A rapid alkaline extraction procedure for screening recombinant plasmid DNA

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ABSTRACT

A procedure for extracting plasmid DNA from bacterial cells is described. The method is simple enough to permit the analysis by gel electrophoresis of 100 or more clones per day yet yields plasmid DNA which is pure enough to be digestible by restriction enzymes. The principle of the method is selective alkaline denaturation of high molecular weight chromosomal DNA while covalently closed circular DNA remains double-stranded. Adequate pH control is accomplished without using a pH meter. Upon neutralization, chromosomal DNA renatures to form an insoluble clot, leaving plasmid DNA in the supernatant. Large and small plasmid DNAs have been extracted by this method.

INTRODUCTION

Bacterial plasmid DNAs are widely used as cloning vehicles in recombinant DNA research. After new plasmids are constructed, they may be isolated and characterized with respect to their size and restriction enzyme pattern by gel electrophoresis. One method for preparing plasmid DNA in a highly purified form involves gently lysis of bacterial cells, centrifugation to remove the bulk of the chromosomal DNA, and then banding of residual DNA in a cesium chloride gradient in the presence of ethidium bomide. Covalently closed circular (CCC) DNA binds a different amount of the dye than does open circular (OC) or linear DNA and is readily separated from the latter two forms of DNA (1). A second, more rapid, method for preparing plasmid DNA employs hydroxyapatite chromatography (2); this also produces highly purified DNA. However, for many purposes including analysis by gel electrophoresis, less purified DNA can be used. Under favourable conditions, a colony of cells can be lysed and plasmid DNA can be detected in crude extracts after electrophoresis (3-6); this permits the analysis of many clones and makes screening possible. In this report we describe another method for plasmid DNA extraction which is both simple enough to permit screening of many small samples, yet

yields plasmid DNA in a form sufficiently pure to be digestible by restriction enzymes or to be used to transform other cells. In these respects, the procedure we describe is more versatile than other rapid extraction methods. Plasmid DNA can be detected in as little as 0.1 ml of non-amplified liquid culture or in a colony of cells scraped from a plate.

PRINCIPLE OF THE ALKALINE EXTRACTION METHOD

Previous workers have shown that there is a narrow range of pH (about 12.0-12.5) within which denaturation of linear DNA but not CCC-DNA occurs and that this property can be used for purifying CCC-DNA (7-10,20,21). We have utilized this approach for developing a rapid extraction method for plasmid DNAs. Plasmid-containing cells are treated with lysozyme to weaken the cell wall and then lysed completely with sodium dodecyl sulfate (SDS) and NaOH. By choosing the ratio of cell suspension to NaOH solution carefully, a reproducible alkaline pH value is obtained without the necessity of monitoring the pH with a meter; further pH control is obtained by including glucose as a pH buffer. Chromosomal DNA, still in a very high molecular weight form, is selectively denatured and when the lysate is neutralized by acidic sodium acetate, the mass of chromosomal DNA renatures and aggregates to form an insoluble network. Simultaneously, the high concentration of sodium acetate causes precipitation of protein-SDS complexes (11,12) and of high molecular weight RNA (13). In this way, most of the three major contaminating macromolecules are co-precipitated and may be removed by a single centrifugation in a bench-top centrifuge. Plasmid DNA (and residual low molecular weight RNA) are recovered from the supernatant by ethanol precipitation. Plasmid DNA may be analyzed by gel electrophoresis either intact in the CCC form or after digestion with a restriction enzyme.

MATERIALS AND METHODS

Cell strains and Media : Escherichia coli strains HB101 (14), SK1592 (15) and RRI (16) were used for most of the work reported here. E.coli F' strains GM218 (thr$^-$, leu$^-$, arg$^-$, ilv$^-$, thy$^-$, ura$^-$, his$^-$, recA$^-$/F'his$^+$) and JM856 (thyA$^-$, lysA$^-$, pheA$^-$, arg$^-$, relA$^-$, recA$^-$/F' thy$^+$, lys$^+$, phe$^+$, arg$^+$, relA$^+$, tif) from J. George were grown in minimal medium supplemented with the appropriate nutritional requirements at 30°C. Otherwise, L-broth was used throughout. When appropriate, Ampicillin (Ap) was added at a concentration of 100 ug/ml for liquid medium or 40 ug/ml for plates. Tetracycline (Tc) was used

for agar plates at a concentration of 10 ug/ml.

