ASSOCIATION OF R6K PLASMID REPLICATIVE INTERMEDIATES WITH THE FOLDED CHROMOSOME OF ESCHERICHIA COLI

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Summary: When Escherichia coli strain CSH50(R6K) is lysed so as to preserve the folded chromosome structure approximately 9 of the 11 R6K molecules maintained per chromosomal equivalent cosediment with the host nucleoid on a neutral sucrose gradient; the remaining 2 plasmids sediment at their normal rate. When cells are briefly labeled with [^{3}H]thymidine, the majority of plasmid replicative intermediates and nascent mature plasmids are found in the plasmid subpopulation that cosediments with host folded chromosomes. This finding suggests that plasmid replication occurs in a restricted cellular locus, perhaps even while in association with its host's folded chromosome.

We have recently shown that the autonomous F plasmid associates (not by linear insertion) with its host's chromosomes (1). The basis for this conclusion is the observation that the majority of covalently-closed circular (CCC) F plasmids ($S^\circ_{20,w}$=80) from a cell population cosediment on neutral sucrose gradients with host nucleoids at rates of 1100 to 4000S. We have also shown that many other plasmid genotypes associate with their host's folded chromosomes (2). In particular, R6K, which is maintained at a level of about 11 copies per chromosomal equivalent, binds about 83% of its population to host chromosomes. In an effort to shed light on the physiological significance of plasmid chromosome complexes, we have examined plasmid replication in a R6K bacterial host. The results presented in this paper show that replicative intermediates and nascent monomers are associated preferentially to folded chromosomes.

Methods: Bacterial strain CSH50 was used in these studies. It is described by Miller (3). We find CSH50 is plasmid free by dye-CsCl analysis. Plasmid R6K was introduced into CSH50 by transformation (4). Methods for isotopically la-

beling DNA in M9-casamino acid-deoxyadenosine medium (1) have been described. The [^{14}C]thymine used for continuous labeling of bacteria was used at a specific activity of 110 mC/mmole. The [^{3}H]thymidine used for pulse labeling was used at a specific activity of about 60 C/mole and was added to broth medium at a level of 40 μC/ml, final concentration. Purification of plasmid-folded chromosome complexes is as previously described (1) except that 1M NaCl was present throughout the lytic procedure and cells were exposed to detergent at 22-25°C for 30 min.

Results and Discussion: Intracellular distribution of R6K CCC DNA. The intracellular distribution of R6K CCC DNA has been determined by sedimenting the material in a cell lysate to separate the folded chromosome-bound from -free plasmids (1,2). In three separate determinations, we found (2) that 75, 82, and 92% or an average of 83% of the total R6K was bound to folded chromosomes. Since the average amount of CCC DNA associated with folded chromosomes is about 9% of the chromosomal DNA, we calculate (after correcting for 83% plasmid binding to chromosomes) that these cells contain about 11 plasmid copies per chromosomal equivalent.

Intracellular distribution of R6K replicative intermediates. Plasmid replicative intermediates are found in many conformations, one of which is circular dimers (5,6). To show that circular dimers have the properties expected of replicative intermediates, we examined this class of molecules to see if they became preferentially labeled during a brief exposure to [^{3}H]thymidine. Accordingly, a culture was prelabeled with [^{14}C]thymine for 3 to 4 generations, then pulse labeled with [^{3}H]thymidine for 5 min. The cells were lysed and the crude lysate centrifuged in a dye-CsCl gradient to separate supercoiled from linear DNA forms. Finally, the supercoiled DNA was sedimented through an alkaline sucrose density gradient (Fig. 1). The ^{3}H/^{14}C ratio in the circular dimer peak at fraction 7 is about 3.5 times greater than the ratio in the monomer peak at fraction 13. The peak at fraction 7 represents either catenated or concatenated dimers (7) or a mixture of both. Some partially relaxed catenated dimers (5,6) are present as evidenced by the small peak of [^{3}H]thymidine at fraction 17. The peaks at both fractions 7 and 17 have ^{3}H/^{14}C ratios greater than monomer R6K and are typical of replicative intermediates.

