP4 plasmid (MP304). The DNA used as the probe in DNA-DNA annealing assays was obtained from Mu phage grown on *Proteus mirabilis* RP4::Mu (AT3557) or from λ phage grown on AT4511 as described previously (25).

Cells were routinely grown on T medium (27) containing 2 μg of thymidine and thymine per ml, 200 μg of uridine and deoxyadenosine per ml, and 50 μCi of [^{3}H]thymidine (specific activity, 40 to 60 Ci/mmol) per ml. Cells were grown at 28°C and induced by shifting to 42°C. The onset of lysis occurred 38 to 40 min after induction.

Precipitation of high-molecular-weight DNA. The procedure of Hirt (9) as modified by Guerry et al. (7) was used to precipitate high-molecular-weight

DNA. Portions (2 ml) of bacterial cultures were centrifuged and suspended in 0.5 ml of 25% sucrose in 0.05 M Tris (pH 8.0). Lysozyme (0.1 ml of a 5-mg/ml solution in 0.25 M Tris, pH 8.0) was added at 4°C, and after 5 min, 0.2 ml of EDTA (0.25 M, pH 8.0) was added. After an additional 5 min, sodium dodecyl sulfate (SDS) was added to a final concentration of 1%, and 5 M NaCl was added with gentle mixing to a final concentration of 1 M. After overnight storage at 4°C, the samples were sedimented at 17,000 × *g* for 30 min, and portions of the supernatants were precipitated with 10% trichloroacetic acid. Precipitates were collected on membrane filters, washed with 10% trichloroacetic acid and then with hot water, and counted in a toluene-based scintillation mixture.

Preparation of nucleoids. Preparation of nucleoids was similar to the method of Hecht et al. (8) except that more rigorous lysis conditions were required for cells induced for phage production. Portions (2 ml) of cultures were sampled into 10 ml of buffer A (0.01 M Tris, pH 8.1, 0.1 M NaCl, and 0.1 M sodium azide) and centrifuged at 10,000 rpm for 7 min in a Sorvall SS34 rotor. Pellets were suspended in 0.2 ml of buffer B containing 1 M NaCl–0.01 M Tris (pH 7.6 for uninduced cells or pH 8.1 for induced cells); 0.05 ml of lysozyme (10 mg/ml in 0.05 M EDTA, 0.1 M Tris) at the appropriate pH was added. Measurements of pH were performed at room temperature; pH 7.6 Tris is approximately pH 8.1 at 4°C. Uninduced cells were incubated for 1 min at 4°C before addition of 0.25 ml of a lysis mixture containing 1 M NaCl, 0.01 M EDTA, and 1% each of the detergents Sarkosyl, Brij 58, and deoxycholate (the latter being added immediately before use). Induced cells were incubated for 4 min at 24°C before addition of the lysis mixture. Samples then were incubated at 24°C for 5 to 10 min to allow lysis before chilling.

Sedimentation of nucleoids. To sediment folded nucleoids, samples of the unfractionated lysates were layered gently onto 10 to 30% sucrose gradients containing 1 M NaCl, 0.01 M Tris, and 0.001 M EDTA, pH 7.6, and sedimented in an SW50.1 rotor at 17,000 rpm for 15 to 20 min at 4°C. ^{32}P-labeled T4 phage were included as a sedimentation marker. Portions were sampled from a puncture at the bottom of the tube.

To obtain unfolded nucleoid DNA, samples from unfractionated lysates or folded nucleoids from primary gradients were diluted 10-fold into 0.05 M LiCl, 0.01 M Tris, 0.001 M EDTA, pH 8.1, with or without 50 μg of RNase per ml, and gently layered on top of 5 to 20% sucrose gradients in 0.05 M LiCl, 0.01 M Tris, 0.001 M EDTA, pH 8.1, and 1 mM β-mercaptoethanol with or without 0.3% SDS. When the samples were to be heated before sedimentation, they were overlaid with mineral oil, heated to 70°C, and then cooled to room temperature. Sedimentation in an SW27 rotor was for 40 to 45 h at 20°C and 3,500 rpm to avoid the rotor speed effects described by Hecht et al. (8). ^{32}P-labeled DNA from phage T4, λ, or intact λ phage was added as a sedimentation marker.