Equipment and Reagents for Plasmid Extraction : Eppendorf-type (1.5 ml) polypropylene tubes and a bench-top centrifuge capable of generating 8-10,000 x g were used. A rack holding 60 Eppendorf tubes speeds up handling of larger numbers of samples. Several racks can be prepared by stacking 5 sheets of 2 mm-thick aluminum (or other metal) and drilling 11 mm-dia. holes at 15 mm spacing through all simultaneously; the edges of each sheet are folded as legs. For complete removal of supernatants following centrifugation in Eppendorf tubes, a Pasteur pipette drawn out to a fine tip is used.

Reagents : I. Lysozyme solution - 2 mg/ml lysozyme, 50 mM glucose, 10 mM CDTA, 25 mM Tris-HCl (pH 8.0). Prepare fresh daily from crystalline lysozyme and stock solutions of the other components. Store at 0°C. II. Alkaline SDS solution - 0.2 N NaOH, 1% sodium dodecyl sulfate (SDS). Store at room temperature; stable for about 1 week. III. High salt solution - 3 M sodium acetate (pH 4.8). Prepare by dissolving 3 moles of sodium acetate in a minimal volume of water, adjusting to pH 4.8 with glacial acetic acid, and then adjusting volume to 1 l. Store at room temperature. CDTA (cyclohexane diamine tetracetate) is a chelating agent which is more soluble in alcohol and forms stronger complexes with metal ions than does EDTA. EDTA can be substituted for applications listed here. CDTA from Sigma or Aldrich Chemical Company was used. A stock solution of RNAse A (1 mg/ml in 5 mM Tris-HCl, pH 8.0) is treated by heating at 100° for 10 min.

Standard Procedure for Extraction of Plasmid DNA from Small Volumes of Cell Cultures : (Footnotes refer to comments below.) The method has been used principally with plasmid pBR322 (ref. 16) and its derivatives in E.coli strains HB101, RRI and SK 1592[1]. Selected clones (Ap^R, Tc^S in the case of hybrid plasmids with inserts at the Hind III site of pBR322) are grown in 2.5 ml of L-broth containing 100 ug/ml Ap in 6-ml vials[2]. After 18 h incubation, 0.5 ml of culture is transferred to a 1.5 ml Eppendorf tube for plasmid extraction, and the remainder is stored at -20°C after the addition of glycerol to 40%. All manipulations are carried out at room temperature unless otherwise indicated. The tube is centrifuged for 15 seconds[3]. The supernatant is carefully removed with a fine-tip aspirator and the cell pellet is thoroughly suspended in 100 ul of solution I. After a 30 min. period of incubation at 0°C, 200 ml of solution II is added and the tube is gently vortexed. The suspension should become almost clear and slightly viscous. The tube is maintained for 5 min. at 0°C and then 150 ul of solution III is

added. The contents of the tube are gently mixed by inversion for a few seconds during which time a clot of DNA forms. The tube is maintained at 0°C for 60 min. to allow most of the protein, high molecular weight RNA and chromosomal DNA to precipitate. Centrifugation for 5 min. yields an almost clear supernatant. Four-tenths of a ml of the supernatant is removed and transferred to a second centrifuge tube[4]. Small amounts of floating material may be carried over at this time. One ml of cold ethanol is added and the tube is held at -20°C for 30 min. The precipitate is collected by centrifugation for 2 min. and the supernatant removed by aspiration. The pellet is dissolved in 100 ul of 0.1 M sodium acetate/0.05 M Tris-HCl (pH 8) and reprecipitated with 2 volumes of cold ethanol. After 10 min. at -20°C, the precipitate is again collected by centrifugation as before. The pellet is dissolved in 40 ul water and then 10 ul of 5 x sample buffer is added. 10-20 ul is applied to an agarose gel for electrophoretic analysis.

Modifications required when plasmid DNA is to be used for bacterial transformation or is to be treated by restriction enzymes : The pellet at the last step is redissolved once more in 100 ul of 0.1 M sodium acetate/0.05 M Tris-HCl (pH 8) instead of water and precipitated with 2 volumes of ethanol as before. For transformation, the pellet may be dissolved in 40 ul of water or dilute buffer and a few microliters added to competent cells. For restriction enzyme analysis, the pellet is dissolved in 36 ul H_2O and 4 ul of pancreatic RNase (1 mg/ml) is added. After 30 min. incubation at 37°C, concentrated restriction enzyme buffer and 1 unit of enzyme are added. Following a period of digestion (37°, 60 min.), the sample is mixed with concentrated sample buffer and part of it applied to a gel for electrophoretic analysis.

