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A new method for the purification and identification of covalently closed circular DNA molecules

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ABSTRACT

A new technique has been developed for the rapid isolation of covalently closed circular DNA molecules. The procedure is a selective extraction based on differences in the partitioning of covalently closed circular DNA molecules and noncovalently closed species between phenol and water at acid pH and low ionic strength. Under the conditions described, linear as well as nicked circular DNA is extracted into phenol, while covalently closed circular DNA molecules remain in the water phase. The method permits the quantitative isolation of covalently closed circular DNA from either total cellular DNA or partially purified preparations, to a degree of purity comparable with buoyant density procedures.

INTRODUCTION

The isolation of closed circular DNA free of contaminants has required considerable experimental effort (1-14). Such purification is essential in the preparation of bacterial plasmids (including those containing recombinant DNA) (15, 16), the DNA of animal tumor viruses (1, 3), the replicative forms of certain prokaryotic and eukaryotic viruses (17, 18), and the closed circular DNA present in subcellular organelles (19). The purification requires removal of contaminating host cellular DNA, as well as circular DNA that has been nicked.

In this paper a simple and highly reproducible method for the purification of closed circular DNA is described. The method is rapid and quantitative and appears to be applicable to DNA molecules at least within the range of molecular weights between 2.6 and 25 x 10^6. The purification is effective even when as little as 0.01% of the DNA is in the closed circular form.

RESULTS

The acid-phenol extraction method. The procedure is based on the use of phenol for the selective extraction of DNA species other than

covalently closed DNA at acid pH and low ionic strength. The method is outlined as follows:

1. A DNA preparation containing closed circular DNA is purified free of RNA and protein by standard procedures.
2. The DNA sample is concentrated by ethanol precipitation and resuspended in a buffer containing 50 mM sodium acetate, pH 4.0 and 75 mM NaCl.
3. The sample is extracted with an equal volume of redistilled phenol equilibrated with 50 mM sodium acetate, pH 4.0. In the first extraction more than 95% of the nicked circular species, and linear DNA molecules greater than about 1500 base pairs in length, are cleared from the water phase; after three extractions, more than 99% of these contaminants are removed. In contrast, closed circular DNA remains quantitatively in the water phase.

In the following sections we describe a series of purifications as examples of the range of applicability of the method.

Purification of closed circular Col E1 DNA from total cellular DNA

In this section we describe the purification of closed circular Col E1 DNA by the acid-phenol extraction of a standard preparation of total E. coli cellular DNA (see Methods). Col E1 is a covalently closed, supercoiled, circular duplex molecule (28) of molecular weight about 4.4×10^6 (H. Ohmori and J. Tomizawa, personal communication). The purification of this plasmid is demonstrated starting from E. coli grown either under normal conditions, in which the plasmid represents about 1-2% of the total DNA, or in the presence of chloramphenicol, where about 30% of the total cellular DNA is of plasmid origin (28).

An electrophoretic analysis of the purification of Col E1 DNA from total cellular DNA by means of acid-phenol extraction is presented in Figure 1. An EcoRI digest of bacteriophage λcI857 DNA is shown in channel 9, and provides a set of reference fragments. In channel 1 about 8 μg of DNA isolated from E. coli harboring about 20 copies of the plasmid per cell is shown; in channel 5 the same amount of DNA is displayed, but from cells in which the plasmid has been amplified by chloramphenicol treatment to about 3000 copies per cell. Both DNA samples are seen to contain high molecular weight cellular DNA of heterogenous size (present at the gel origin as well as at the position near fragment A), a broad band of nicked circular Col E1 DNA (between fragments A and B), a sharp band of linear plasmid (between fragments B and C), and a dense band of closed