Recovery of labeled DNA from sucrose gradients was monitored; 80 to 100% of radioactively labeled input material routinely was recovered.

DNA-DNA annealing assay. Quantitation of Mu or λ DNA was obtained with the DNA-DNA annealing assay described previously (25, 28).

RESULTS

Separation of high- and low-molecular-weight DNAs. Our previous results (28, 29) showed that extensive Mu-specific DNA synthesis is initiated 6 to 8 min after induction of a Mu lysogen. By 20 min after induction, Mu DNA constitutes approximately 15 to 20% of the DNA in the cell, and by the onset of lysis at about 40 min postinduction, more than one-half the DNA in the cell is Mu DNA. To determine the cellular location of the Mu DNA replicas formed during the lytic cycle, we first employed a separation of high- and low-molecular-weight DNA by the procedure of Hirt (9) as modified by Guerry et al. (7). The separation is based on the precipitation of high-molecular-weight DNA in the presence of 1 M NaCl and 1% SDS. A lysogen carrying a Mu prophage with a thermolabile repressor was grown at 28°C in the presence of [^{3}H]thymidine for several generations to fully label cellular DNA. Samples were taken before and at intervals after induction at 42°C. Lysates were prepared, and, after overnight precipitation, the distribution of labeled DNA between precipitate and supernatant was determined. Table 1 shows the percent DNA in the supernatant. Essentially all of the DNA in the cells precipitated as high-molecular-weight material until shortly before the onset of lysis, whereas most of control ^{32}P-labeled λ DNA was found in the supernatant.

Isolation of folded nucleoids from Mu lysogens. Since molecules containing Mu DNA may be extremely variable in size, as is true for Hc DNA (29), a more precise definition of high-molecular-weight DNA was desired. We decided to study isolated nucleoids (intact host chro-

TABLE 1. *Distribution of labeled DNA*[a]

Min after induction	Labeled material in supernatant (%)
0	1.5
10	0.6
20	0.8
30	0.8
35	3.1
40	18.3

[a] *E. coli* MP300, lysogenic for Mu*cts*62, was grown for several generations in medium containing [^{3}H]thymidine to fully label host chromosomes. Samples were removed before (0 min) and at intervals after induction. "Hirt supernatants" were prepared as described in the text, and the percent of the total label present in the supernatant (low-molecular-weight DNA) was determined.

mosomes) to make a distinction between Mu DNA contained in the host nucleoid and any extrachromosomal form of DNA containing Mu.

Lysis of cells at 37°C in 1 M NaCl in the presence of neutral detergents including Sarkosyl can produce membrane-free nucleoids as described by Pettijohn and co-workers (8), which sediment in neutral sucrose gradients at 1,600S to 1,800S. By employing the conditions of Pettijohn and co-workers, which included 45 s of incubation in lysozyme at 4°C before shifting to 24°C and addition of detergents, we observed a broad nucleoid peak sedimenting at about 4,000S, characteristic of membrane-bound nucleoids. Increasing the incubation time to 2 min in lysozyme gave nucleoids sedimenting at 1,600S to 2,000S as expected for membrane-free nucleoids. More rigorous lysis conditions did not alter the sedimentation value of the nucleoid peak, but did lead to increased breakage of nucleoids as evidenced by an increase of material at the top of the gradient. When the same lysis conditions were used on cells after induction at 42°C, extremely poor lysis was observed. To achieve satisfactory lysis, lysozyme treatment was carried out for 4 min at 24°C before addition of the detergent mixture. Again, more rigorous lysis conditions resulted in increased breakage of the nucleoids. Figure 1 shows the sedimentation profile for DNA from samples isolated before and 20 and 30 min after induction at 42°C.

