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Characterization of the Inhomogeneous DNA in Virions of Bacteriophage Mu by DNA Reannealing Kinetics

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The DNA of bacteriophage Mu has been studied to characterize a region of inhomogeneous sequence that occurs at one end of the molecule. The kinetics of reassociation of tracer amounts of labeled host DNA in the presence of Mu DNA show that Mu DNA contains a complete selection of host sequences. These host sequences are shown to be covalently attached to phage-specific sequences and are present at a concentration that accounts for the inhomogeneity observed in the electron microscope. The significance and possible function of the host DNA attachment is discussed.

The DNA extracted from purified virions of bacteriophage Mu is not completely homogeneous with respect to base sequence. When the DNA is denatured, reannealed, and observed in the electron microscope, virtually all the reassociated structures have single-stranded split ends at one end of the molecule measuring between 2 and 5% of the total length of the molecule (thus between 500 and 1,000 base pairs) (8). These heteroduplex structures indicate that Mu DNA carries a large assortment of sequences at one end, differing from molecule to molecule. This inhomogeneity is present whether Mu is grown by induction from a prophage in a cloned lysogen, or grown by lytic infection.

A likely source of the inhomogeneous DNA attached to phage-specific DNA is the host chromosome. This is a particularly attractive hypothesis since Mu is known to integrate its DNA randomly throughout the genome of its host, *Escherichia coli* (3, 6, 10).

A precise way of characterizing nucleic acid sequences as to source, specificity, and frequency of occurrence is by studying kinetics of reassociation (5). The presence of specific sequences within a large genome can be detected and quantitated by annealing with labeled, specific sequences as a probe (11). By using these techniques, we were able to show that Mu DNA contains *E. coli* sequences covalently attached to Mu-specific sequences, and that these sequences represent the entire *E. coli* chromosome in an apparently random selection. An estimate of the fraction of Mu DNA made up by these host sequences is consistent with their being in the split ends observed in heteroduplexes.

MATERIALS AND METHODS

Preparation of Mu DNA. Mu phage was obtained by induction of Mu c*Its*62 (12, 13) from a cloned lysogen. The site of integration was not determined, but reannealing data indicates that the strain used is a monolysogen (Daniell, unpublished data). The phage was precipitated with polyethylene glycol and purified by banding in cesium chloride (9). The phage were then dialyzed against 0.01 M Tris (pH 7.5) and 0.01 M Mg^{2+}, and was extracted three times with freshly distilled, water-saturated phenol and once with chloroform, and the DNA was dialyzed against 10^{-3} M EDTA.

Preparation of E. coli DNA. A nonlysogenic strain of *E. coli* W3110, the parent strain of the host from which Mu was induced, was grown to stationary phase in LB broth (3). The cells were centrifuged and washed twice in 0.1 M Tris and 0.1 M NaCl buffer, and the DNA was extracted by a modification of the procedure of Kohne (16). The cells were centrifuged, washed twice with Tris (pH 7.5) and 0.1 M NaCl, resuspended in 2 to 3 ml of 1% sodium dodecyl sulfate and 0.1 M EDTA, and incubated for 1 h at 37 C. Sodium perchlorate was added to a concentration of 1 M and the nucleic acid was extracted with a mixture of equal volumes of phenol and chloroform, then with chloroform alone. The nucleic acid was precipitated by addition of 2 volumes of EtOH. The precipitate was resuspended in 0.2 M NaCl and 10^{-3} M EDTA (pH 7) and was treated with 100 μg of RNase per ml for 1 h at 37 C. Phenol and chloroform extraction was repeated, and the DNA was precipitated and resuspended in 0.005 M EDTA (pH 7).

Preparation of ^{32}P-labeled DNA of W3110. Cells were grown to stationary phase in low phosphate medium (18) with only 10^{-5} M added phosphate and 10 mCi of ^{32}P in 50 ml of culture. DNA was purified as

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above, through the second ethanol precipitation. The pellet was resuspended in 8 M urea and 0.24 M phosphate buffer pH 6.8 (16), and the DNA was passed over an A1.5 M (Agarose, Bio. Rad.) column equilibrated in the same buffer. The excluded volume, which contained 20% of the radioactivity loaded on the column, was diluted fivefold, loaded onto an hydroxyapatite column, washed with 0.01 M phosphate buffer, and eluted with 0.3 M phosphate buffer. This material was 100% acid precipitable and greater than 95% DNase sensitive. Hybridization to unlabeled *E. coli* DNA as assayed on hydroxyapatite was greater than 95% complete.