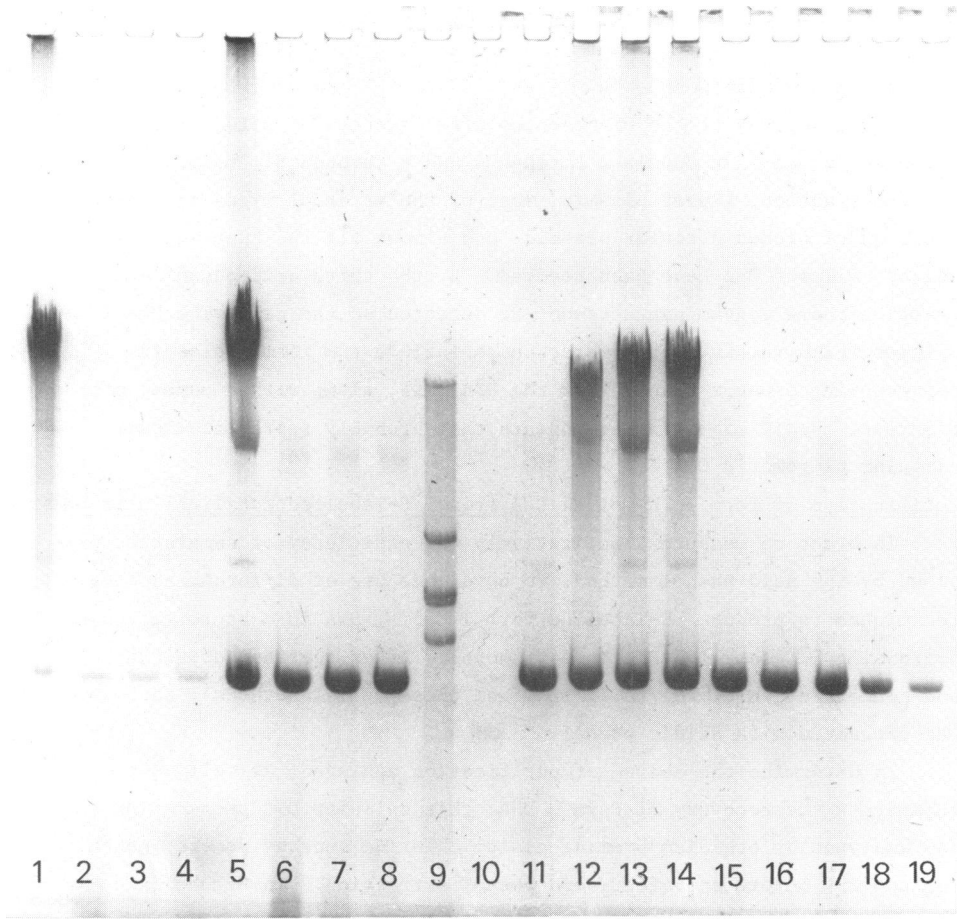

Figure 1. Purification of covalently closed circular Col E1 DNA by acid-phenol extraction. 20 µl samples of the DNA preparations are displayed. Details are described in the text. (1) E. coli cellular DNA containing unamplified Col E1; (2-4) water phase after 1, 2, and 3 acid-phenol extractions; (5) E. coli DNA containing amplified Col E1 and (6-8) the water phase after 1, 2, and 3 acid-phenol extractions; (9) λcI857 DNA EcoRI digest, 1 µg displaying fragments A through F, of molecular weight 13.7, 4.74, 3.73, 3.48, 3.02, and 2.13 x 10^6, respectively (31); (10-14) sample shown in (5) after 3 extractions at 75 mM NaCl and 50 mM sodium acetate at pH 3, 4, 4.5, 5, and 6, respectively; (15-19) sample shown in (5) after extraction at 50 mM sodium acetate, pH 4, in the presence of either 75, 100, 125, 150, or 200 mM NaCl.