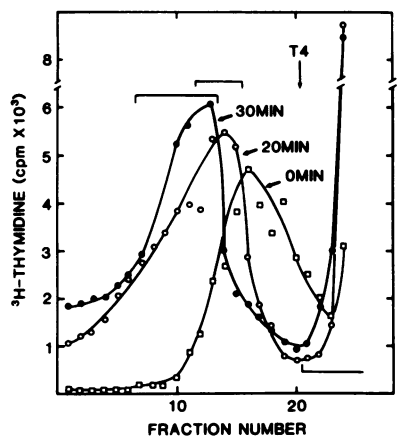

FIG. 1. *Folded nucleoids from a Mu lysogen before and after induction. E. coli MP300 (Mu) was labeled with [^{3}H]thymidine for several generations to fully label host chromosomes. Samples were removed before (□), 20 min after (○), and 30 min after (●) induction at 42°C. Lysates were prepared as described in the text, and folded nucleoids were sedimented on 10 to 30% sucrose gradients for 20 min at 17,000 rpm in an SW50 rotor. ^{32}P-labeled T4 phage was added as a sedimentation marker (S = 1,025).*

The physical integrity of the nucleoids was maintained in all of the samples, although the sedimentation rate of the nucleoids was altered as a function of time after induction; a progressive increase in this value was observed. Linear or nicked circular Mu DNA would have sedimented at less than 40S as estimated from empirical formulas relating S value and molecular weight (4); molecules up to 10 times the molecular weight of Mu would have sedimented at about 100S. Such molecules would have been readily separated from the nucleoids sedimenting at 1,800S or greater.

Table 2 shows approximate S values for the nucleoids shown in Fig. 1 and annealing data on the percent Mu DNA in the unfractionated DNA, the nucleoids and the material in the upper portions of the gradients. Release of any sizeable amount of Mu DNA from the nucleoid would result in an enrichment for Mu DNA in species sedimenting more slowly than the intact nucleoid, with a concomitant decrease in Mu DNA in the nucleoid region. Random breakage of nucleoid DNA would increase the amount of DNA sedimenting more slowly than intact nucleoids, but would not in itself alter the percent Mu DNA contained in the various fractions. We chose to concentrate on samples isolated 20 min after induction, when Mu DNA accounts for approximately 15 to 20% of the total cellular DNA. Numerous (>10) experiments similar to that of Fig. 1 were performed in which samples from across the gradients were pooled and analyzed by DNA-DNA annealing for the percentage of Mu DNA, and the values were compared with the percentage of Mu DNA in the unfractionated lysates. In all of the experiments performed, little or no enrichment for Mu DNA was seen in fractions which sedimented slower than nucleoids, indicating that all or most of the Mu DNA cosedimented with the nucleoids.

Folded nucleoids from λ lysogens. As a

TABLE 2. *Sedimentation and annealing data for Mu DNA*[a]

Min after induction	Peak S value	Mu DNA (%)		
		Lysate	Peak	Top
0	1,800	0.85		
20	2,200	22.60	18.6	19.6
30	2,600	45.40	46.8	42.5

[a] Folded nucleoids were prepared from *E. coli* MP300, fully labeled with [^{3}H]thymidine, before and at intervals after induction. Fractions of the gradients shown in Fig. 1 were pooled as indicated by the bars, and the percent Mu DNA was determined by DNA-DNA annealing. Approximate S values were calculated by using T4 phage as a marker, with S = 1,025.

comparison, we studied the sedimentation of folded nucleoids from strains lysogenic for λ, before and after induction of the λ prophage. Prophage DNA is excised from the host chromosome at 10 to 15 min after induction (5, 13). Nucleoids were prepared 18 min after induction, when λ DNA comprises 15 to 20% of the total cellular DNA. Figure 2 shows the sedimentation profile of the DNA in neutral sucrose gradients. Fractions were pooled as indicated, and the percent λ DNA in each fraction was determined by DNA-DNA annealing. The values shown in Fig. 2 correspond to approximately 40% of the λ DNA sedimenting independently of the nucleoids; in several experiments of this kind, up to 50% of the λ DNA sedimented independently of the folded nucleoids. The cause of the cosedimentation of 50% or more of the λ DNA with the folded nucleoids is unclear (see below). No increase in S value of the nucleoids isolated after induction of λ lysogens was observed.