After shearing this DNA was denatured by heating to 120 C, diluted into 0.14 M phosphate buffer, and passed over hydroxyapatite at 60 C immediately to remove a 5% fraction which adsorbed to hydroxyapatite with no renaturation.

Preparation of ^{32}P-labeled Mu DNA. Strain W3110 was grown on low phosphate medium with 10^{-4} M added phosphate and 20 mCi of ^{32}P in 50 ml of medium to a cell density of 2×10^8/ml. The cells were centrifuged and resuspended in one-fifth volume of low phosphate medium plus 5×10^{-3} M Mg^{2+} and 5×10^{-3} M Ca^{2+}. The suspension was aerated 15 min, then Mu^+ phage was added at a multiplicity of infection of 0.05. The phage were allowed to adsorb without aeration for 15 min at 37 C. Then the labeled medium was added back and the culture was aerated at 37 C until lysis was visible. The culture was chilled and chloroformed, and the debris was centrifuged out. The phage were purified by banding in CsCl and DNA was extracted as for unlabeled phage. The specific activity of phage DNA was approximately 2×10^5 counts/min per μg.

Shearing and sizing of DNA. Unless otherwise specified, DNA was sheared by forcing through a 25-gauge syringe needle several times, followed by sonication with a Branson sonifier (LS 75) at highest power. Sonication was performed in an ice bath for three cycles of 15 s each, spaced 1 min apart to prevent overheating of the DNA. The size of the resulting fragments (300 to 500 base pairs) was checked by electron microscopy.

DNA-DNA reassociation kinetics. The theory and practice of DNA reannealing is fully described in Kohne and Britten (17). Details of different experiments are given in figure legends. DNA from both single- and double-strand aliquots was collected and radioactivity was counted. When eluted DNA was to be reused, sodium dodecyl sulfate was omitted from the eluting buffer.

S_1 endonuclease. S_1 endonuclease was the kind gift of Roy Britten. Properties of the enzyme and conditions for its use are described in Britten et al. (4).

RESULTS

Reassociation of ^{32}P-labeled E. coli DNA in the presence of Mu DNA. Figure 1 shows the kinetics of reassociation of radioactively labeled *E. coli* DNA under conditions such that the reassociation must be driven by *E. coli* sequences in the Mu DNA present in the renaturation mixture at high concentration. The simple nature of the log C_0t plot indicates that most of the species reassociating are present at the same concentration; if there is any rapidly reassociating fraction (indicating preferential occurence of particular host sequences in the Mu DNA), it represents less than 10% of the *E. coli* genome. The reaction is complete with 96% of *E. coli* DNA reassociated; this is the extent to which this labeled DNA reanneals with cold bacterial DNA from the same strain. Thus we can say that all or nearly all sequences present in *E. coli* DNA are present in Mu and are found with equal frequency. A control shows that the labeled *E. coli* DNA has not reassociated at the same C_0t in the absence of Mu DNA. When annealed sequences were melted by stepwise temperature elevation, the ^{32}P-labeled *E. coli* DNA reannealed in the presence of Mu DNA