Electrophoresis was conducted at 10 volts/cm for 10 hours in 1% agarose (Miles, LE grade). The buffer system contained, per liter solution: Tris base, 4.8 gm, Na_3EDTA, 0.37 gm, sodium acetate, 0.68 gm, glacial acetic acid, 1.8 ml. DNA staining and photography were performed as described previously (32).

circular Col E1 DNA (between fragments E and F). As is evident in the figure, the plasmid is present in these two DNA preparations in about the expected proportions.

Equal volumes of the DNA samples after successive acid-phenol extractions are shown in channels 2 through 4 and 6 through 8. Following the first extraction, linear plasmid, most of the material migrating at the position of nicked circular plasmid, and almost all the high molecular weight cellular DNA have been removed. By the third acid-phenol extraction these contaminants cannot be detected on the gel. The DNA species which remain in the water phase include the form I plasmid, representing greater than 95% of the DNA mass, along with a number of discrete, slowly migrating components that probably represent closed circular oligomeric Col E1.

Purification of form I ^{3}H-Col E1 DNA from ^{14}C-labelled total cellular DNA

In order to measure quantitatively the efficiency of separation provided by the acid-phenol method, we have made use of differential labelling techniques to produce ^{3}H-labelled form I Col E1 DNA in a bacterial host containing ^{14}C-labelled DNA (see Methods). After routine purification and concentration of the total DNA from these cells (Methods), the sample was extracted with acidic phenol.

To determine the degree of purification achieved, as well as to quantitate the recovery of form I DNA, this cellular DNA preparation was analyzed by ethidium bromide-cesium chloride buoyant density centrifugation (22) before and after acid-phenol extraction. In Figure 2a a sample of total cellular DNA is shown; most of the ^{14}C-labelled *E. coli* DNA bands at fraction 16, at the density expected for linear bacterial DNA. The small additional peak of ^{14}C-labelled material centered at fraction 8 represents supercoiled Col E1 DNA labelled during exposure to ^{14}C-thymine. The peak of ^{3}H-labelled DNA centered at fraction 8 is supercoiled Col E1 DNA, while the other ^{3}H-labelled peak, at fraction 16, contains both nicked Col E1 and cellular DNA.

In Figure 2b is shown an aliquot of the same volume of the DNA preparation, following three acid-phenol extractions. All of the ^{14}C-labelled DNA has been extracted, except for the small peak banding at the position of supercoiled DNA. Of the ^{3}H-labelled DNA, about 3% can be detected at the density of nicked Col E1. Greater than 95% of the ^{3}H-labelled DNA that banded at the position of form I DNA in the starting sample is recovered at that position following acid-phenol extraction.