Isolation of unfolded nucleoid DNA. The attachment of Mu DNA to the folded nucleoid (see above) may be through covalent bonds to the host chromosome or it may involve an interaction with the elements that maintain the compact structure of the folded nucleoid. RNA or protein molecules or both have been implicated in maintaining the folded nucleoid structure (8, 32), and these constraints can be released by incubation in the presence of RNase or SDS or by heating to 65°C (8). The intact, unfolded nucleoid DNA then can be isolated on sucrose gradients. Extreme care is necessary as the unfolded nucleoid DNA is very sensitive to shearing; hence, treatment with the unfolding agents is performed on top of the sucrose gradients to eliminate pipetting of unfolded DNA. In addition, sedimentation of unfolded nucleoid DNA is subject to rotor speed effect; i.e., a dependence of S value on the strength of the centrifugational field. When sedimented at low rotor speeds (about 3,000 rpm) the fully unfolded nucleoid DNA sediments at about 200S to 250S (8, 15).

Folded nucleoids present either in unfractionated lysates or in purified form after sedimentation in sucrose gradients (Fig. 1) were subjected to various combinations of unfolding treatments. The most rigorous regime consisted of incubation in RNase and heating to 70°C in 0.3% SDS for 1 h. Figure 3 shows the sedimentation patterns for a Mu lysogen (A) and a λ lysogen (B). Extracts of cells isolated before, and at the indicated times after, induction at 42°C were layered onto neutral sucrose gradients containing 0.3% SDS and heated to 70°C for approximately 1 h before cooling to room temper-

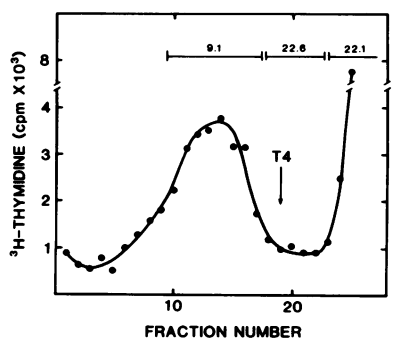

FIG. 2. *Folded nucleoid from a λ lysogen. A lysate of E. coli AT4511 (λ) was prepared 18 min after induction at 42°C and heated as described for Fig. 1. λ DNA in the lysate was 15.2% of the total DNA present.*

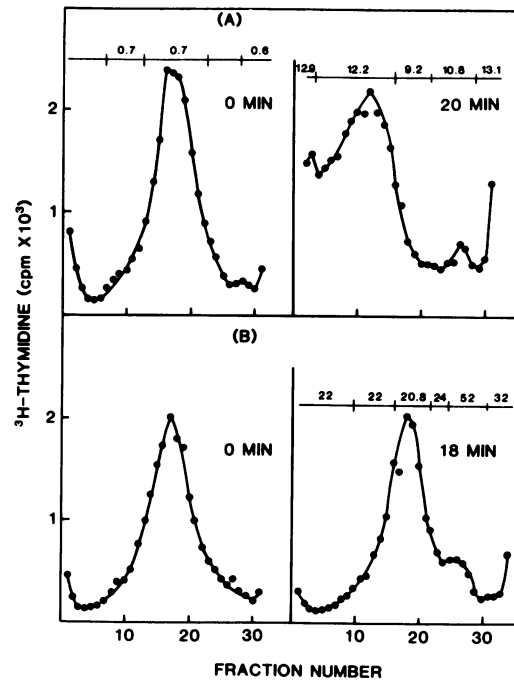

FIG. 3. *Unfolded nucleoid DNA from Mu and λ lysogens. (A) Lysates of fully labeled MP300 were prepared before (0 min) and 20 min after induction at 42°C. Samples were layered onto 5 to 20% sucrose gradients containing 0.3% SDS and heated to 70°C. After cooling to room temperature, the gradients were sedimented at 3,000 rpm for 40 h in an SW27 gradient. Fractions were pooled as indicated, and the percent Mu DNA was determined with the DNA-DNA annealing assay. Mu DNA in the lysates was 0.6 and 12.5% of the total before and 20 min after induction, respectively. (B) As above, except the λ lysogen AT4511 was sampled before (0 min) and 18 min after induction at 42°C. λ DNA in the lysate was 24% of the total 18 min after induction.*