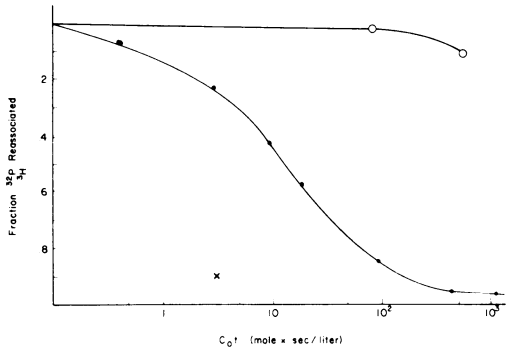

FIG. 1. *Reassociation of ^{32}P-labeled E. coli DNA in the presence of Mu DNA. Nineteen optical density units per milliliter of unlabeled, sonicated Mu DNA and 5×10^{-4} optical density units per ml of ^{32}P-labeled E. coli DNA were mixed and denatured. A small amount of ^{3}H-labeled Mu was present so that completeness of denaturation at zero time and rapid renaturation of Mu could be monitored easily. The denatured DNA was incubated at 70 C in 0.48 M PO_4 buffer, with aliquots taken at specified times and diluted into cold 0.14 M PO_4 buffer (3 ml). The fraction of reannealed, labeled DNA in these diluted aliquots was measured by binding of the DNA to hydroxyapatite at 60 C. The cross (×) represents the reassociation of ^{3}H-labeled Mu DNA at the first time point (20 min = 3.2 C_0t Mu DNA). Closed circles (●) represent the reassociation of ^{32}P-labeled E. coli DNA driven by the unlabeled Mu DNA present. Open circles (○) show the reassociation of the same amount of E. coli DNA in the presence of sheared, denatured chick DNA. Aliquots taken at zero time had ^{32}P-labeled E. coli less than 1% associated and ^{3}H-labeled Mu less than 4%. These time points represent the results of one experiment. Results of two duplicate experiments agree closely. The C_0t value is for Mu DNA in the reactions represented by the cross and by the closed circles, and for chick DNA in the reaction represented by open circles.*

gave the same profile as ^{32}P-labeled *E. coli* DNA reannealed with unlabeled *E. coli* DNA.

Approximate fraction of Mu virion DNA consisting of host sequences. Under a given set of conditions of renaturation (salt concentration, temperature, DNA fragment length), DNA of a given complexity has a characteristic C_0t at which half the DNA is reassociated ($C_0t_{1/2}$) (5). To determine the fraction of *E. coli* DNA in Mu DNA from the reassociation of labeled bacterial DNA, we analyzed the kinetics of reassociation of labeled bacterial DNA in the presence of a measured amount of cold bacterial DNA sheared and renatured under conditions as much as possible like those in the experiment with phage DNA. The results are shown in Fig. 2. The $C_0t_{1/2}$ is taken from the graphed results to be 0.7 (mole times seconds/liter). In the reassociation driven by Mu DNA, at the point where 50% of ^{32}P-labeled *E. coli* has associated, the C_0t of Mu DNA was 14 mol × s/liter (Fig. 1). Because the rate of reassociation of a particular species of DNA under fixed conditions is a fixed parameter, the C_0t of *E. coli* sequences in the mixture at this point *must be* 0.7 mol × s/liter. The fraction of Mu DNA renaturing as *E. coli* sequences is 0.7/14 or 5%. This agrees, within our experimental accuracy, with the length measurements of the split ends in electron microscopy preparations of Mu heteroduplexes (10).

Covalent attachment of the host sequences to Mu DNA. The aim of these experiments was to characterize the DNA appearing as split ends in heteroduplexes. It was necessary to assure that the host sequences we detected in Mu by C_0t analysis were covalently attached to phage-specific DNA. Since Mu is a generalized transducing phage, we had to consider another source for these *E. coli* sequences. Co-transduction frequencies suggest that much of the transduction is accomplished by pieces of DNA the length of the whole Mu genome (13), presumably in the form of pseudovirions (host DNA packaged without Mu DNA).

In the case of generalized transducing phage P1, 0.3% of phage particles contain host DNA as pseudovirions. We might expect Mu to have fewer such particles, as transduction frequencies per host marker are 100- to 1,000-fold lower for Mu than for P1. However, as we do not have a direct measure of pseudovirions in Mu, we wished to rule this out as the major source of host sequences. To do so, we made use of the fast reassociation of Mu DNA compared to *E. coli* DNA and of the S_1 nuclease from *Aspergillus oryzae* which digests single-stranded DNA specifically, leaving double-stranded DNA in-