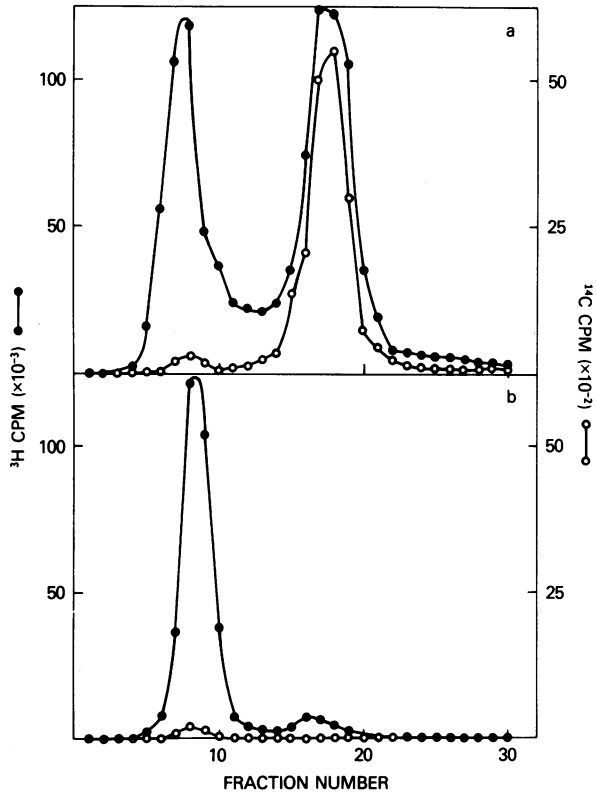

Figure 2. Ethidium bromide-cesium chloride density gradient analysis of total cellular DNA isolated from E. coli harboring Col E1 before and after acid-phenol extraction. A, 50 µl of the total cellular DNA preparation described in the text before acid-phenol extraction, containing about 3 x 10^6 cpm in ^{3}H (•-•) and 1 x 10^5 cpm in ^{14}C (o-o); B, the same volume of the DNA preparation after three acid-phenol extractions, containing about 1.6 x 10^6 cpm in ^{3}H and 1 x 10^3 cpm in ^{14}C. The DNA samples were added separately to two tubes containing 5.65 gm CsCl, 5.75 gm TE buffer, and 0.25 ml ethidium bromide (10 mg/ml in water). The solutions were overlaid with dodecane and centrifuged in the Beckman 65 rotor at 40,000 rpm for 80 hours at 20°. 0.2 ml fractions were collected and 100 µl portions counted in 10 ml of Aquafluor (New England Nuclear). ^{3}H was counted at 20% and ^{14}C at 40% efficiency; cross-contamination of ^{14}C into the ^{3}H channel amounting to 15% and ^{3}H into the ^{14}C channel at 0.2% has been subtracted from the data.

Purification of form I plasmid pML21 from total cellular DNA

Purification of high molecular closed circular DNA molecules presents a difficult experimental problem (9, 13). Enrichment of the plasmid species by preparation of a cleared lysate or by application of the Hirt extraction procedure (3, 11) is variably successful (9, 13). To