Comments and other modifications of the method : 1. CCC-DNA in other cell types has been successfully extracted by the above method. Incubation at room temperature or 37°C with solution I may be necessary to disrupt Bacillus subtilis (S.D. Ehrlich, G. Rapoport - personal communication). A band corresponding in size to the 2 μ circle of yeast cells was detected by gel electrophoresis when alkaline extracts of Saccharomyces cerevisiae strain GRF18 protoplasts (kindly prepared by A. Hinnen) were examined. 2. One-half ml cultures have been grown directly in 1.5 ml Eppendorf tube ; alternatively, colonies (3-4 mm dia.) can be scraped from the surface of an agar plate and resuspended in 100 ul of solution I. 3. If the cell number is very low, a longer centrifugation time may be required for the pellet to stick adequately to the wall of the tube or 0.2 ml of carrier cells can be added. 4. If the

cell number is low, 25 ug of tRNA can be added as carrier to assist precipitation of plasmid DNA.

Gel electrophoresis : 0.8% agarose gels were used, either as 6-mm dia. tubes or as 3-mm thick vertical slabs. Electrophoresis buffer contained 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, adjusted to pH 7.8 with acetic acid. 5 x sample buffer contained 25% sucrose, 5 mM sodium acetate, 0.05% bromophenol blue, 0.1% SDS. After electrophoresis, gels were stained with ethidium bromide (1 ug/ml) and photographed under UV illumination.

Restriction Enzymes : Most were obtained from Biolabs and used under conditions described by the manufacturer. Hind III was prepared at this institute by A. Meier.

All experiments involving recombinant DNA were carried out with the permission of the French Commission on Recombinant DNA according to the French Guidelines.

RESULTS AND DISCUSSION

Treatment of plasmid DNA with alkali : The extraction method we have described involves exposing a crude extract to alkaline pH to denature chromosomal DNA. However, when covalently closed circular DNA is exposed to > pH 13, it gives rise to a denatured form which is not readily renaturable (8,9). The generation of this "irreversibly denatured" form (which is to be avoided in preparing plasmid DNA) is illustrated in Fig. 1. Fig. 1a shows the electrophoretic mobility of pBR322 DNA, purified by conventional methods (1). Band 4 is the supercoiled (CCC form). A small amount of OC form (band 3) and CCC-dimer (band 2) are also visible. When treated with 0.1 N NaOH, neutralized, and run on a neutral gel, a faster moving band (band 5), corresponding to the "irreversibly denatured" form, can be seen (Fig. 1b). Two additional minor bands which migrate close to bands 4 and 5 are also seen. It is likely that these represent two single-stranded forms of plasmid DNA (linear and circular) which arise by denaturation of the OC form. This was verified (Fig. 1c and 1d) by treating the CCC form with nuclease S1 to introduce a few single-strand nicks and then treating with alkali as in Fig. 1b. Bands 4 and 5 diminished and the bands of single-stranded plasmid DNA increased in intensity. This experiment allowed us to identify the position of "irreversibly denatured" CCC-DNA on gels, as well as to establish the approximate position of single-stranded plasmid DNA molecules.