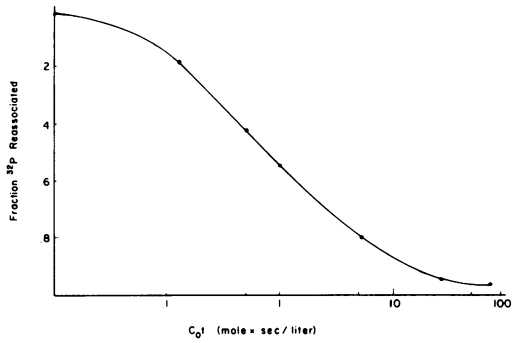

FIG. 2. *Reassociation of ^{32}P-labeled E. coli with unlabeled E. coli DNA. One optical density unit per milliliter of unlabeled, sonicated E. coli DNA and 5 × 10^3 counts/min of ^{32}P-labeled E. coli DNA (10^6 counts/min per gamma) were denatured, and reassociation kinetics were measured under the conditions of Fig. 1. The actual zero time measurement was 1% and the final point (70 C_0t) was 98% reassociated. The $C_0t_{1/2}$ for E. coli DNA reassociation under these conditions, read from the graph of the data, is 0.7 (1.4-h incubation at this concentration).*

tact. The experiment is outlined schematically in Fig. 3. Mu DNA was fragmented by sonication at very low power, such that the average piece size was about 10^6 molecular weight, about twice the length of the inhomogeneous ends. (This was single-strand molecular weight. Fragments were spread for electron microscopy in the presence of full-length Mu as a length standard. One hundred fragments were traced and measured; the length in relation to Mu was 8 ± 3%.) This DNA was denatured and reannealed to a C_0t at which 75% of Mu DNA was renatured (as measured by reassociation of ^{3}H-labeled tracer Mu), but less than 1% of ^{32}P-labeled *E. coli* DNA in the mixture had renatured. The fraction of DNA which had double-stranded regions was recovered from hydroxyapatite. Any non-renatured sequences attached to renatured (phage) sequences would be expected to be recovered in this fraction. Unrenatured host sequences of the pseudovirion type, unattached to phage sequences, would be lost in the single-strand eluant. After lengthy dialysis to remove phosphate, half of the reannealed fraction was digested with S_1 and the other half was incubated without enzyme. Both samples were then purified away from enzyme and digested fragments by phenol extraction and tested for their ability to hybridize labeled *E. coli* sequences. Results of several determinations are in Table 1. Ninety-five percent of the *E. coli* DNA probe hybridizes to this rapidly reannealing Mu DNA fraction. This hybridization is reduced to low levels by diges-

TABLE 1. *Maximum percentage of host DNA annealing to Mu DNA which has been denatured and reannealed to low C_0t*[a]

Expt no.	% Hybridized to undigested fraction	% Hybridized to S_1 digested fraction	$C_0t_{\frac{1}{2}}$[b]
1	85	8	
2	90	4	
3	95		15 (±3)

[a] See text and Fig. 3 for experimental detail.

[b] Amount of Mu DNA, as in Fig. 1. The error arises from the small number of data points taken here.

tion of the reannealed Mu DNA with S_1 nuclease. The rough (only a few time points were taken in these experiments due to lack of material) determination of a $C_0t_{\frac{1}{2}}$ value for the denatured, reannealed fraction of Mu is in agreement with the value obtained for total extracted Mu DNA.

Most of the annealing of *E. coli* sequences, then, can be accounted for by sequences which are covalently attached to rapidly renaturing sequences and are not themselves rapidly renatured. Thus it appears that the inhomogeneous DNA present on one end of each molecule of Mu DNA is host specific in sequence, and that the sequences in a population of Mu represent the entire host chromosome in random selection.

It is possible that host DNA contains phage-specific sequences, which would hybridize to virion DNA. (As observed for lambda by Cowie and McCarthy, 7.) It should be noted that the presence of Mu sequences in host DNA at an equivalence of 1 or less per cell will have no bearing on the results concerning host sequences in Mu DNA. The entire Mu genome is only 1% of the length of the *E. coli* chromosome; this amount of homology could not have shown up in the annealing of labeled host in the presence of Mu. Hybridization of ^{32}P-labeled Mu DNA to host DNA showed that in fact Mu-specific sequences are represented in less than 1 copy per cell in a nonlysogen (Daniell, unpublished data). This agrees with results obtained by Bird et al. (2).