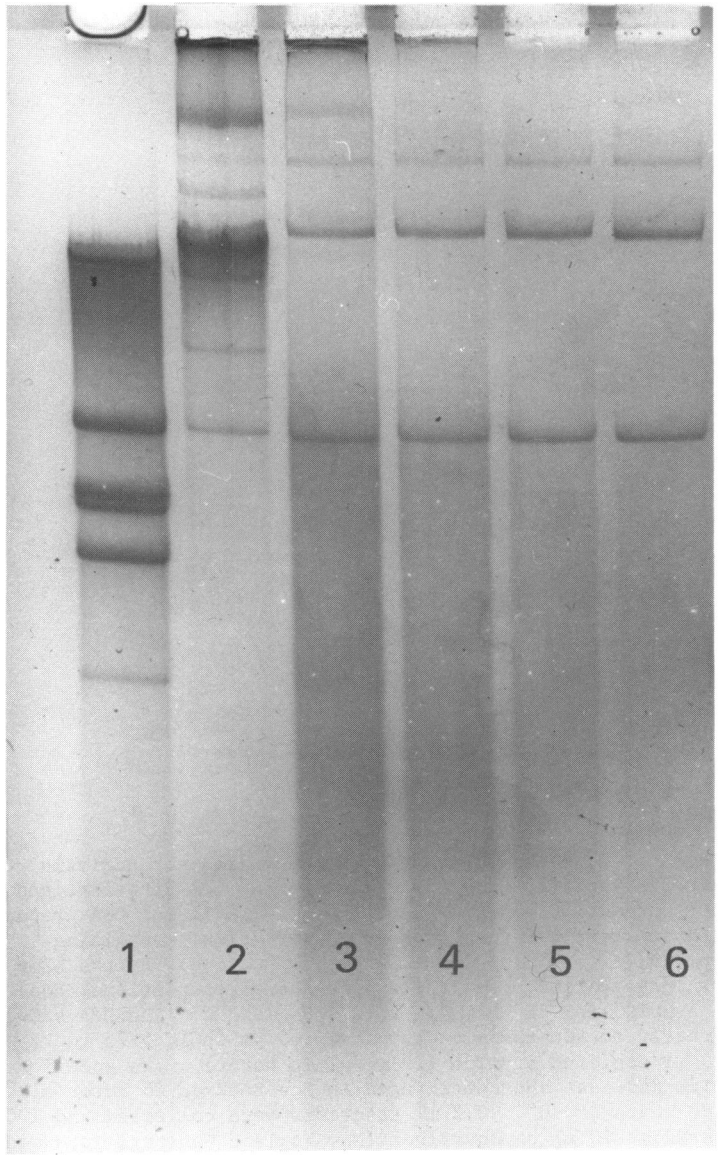

Figure 3. Purification of the plasmid pML21 from total cellular DNA by acid-phenol extraction. (1) 5 µg of λcI857 DNA EcoRI digest; (2) 10 µl of total cellular DNA containing pML21; (3-6) 25 µl of the preparation in (2) after 1, 2, 3, or 4 acid-phenol extractions. Conditions of electrophoresis are as described in Figure 1.

explore the size range of closed circular DNA that can be isolated by means of acid-phenol extraction, we have purified the plasmid pML21 from a total E. coli cellular DNA extract. pML21 is a derivative of Col El

containing an R factor insertion conferring kanamycin resistance (24). The plasmid is approximately 4 μm in contour length (24), which corresponds to a molecular weight of about 8 x 10^6, based on the contour length of 2.1 μm for Col E1 (25), and a molecular weight for Col E1 of 4.4 x 10^6.

A dimeric plasmid appears in roughly equal proportions to monomer, while trimers can be isolated at about one third the concentration of the two smaller species (Dr. J. Tomizawa, personal communication).

In Figure 3, channel 2, an aliquot of the crude cellular DNA is shown. Numerous discrete DNA species are evident in addition to the bulk cellular material. Following acid-phenol extraction, three species remain in the water phase in invariant ratios through the course of four successive acid-phenol extractions (channels 3-6). These species represent the monomer, dimer, and trimer in the expected mass ratios.

Although we have not determined the upper limit of the size of closed circular DNA molecules that partition quantitatively in the water phase under the conditions of the acid-phenol extraction, it is clear from Figure 3 that plasmids as large as 25 x 10^6 can be purified quantitatively by this simple procedure. At the other extreme, the smallest plasmid that we have purified is pNT1, about 58% the size of Col E1 or 2.55 x 10^6 in molecular weight (26).

Figure 3 also demonstrates another powerful application of the acid-phenol extraction method, namely as a procedure to identify closed circular DNA molecules in a complex mixture. Of the many discrete species that are noted in the total cellular DNA sample, some of which represent nicked circular and linear molecules, the subset of closed circular species are readily identified by virtue of their persistence in the water phase. The closed circular dimer, in fact, cannot be identified at all in the crude cellular preparation because of the dense cellular contamination. This plasmid emerges distinctly upon acid-phenol extraction.

DISCUSSION

In this report we describe a new, rapid, and simple method based on the quantitative extraction from aqueous solution of all DNA species except closed circular structures. The physical basis underlying the selective extraction by phenol at acid pH and low ionic strength is not as yet understood. The method, however, provides a highly reproducible approach to the purification of closed circular DNA, circumventing the need for such standard procedures as ethidium bromide-cesium chloride density gradient centrifugation (23), alkaline sucrose velocity sedimen-

tation (6), or selective nitrocellulose adsorption (4, 10).

We have applied the acid-phenol extraction procedure to the purification of DNA plasmids ranging in molecular weight between about 2.6 and 25 x 10^6. Neither the upper nor lower limits in the size of the closed circular forms amenable to the procedure has yet been determined.