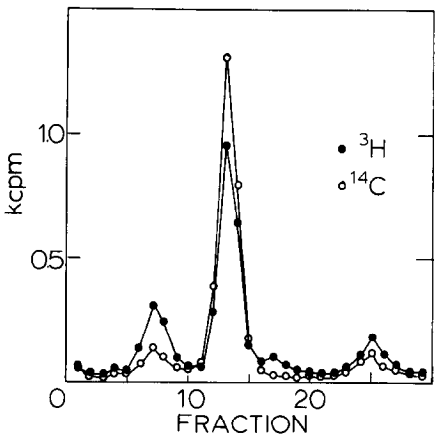

Figure 1. Alkaline sucrose gradient centrifugation of R6K CCC DNA. A ^{14}C pre-labeled culture was pulse labeled with [^{3}H]thymidine 40 μC/ml for 5 minutes. The DNA was then released from cells (10) and the entire lysate centrifuged to equilibrium in a CsCl-ethidium bromide gradient. The supercoiled DNA was then pooled and further analyzed on a 5 ml, 20-31% alkaline sucrose gradient containing 0.3M NaOH, 1M NaCl and 0.005M ethylenediamine-tetraacetate. Centrifugation conditions were 45,000 rpm for 60 minutes in a Spinco SW50.1 rotor. Sedimentation is from right to left and the peaks at fractions 7 and 13, respectively, represent dimer and monomer plasmid forms.

Our next objective was to determine the intracellular "location" of the circular dimers. To achieve this objective we examined, on alkaline sucrose gradients, the relative amounts of CCC dimers found as chromosome bound or free plasmids. In the chromosome bound population, we find that 9-12% of the total CCC plasmid DNA is in the dimer form (data not shown). Since there are about 9 monomers per chromosome, this means there is about 1 dimer per chromosome. In the free plasmid population we find only 3-4% of the total CCC DNA is in the dimer form. Since there are only two free R6K monomers per chromosome, this means there is less than 0.1 free dimers per chromosome. Thus dimers are mostly folded chromosome associated.

The data in Table 1 shows the *in vivo* flow of ^{3}H pulse labeled into ^{14}C pre-labeled monomer and dimer R6K plasmids. To obtain this data, we followed the same labeling procedures as in the experiment described in Fig. 1. Note that in Experiment 1, Table 1 both bound and free dimers have greater $^{3}H/^{14}C$ ratios

TABLE I

Distribution of Pulse Label in R6K Plasmids

PLASMID SUBPOPULATION		TIME	$^{3}H/^{14}C$ DIMER	MONOMER
Experiment I	Free	1.5 Min.	0.4	0.1
	Bound	Pulse	1.0	0.8
Experiment II	Free	5 Min.	2.4	0.7
	Bound	Pulse	2.1	0.8
	Free	10 Min.	1.1	1.4
	Bound	Chase	1.2	1.4

A culture prelabeled with [^{14}C]thymine was pulse-labeled with [^{3}H]thymidine. Samples were withdrawn at 1.5 and 5.0 minutes and the remaining culture was incubated for 10 minutes more after adding a thousandfold excess of unlabeled thymidine (chase). Next 1800S plasmid-folded chromosome complexes were separated from free plasmids on neutral sucrose gradients (1). Finally, the $^{3}H/^{14}C$ ratios for the CCC monomer and CCC dimer forms were determined as in Fig. 1 for the bound and free plasmids.

than the equivalent free monomer species which again, indicates dimers are precursors to monomers. Likewise, the bound dimer has a 2.5 fold greater $^{3}H/^{14}C$ value than the free one. Correcting this latter value for the fact that there is a 10 fold greater concentration of ^{14}C prelabeled bound dimers as compared to free ones, we calculate the bound dimer is synthesized at least 25 times faster than the free one. A comparable argument exists for the rate at which bound monomers arise, that is, this rate is about 36 times faster since the bound monomers outnumber the free ones 4.5 to 1. The data of the second experiment shows that during a subsequent incubation with an excess of cold thymidine the pulse label in dimers decreases with a concomitant shift of the label into monomers.