DISCUSSION

We have shown that approximately 5% of the DNA extracted from Mu virions consists of *E. coli* sequences covalently attached to phage-specific sequences. This can completely account for the heterogeneous sequences at the end of the Mu DNA molecule observed by electron microscopy. The selection of host sequences is essentially random.

The host DNA acquired and packaged by Mu introduces new complexities into the picture of phage integration and excision. When Mu integrates, it does so without the attached host sequences (14). One step in integration must then be the detachment of these sequences. Since the population of DNA molecules in a single burst from a lysogen has a large selection of host sequences, we also must suppose that Mu DNA attaches different host sequences in the course of normal replication.

Schroder and van de Putte (19) and Bukhari and Razzaki (personal communication) have obtained genetic evidence that there is considerable integration of Mu DNA during phage development. Supercoiled circles of DNA of varying size and containing varying amounts of phage and host DNA have been detected intracellularly (20; W. Schroder, submitted for publication; W. Schroder et al., Virology, in

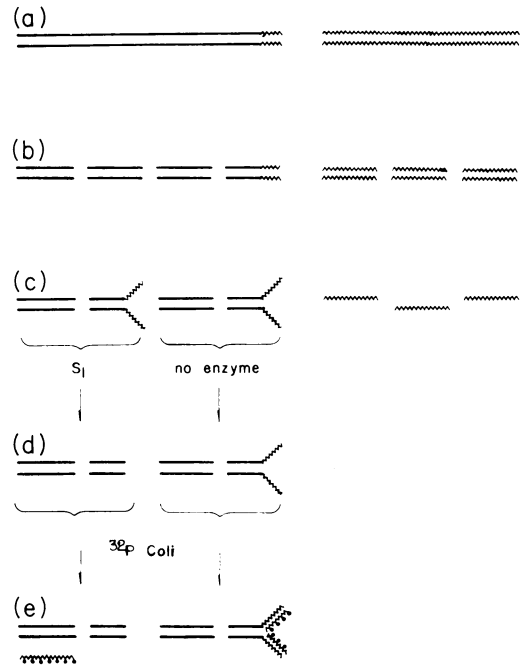

FIG. 3. *Outline of an experiment to show that host sequences in Mu DNA are covalently attached to phage-specific sequences. Symbols:* ——, *phage-specific sequence;* ∿∿∿, *host-specific sequence;* ∿∿∿, *labeled host DNA. (a) A population of whole Mu molecules, having some pseudovirions present. (b) Fragmentation to sized longer than the inhomogeneous ends. (c) Denaturation and renaturation at low C_0t (host sequences unattached to phage-specific sequences have not renatured). Reannealed sequences are separated from unrenatured sequence. (d) S_1 enzyme removes single-strand tails (nonhomologous sequences) from reannealed Mu. (e) Labeled host DNA reanneals only with rapidly renatured fraction which has not been digested with S_1 nuclease.*

press) after induction of Mu cI*ts* and after lytic infection. They apparently include the replicating DNA. Host DNA in the virion may be a result of packaging from the Mu-host intracellular forms.

Loss and acquisition of new host sequences during phage multiplication can explain the failure of attempts to isolate specialized transducing phages of Mu. Analysis of generalized transduction frequencies has been done in hopes of determining whether the original molecules excised from the chromosome on induction of the prophage retain host sequences, but the results are ambiguous (M. Howe, personal communication). Physical studies may clarify this.

Do the host sequences have a function in integration? The site of integration of Mu is not directly determined by homology with the host sequences, as an infecting Mu phage will integrate at a normal frequency into regions completely missing in the host on which the phage was previously grown (E. Daniell, unpublished data; M. Howe, personal communication). More direct experiments are needed to ascertain possible involvement of host sequences in integration. The DNA of other lysogenizing phages has been shown to be infectious and capable of integration. If this is true for whole Mu DNA, it would be of great interest to see whether selective removal of host sequences (by denaturation, reannealing, and use of single-strand nuclease) would affect the ability of the DNA to lysogenize.

Evidence that the inhomogeneous ends of Mu consist of host-specific sequences has also been obtained by Ernesto Bade (Fed. Proc. **33**:1487, 1974).

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