From reconstitution experiments with ^{14}C-labelled bacterial DNA and ^{3}H-labelled Col E1 DNA we can demonstrate quantitative purification of Col E1 DNA present at a mass ratio with cellular DNA as low as 0.01% and at a concentration in the water phase as low as 0.05 µg/ml. Thus, the acid-phenol extraction procedure should be of use in the purification of circular DNA molecules present as only minor components relative to the cellular genome. Recently (unpublished data) we have extended the procedure without difficulty to the purification of closed circular SV40 DNA from total cell extracts, following essentially the protocol outlined below for the purification of bacterial plasmids (omitting lysozyme).

Since closed circular DNA species within the size range that we have studied remain in the water phase at a constant concentration through successive acid-phenol extractions, we suggest that the method may have general application not only for purification, but also as a rapid screening procedure for the identification of closed circular DNA molecules.

METHODS

Reagents

Distilled phenol is used for the acid extraction procedures. Commercial undistilled phenol is not used since the reagent contains variable amounts of acidic impurities, which upon equilibration with water may generate a solution with a hydrogen ion concentration as high as 0.1 N. Adjustment of such an impure phenol solution to pH 4.0 introduces a variable, the ionic strength, that has considerable effect on the efficiency of the extraction (see below). Although we routinely distill phenol under nitrogen, we have used phenol distilled under the ambient atmosphere with the same results.

Bacteriophage λcI857 DNA was a generous gift of Drs. K. Mizuuchi and M. Gellert. EcoRI (31) was generously provided by Dr. Jim McGhee. EcoRI digestion of λcI857 DNA was conducted under the conditions described by Thomas and Davis (31).

The acid-phenol extraction method

The acid-phenol extraction is conducted in the cold (0-4°) to reduce the rate of acid-catalyzed depurination (20), although the selectivity of the

extraction method is unchanged at higher temperatures (22°). DNA containing covalently closed circular duplex species is prepared by standard procedures (see below). It is essential that contaminating protein be eliminated rigorously from the DNA preparation, since its presence will lead to non-selective losses of DNA from the aqueous phase during the subsequent acid-phenol extraction. The DNA sample is concentrated and freed of inorganic salts by ethanol precipitation. The DNA precipitate is resuspended in a buffer of low ionic strength, such as 1 mM Tris-Cl, pH 8.0 -1 mM sodium EDTA, pH 8.0. For preparative purposes the concentration of the DNA sample to be acid-phenol extracted is adjusted to about 10 A_{260} units/ml. Non-covalently closed DNA contaminants are extracted with lower efficiency from solutions of higher DNA concentration. The lower limits on the concentration of the DNA sample to be extracted are determined solely by the desired final concentration of the closed circular species that will remain in the water phase.

To the DNA solution is added 1/20 volume of 1 M sodium acetate, pH 4.0 (57.2 ml glacial acetic acid and 6.2 gms sodium hydroxide per liter solution) and 1/20 volume of 1.5 M NaCl. One volume of redistilled phenol, equilibrated with 50 mM sodium acetate, pH 4.0 is added and the mixture shaken vigorously for 2-3 minutes. The white emulsion is centrifuged at 6000 x g for 5 minutes in an angle head or swinging bucket rotor. The phases separate distinctly, leaving a clear upper water phase. The appearance of the lower phenol phase is quite variable in our experience; the turbidity is dependent in part on the concentration of DNA contaminating the closed circular species in the starting sample. The phenol phase may appear as a plastic, white emulsion throughout or may contain only a narrow band of white emulsion near the phase boundary.

Between one and four acid-phenol extractions will yield a preparation of closed circular DNA free of greater than 95% of the DNA contaminants present in the original sample. The number of extractions required depends on the proportion of contaminating DNA to closed circular species. Starting from a cleared lysate (7) in which about 80% of the DNA is closed circular, one acid-phenol extraction will achieve this degree of purity; starting from a total cellular DNA preparation (see below), in which the form I species may represent 1% or less of the mass of the population, three extractions may be needed to achieve comparable purification.