The use of the alkaline sucrose gradient technique precludes examining early replicative intermediate forms since many of these forms are not covalently closed. Such forms band at densities intermediate to those of CCC and linear DNA in dye-CsCl gradients (8). To examine these replicative forms, we again pulse labeled a ^{14}C prelabeled R6K^{+} culture with [^{3}H]thymidine, lysed

the cells, resolved the folded chromosome-bound and free plasmids on neutral sucrose gradients, and then banded the resolved DNA's in a propidium diiodide-CsCl gradient (1).

Typical results are shown in Fig. 2. These data show a peak of intermediate density located between the positions of linear and CCC DNA. The intermediate density peak is enriched for ^{3}H counts compared to either the CCC plasmid or linear chromosome peak. Also, the absolute amount of DNA in the intermediate peak is much greater from the folded chromosome pool as compared to the free plasmid pool. The relative amount of replicative intermediates associated with folded chromosome as shown in Fig. 2 is about 6 times greater than found in the free plasmid population.

Replicative intermediates with dye-buoyant density properties identical to those shown in Fig. 2 have been found by others (8) to represent a mixture of dimers (consisting of interlocked supercoiled and open circular monomers) and partially replicated monomers (early forms). Hence, we conclude from the data of Table 1 and Fig. 2 that both early and late R6K replicative intermediates preferentially copurify with that fraction of the R6K population which is bound to folded chromosomes. As expected, examination of DNA's from CSH50 $R6K^{-}$ cultures for both early and late replicative intermediates gave negative results of alkaline sucrose and dye-CsCl gradient centrifugation, respectively (data not shown).

Plasmid R6K segregates into minicells and replicates therein (9). Based on our correlation that plasmid systems which do not associate completely with folded chromosomes do segregate well into minicells (2), we expected to find that nascent R6K molecules would be detected first in "free" plasmid population; to the contrary, we found the opposite. Nonetheless, we do find a slight amount of early and late replicative intermediates among the free R6K molecules which indicates that R6K might replicate at more than on intracellular site. We feel, however, that it is more likely R6K replicates at just one site. This conclusion is based

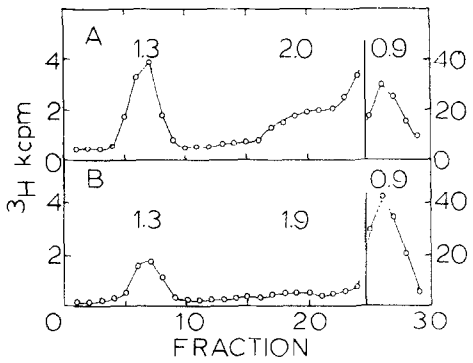

Figure 2. Dye-CsCl isopycnic analysis of pulse-labeled R6K DNA. An R6K was pulse-labeled as in Fig. 1; folded chromosome-bound (A) and -free (B) R6K plasmids were isolated; pooled and then centrifuged to equilibrium in a propidium diiodide CsCl gradients (1) contained in a Spinco Ti 50 rotor. Centrifugation was for 40 hours at 40,000 rpm and 15°C. Gradients were fractionated, counted, and the ^{3}H pulse label plotted. The ^{3}H cpm have been corrected for spillover of ^{14}C cpm. The number over the indicated regions of the gradients indicate the measured ^{3}H/^{14}C ratios. Density increases from right to left and the peak at fraction 7 represents CCC plasmid DNA. The verticle line between fractions 24 and 25 represents a tenfold change of scale on the ordinate.

on our findings (data not shown) that the degree of chromosome breakage (with putative release of plasmids) exactly parallels the amount of free R6K replicative intermediates detected in our experiments.

Strictly speaking, we cannot distinguish if R6K is replicated in continuous association with a folded-chromosome or if it is replicated free of chromosome but at a site close enough to promote efficient formation of nascent plasmid-chromosome complexes. In either case, a limited or unique cellular location is implied for R6K replication. Resolution of these choices and other obvious questions about the roles of plasmid-chromosome complexes in plasmid maintenance are the objects of current studies.

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Note in added proof: Replicative intermediates of plasmid R6K also have been found to be associated with host cell folded chromosomes by Dr. Ronald Sheehy and coworkers (R. Sheehy, personal communication).

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