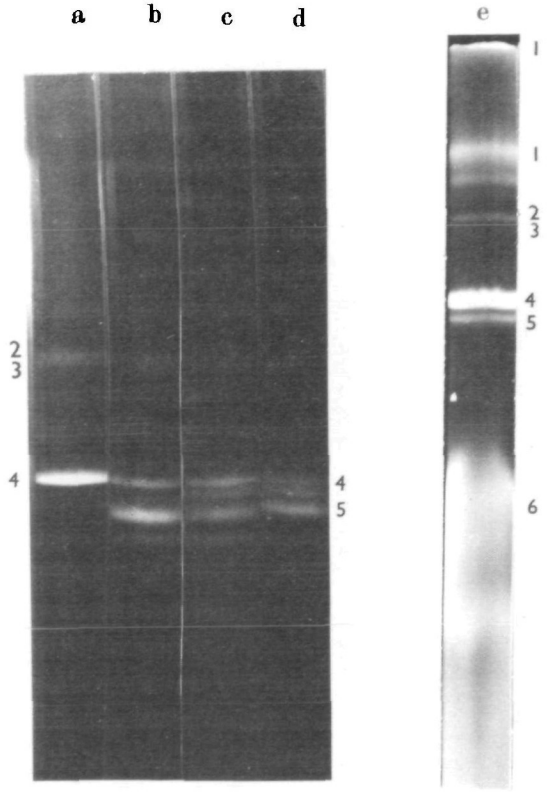

Figure 1. Alkaline treatment of plasmid DNA and cell extracts containing plasmid DNA. a-agarose gel electrophoresis of pBR322, purified by conventional methods. b- as in a, but treated with 0.1 N NaOH for 5 min. at room temperature before electrophoresis. c- as in b, but treated with nuclease S1 for 15 min. before treatment with alkali; d- as in c, but treated for 30 min. with S1. e- alkaline extract of E.coli HB101 cells containing pBR322. This gel is overloaded to demonstrate the position of fluorescent bands other than the CCC-form of plasmid pBR322 (band 4).
Band 1 - chromosomal DNA; band 2 - pBR322 dimer; band 3 - pBR322, OC-form; band 5 - "irreversibly denatured" form of pBR322 CCC DNA; band 6 - region of low molecular weight RNA.

The alkaline extraction method was designed to prevent the generation of the "irreversibly denatured" form; at the same time, the extracts must be alkaline enough for denaturation of chromosomal DNA to occur. Fig. 1e shows an example of such an extract, prepared according to the standard method. The gel is overloaded to indicate the relative positions of minor components. A

small amount of the "irreversibly denatured" form (band 5) runs just ahead of the CCC form (band 4). Contaminating chromosomal DNA (band 1) and a large amount of low molecular weight RNA (band 6) are present. OC DNA (Band 3) and CCC-dimer DNA (band 2) are also detectable. Since these other fluorescent bands may be present to varying extents during routine extractions of plasmid DNA, a knowledge of their relative mobilities is helpful in interpreting gel electrophoresis patterns.

Screening by size of recombinant plasmids prepared by the alkaline extraction method : A commonly used first step in the characterization of a new recombinant plasmid is determination of its size. If it is formed by integration of a fragment of foreign DNA, the size of the fragment may be estimated by agarose gel electrophoresis of either the intact recombinant plasmid or the excised fragment. The alkaline extraction method permits both kinds of analyses. An example of the results of screening recombinant plasmids in a "shot-gun" type of experiment is shown in Fig. 2. Mouse DNA was cleaved

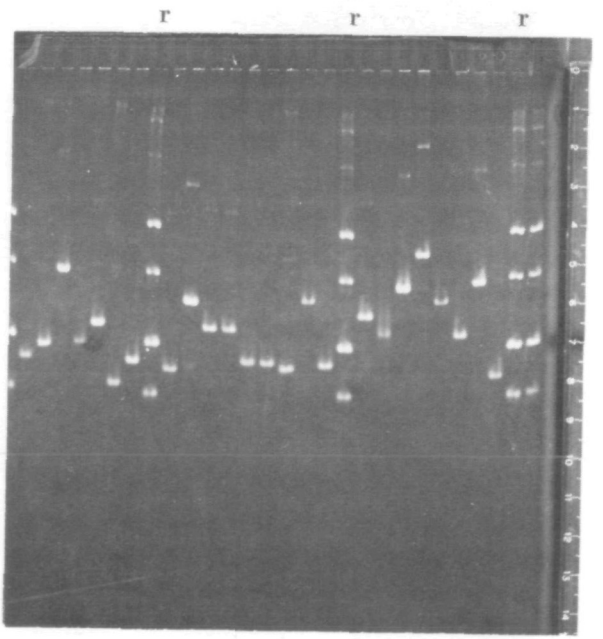

Figure 2. Screening of mouse DNA/pBR322 recombinant plasmids by gel electrophoresis of alkali-extracted plasmid DNA. r - reference mixture of CCC-DNAs; the sizes of reference plasmids (in order of decreasing mobility) are 4.3 kbp (pBR322), 5.7 kbp, 8.5 kbp and 11.2 kbp (pSF 2124). Minor bands are contaminating OC forms in the reference mixture.

by Hind III and inserted into the single Hind III site of pBR322. Ap^R Tc^S clones were selected and plasmid DNA extracted by the standard method. After agarose gel electrophoresis, the principal band seen in each slot has a mobility less than that of the pBR322 CCC-DNA used as a reference (fastest moving band in slot r). The estimated range of integrated DNA fragments, based upon mobility relative to marker CCC-DNAs, was from a few hundred bp up to 3 kbp. A band of plasmid DNA can be detected in each slot although, on a routine screening basis, the recovery of plasmid DNA varied somewhat. We estimate that, on the average, there is about 1 ug of plasmid DNA extracted from 1 ml of original non-amplified culture.