Upon completion of the acid-phenol extraction, 1/20 volume of 1 M Tris-Cl, pH 8.6, is added to bring the plasmid solution to neutral pH. Phenol is eliminated from the solution by four extractions with 5 volumes

of anhydrous ether, and the phases are separated by a brief low speed centrifugation. Ether is then removed under a gentle stream of nitrogen. Alternatively, the DNA sample can be purified free of phenol by dialysis or gel filtration chromatography. It should be noted that small oligoribonucleotides, as well as fragments of DNA less than about 1500 base pairs long, may contaminate the final preparation if present in the sample prior to acid-phenol extraction; these components are not efficiently extracted by this procedure. Should the presence of these contaminants pose a problem, they can readily be eliminated by separation procedures based on size (neutral or alkaline sucrose gradient centrifugation, gel filtration) or buoyant density. Contamination with very small DNA fragments, however, does not pose a problem in the routine purification of form I plasmids from cellular DNA, since fragments of molecular weight less than about 1-2 x 10^6 are not generated by the usual preparative procedures.

The extent of depurination. Depurination of DNA occurs at acid pH in aqueous solutions (20). To determine the extent of depurination that occurs during the acid-phenol extraction, a sample of form I Col E1 DNA purified by this procedure was analyzed by alkaline analytical sedimentation velocity following treatment under conditions in which strand scission of depurinated DNA is observed. A sample of Col E1 DNA which had been maintained at pH 4.0 for 20 minutes (from the initial adjustment of the preparation with sodium acetate to the neutralization with Tris) was incubated at 70° for 15 hours in a buffer containing 0.1 M KCl, .05 M Hepes-KOH, 0.5 mM EDTA, pH 7.4 to induce polynucleotide backbone cleavage at sites of depurination (21). The sample was then diluted with solvent directly in an analytical ultracentrifuge cell to give a final solvent concentration of 0.9 M NaCl, 0.1 M NaOH. Centrifugation (Beckman Model E, ultraviolet absorption optics) revealed two distinct boundaries corresponding to closed circular DNA and to DNA chains that had been cleaved. From the relative absorbance of the two components we determined that less than 10% of the form I Col E1 species had been converted to nicked structures (data not shown). Thus, we conclude that under conditions of the acid-phenol extraction, depurination occurs at a rate of less than one purine per 30,000 bases per hour.

The effect of pH. We have studied the effect of pH on the ability of phenol to extract linear and nicked circular DNA. The results are shown in Figure 1, channels 10 through 14. Total cellular DNA (channel

5) was adjusted to 75 mM NaCl and 50 mM sodium acetate at pH 3, 4, 4.5, 5, and 6. The solutions were extracted three times with equal volumes of phenol equilibrated with 50 mM sodium acetate at the same pH. As shown in Figure 1, extraction at pH 3 results in considerable loss of closed circular DNA from the water phase. In contrast, as the pH of the water phase increases from 4 to 4.5 phenol loses its capacity to extract nicked circular and linear forms; these remain quantitatively in the water phase.

The effect of ionic strength. The influence of ionic strength on the extraction behavior of closed circular Col E1 DNA is shown in Figure 1, channels 15 through 19. Total cellular DNA (channel 5) was adjusted to 50 mM sodium acetate, pH 4.0. The samples were then extracted three times in the presence of 75, 100, 125, 150 or 200 mM NaCl. As the salt concentration is increased from 125 to 200 mM NaCl, selective extraction is abolished, and almost all of the DNA in the sample, including closed circular molecules, is extracted into the phenol from the water phase.

Preparation of total *E. coli* DNA containing Col E1 DNA

An inoculum of *E. coli* A745 $thy^{-}met^{-}$ (Col E1) (30), provided by Dr. J. Tomizawa, was grown to a density of about 0.6 A_{600} units/ml in one liter of minimal glucose medium supplemented with methionine and thymidine, each at 20 µg/ml. In order to amplify the plasmid, a portion of the culture was made 200 µg/ml in chloramphenicol (Sigma) and maintained at 37° for an additional 15 hours.