ature. The gradients were then centrifuged at 3,000 rpm at 20°C in an SW27 rotor. After fractionating the gradients, samples were pooled as indicated, and the percent Mu and λ DNAs were determined. As with the experiments employing folded chromosomes, numerous experiments with Mu lysogens showed the release of no more than a few percent of the Mu DNA from the unfolded nucleoid DNA, probably accounting for less than one molecule per cell. Experiments with unfolded nucleoid DNA from λ lysogens showed that from 20 to 50% of the λ DNA sedimented independently of the nucleoid DNA, most often about 50%. The results were the same with or without RNase treatment and in experiments employing unfolding of nucleoids that were first isolated in the folded form on sucrose gradients. Additional control experiments with *E. coli* MP304 containing an RP4 plasmid showed that approximately 60 to 70% of the plasmid sedimented more slowly than unfolded nucleoid DNA.

Figure 3B shows that the S value for unfolded nucleoid DNA from λ lysogens, approximately 200S to 250S relative to T4 DNA, was essentially the same before and after induction. In contrast, the sedimentation rate of unfolded nucleoid DNA from induced Mu lysogens increased to greater than 300S from the value of 200S to 250S for the nucleoid DNA from uninduced cells. The possible implications of this observation are discussed below.

In some experiments, considerable amounts of DNA sedimenting slower than 200S were observed in lysates from both uninduced and induced cells (approximately 100S relative to T4 DNA at 59S). This could be the result of fragmentation of unfolded, circular DNA to intact, linear molecules as suggested by Levin and Hutchison (12), but even in such gradients (Fig. 4) there was no indication of enrichment for Mu DNA in the upper portions of the gradient.

DISCUSSION

The experiments presented here were designed to determine the intracellular form and location of the copies of Mu DNA synthesized during the lytic cycle. Almost all of the Mu DNA was found associated with host nucleoids, even after the nucleoids were unfolded with RNase digestion and heating to 70°C in the presence of SDS. The most likely explanation for these results is that the Mu DNA copies are integrated into the intact host chromosome and remain there until packaged into mature virions.

Although covalent integration is the most likely cause of the association, other means of association of Mu DNA with nucleoids must be considered. A noncovalent association with the structural components maintaining the compact form of the folded nucleoid is possible. Indeed, Miller and Kline (16) have reported cosedimentation of plasmid DNA with folded nucleoids, but they observed that most of this association could be disrupted by unfolding the nucleoid structure. In all of our experiments in which the nucleoids from induced Mu lysogens were unfolded with various combinations of RNase digestion, incubation in SDS, and heating to 70°C, little or no Mu DNA was released from the unfolded nucleoid DNA. With the same procedures, we observed up to 70% of the plasmid DNA from cells carrying an RP4 plasmid sedimenting independently of the unfolded nucleoid DNA. Approximately 50% of λ DNA from induced lysogens sedimented independently of the nucleoid DNA when there were 15 to 20 copies of λ DNA per cell. Therefore, we consider it unlikely that an association of the form described by Miller and Klein is responsible for the results obtained with Mu lysogens.

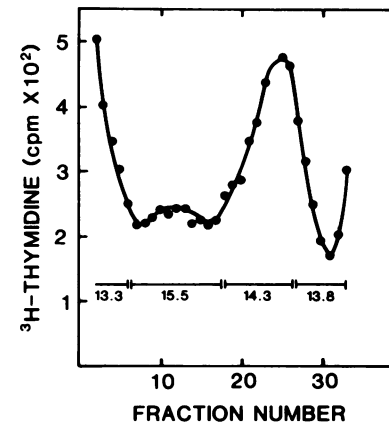

FIG. 4. *Unfolded nucleoid DNA from a Mu lysogen. A lysate of E. coli MP300 was treated as described for Fig. 3 except 50 μg of RNase per ml was added to the layered sample. The Mu DNA in the lysate was 16.1% of the total DNA present.*

An interesting alternative to covalent integration is that Mu DNA and host chromosomes exist as interlocked circular molecules (catenanes). Catenanes have been described as intermediates in replication of supercoiled plasmid DNA (11, 19) and as the products of in vitro excision of λ DNA (18). Resolution of catenanes can be effected by DNA gyrase (10, 17), but unresolved catenanes could persist in vivo. Of the various forms of Mu DNA that could be catenated with the host chromosome, we can eliminate covalently closed circular molecules of Mu DNA devoid of host DNA, since such mol-

ecules apparently do not exist (13, 14), and supercoiled molecules containing Mu and host DNA, since our data with Hc DNA (covalently closed circular forms containing both Mu and host DNA) show that there is less than one molecule of Hc DNA per cell 20 min after induction (29), the time when most of the nucleoid measurements were made. A catenated nicked circular form containing Mu and host DNAs cannot be excluded by our data; however, the most likely explanation for the observed association is actual integration of essentially all Mu DNA into the host nucleoid. The possible role of catenane formation in the inability to release 100% of plasmid DNA or λ DNA from nucleoids requires further investigation.

The persistence of nucleoids after induction of both Mu and λ lysogens is noteworthy, even though more than 95% of the cell population was induced for phage development as judged by decreases in viability and optical density. This contrasts with observations with the virulent bacteriophage T4 which show unfolding of the nucleoid structure early in the T4 lytic cycles, dependent on the T4 *unf* gene, followed by degradation of host DNA (24). Not only are the nucleoids from induced Mu and λ lysogens intact, but the host chromosome appears to remain in a covalently continuous circular form as judged from the sedimentation behavior of the unfolded nucleoid DNA. The effect of double-strand or single-strand breaks of the DNA in folded nucleoids would be to progressively reduce the sedimentation rate due to release of the supercoiling of the DNA in individual domains of the nucleoids (8, 15, 32). Double-strand breaks leading to fragmentation of the host chromosome would result in decreased sedimentation rates after unfolding of the nucleoid structure relative to intact, unfolded molecules. No such decreases were observed with nucleoids from Mu or λ lysogens before or after unfolding of the nucleoid structure. Decreases of S could, of course, be obscured by increase of S due to other factors.

Increases in S values were observed for nucleoids from induced Mu lysogens, both before and after unfolding of the nucleoid structure (Fig. 1 and 3); no corresponding increases were observed with nucleoids from induced λ lysogens. The observed increases were larger than expected for the increase in molecular weight resulting from the integrated copies of Mu DNA. For example, in the experiment shown in Fig. 3A, the S value was calculated by using the following relationship (2) between sedimentation rate (S), distance traveled in the gradient (D), and molecular weight (M).

$$\frac{S_1}{S_2} = \frac{D_1}{D_2} = \left(\frac{M_1}{M_2}\right)^{0.35}$$

The calculated increase was less than 5%, whereas the observed increase was more than 35%. Two explanations that can be envisioned are attachments of membrane to the nucleoids or altered conformation of the nucleoids. As several integrated copies of Mu DNA may be replicating simultaneously during lytic development, multiple interactions of replicating forks and membrane sites could exist; however, it is unlikely that extensive pieces of membrane would persist after treatment with Sarkosyl and heating in SDS. An intriguing explanation for the increase in sedimentation rate envisions an alteration of conformation as a result of coupled processes of replication and integration. The inability to find significant amounts of extrachromosomal Mu DNA and the observed increase of S values are consistent with a model of coupled replication and integration of Mu DNA, in which extrachromosomal replicas of Mu DNA are not the products of replication. Rather, integration of the replica would proceed simultaneously with replication. Our observation that plasmids containing Mu DNA associate with the host chromosome at the time of onset of Mu DNA replication also suggests that integration and replication of Mu DNA are coupled processes (1). Models such as those of Shapiro (23) and Grindley and Sherratt (6) which involve ligation of the ends of the Mu DNA at the site of replication to staggered nicks at the (subsequent) site of integration of the replica could account for the coupling or replication and integration. The details of the postulated replication-integration event and the applicability of such a scheme for other transposable elements remain to be elucidated.

ACKNOWLEDGMENTS

We wish to thank Nancy Tyler and Jayna Karpinski for their skillful technical assistance and David Pettijohn for valuable discussions.

The work was supported by research grant GM24270 from the National Institutes of Health.

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