The cells were harvested by centrifugation at 5000 x g for 15 minutes in the Sorvall GSA rotor. The cells were washed twice in a buffer containing 25% sucrose and 50 mM Tris-Cl, pH 8.0 (ST buffer) by repeated suspension and centrifugation. The pellet was resuspended in ST buffer with a Dounce homogenizer (A-pestle) to a concentration of about 70 A_{600} units/ml and adjusted to 0.1 M NaCl and 20 mM EDTA by addition of 5 M NaCl and 0.4 M sodium EDTA, pH 8.0. Lysozyme (Sigma, 3X crystallized, 10 mg/ml in ST buffer) was added to a concentration of 1.5 mg/ml and the suspension kept on ice for 15 minutes. The preparation was then diluted with four volumes of a buffer containing 1 mM Tris-Cl, pH 8.0 and 1 mM sodium EDTA, pH 8.0 (TE buffer), and made 0.2% in sodium N-lauroyl sarcosinate (Sigma) by addition of a 20% solution (w/w in water). Pancreatic RNase (Sigma, 3X crystallized, 2 mg/ml in 0.1 M sodium acetate, pH 5.0, preheated at 100° for 15 minutes to inactivate DNase) was added

to a concentration of 100 µg/ml and incubated at 37° for 1-2 hours. Proteinase K (E. Merck, crystallized, 5 mg/ml in water) was then added to a concentration of 100 µg/ml and the preparation incubated at 37° for 1-2 hours. The DNA was isolated by extraction with one volume of water saturated phenol (non-distilled, neutralized with Tris base to pH 8.0) and 1/2 volume of chloroform-isoamyl alcohol (24:1). Phases were separated by centrifugation in the Sorvall HB-4 rotor (5000 x g for 15 minutes). The aqueous phase was re-extracted as above, followed by a single extraction with one volume of chloroform-isoamyl alcohol. DNA was precipitated at -20° overnight, or for 1 hour at -70° (29) by addition of 1/5 volume of 1 M sodium acetate, pH 5.0 and 2 volumes of absolute ethanol. DNA was collected by centrifugation in the HB-4 rotor at 5000 x g for 15 minutes. The pellet was drained, air dried, and resuspended in TE buffer to a concentration of 10 A_{260}/ml.

Preparation of ^{3}H-Col E1 DNA and ^{14}C-labelled total cellular DNA

Col E1 DNA labelled with ^{3}H-thymine is grown in an E. coli host which has been prelabelled with ^{14}C-thymine (28).

An inoculum of E. coli A745 thy^{-}met^{-} (Col E1) was grown to a density of about 0.6 A_{600} units/ml in minimal glucose medium supplemented with (methyl-^{14}C) thymine (New England Nuclear) at a concentration of 2 µg/ml and at a specific radioactivity of 1.2 µCi/µg. Cells were harvested by centrifugation at 5000 x g for 15 minutes in the HB-4 rotor. The cells were washed twice by repeated suspension and centrifugation in minimal glucose medium, and then resuspended in 100 ml of the medium containing thymine (2 µg/ml) and chloramphenicol (200 µg/ml). After 15 minutes at 37°, 5 mCi of (methyl-^{3}H) thymine (New England Nuclear) was added and the culture incubated at 37° for 15 hrs.

Total cellular DNA was then isolated as described for the unlabelled preparation above.

Preparation of E. coli DNA containing plasmid pML21

A one liter culture of E. coli W thy^{-}met^{-} (pML21) (24), provided by Dr. J. Tomizawa, was grown to a density of about 0.6 A_{600} units/ml in minimal glucose medium containing 25 µg/ml kanamycin (Sigma). Chloramphenicol was added to 200 µg/ml and the culture maintained at 37° for an additional 9 hours. Total cellular DNA was isolated as described above.

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