Restriction enzyme digestion : A second important method for characterizing recombinant plasmids is restriction enzyme digestion. We have found that the alkaline extraction procedure gives a preparation of plasmid DNA which is pure enough to be susceptible to digestion by several restriction enzymes including Eco RI, Hind III, Bam HI, Hinf I, Ava II, Hinc III, and Pst I (data not shown). By eliminating the requirement for more highly purified DNA, this method should simplify characterization of recombinant plasmids.

Transformation by extracted plasmid DNA : It is often useful to be able to pass plasmid DNA from one cell to other bacterial cell strains or species. Extracts prepared according to the procedure we have described contain DNA suitable for use in transformation. Rapoport et al. (19) have used an earlier version of the method for this purpose and they found that in some cases a relatively low efficiency of transformation was obtained. The yield of transformation has been improved by the washing procedure described in Materials and Methods. We now find that extracts of pBR322 transform competent HB101 cells at levels comparable to highly purified pBR322.

Preparation of large plasmid DNAs : Recombinant plasmids, prepared by the "cosmid" technique (22), are expected to be 38-52 kbp in length. We tested our alkaline extraction method to see if it could be applied to plasmid DNAs in this size range since other methods employing alkali have been used successfully (20,21). E.coli DNA, partially digested with Hind III, was inserted into plasmid pHC79 (from B. Hohn). In vitro packaging of the ligated DNA and transduction into E.coli strain SF8 was carried out under the direction of B. Hohn during an EMBO Course. Several Ap^R clones were selected and plasmid DNA was prepared from two 4-mm dia. colonies as described in Materials and Methods. The DNA was examined by agarose gel electrophoresis

either directly (Fig. 3a and 3e), or after digestion with Hind III (Fig. 3b and 3f). Linear cosmid DNA, generated by digestion with Hind III, migrates at a rate very close to that of the λ 7.0 kbp fragment. The slowly migrating bands in 4a and 4e are presumed to be large CCC-DNAs. Digestion with Hind III generated the expected pHC79 sized fragment as well as several other fragments, including some very large ones. Although digestion with restriction enzyme may not have been complete in this experiment, it is evident these large plasmids may be prepared by the alkaline extraction method. Preliminary results suggest that F'-plasmids (60-120 x 10^6 daltons) can also be extracted from appropriate strains of E.coli (kindly provided by R. d'Ari).

CONCLUSIONS

We have described a procedure for extracting partially purified

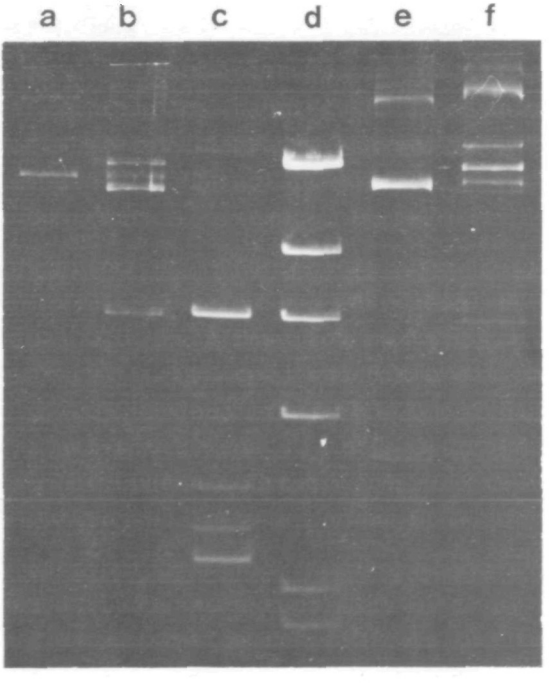

Figure 3. Analysis of two "cosmid" DNAs prepared by the alkaline extraction method. a and e- undigested plasmid DNAs; b and f- digested with Hind III; c- reference CCC-DNAs, in order of decreasing mobility: 4.3, 4.9, 5.7 and 11.2 kbp; d- reference linear DNA (Hind III digest of phage λ DNA): 2.2, 2.5, 4.8, 7.0, 10.0 and 24.0 kbp.

plasmid DNA which we believe to be a useful addition to the list of other methods currently available. The method is simple and reliable and has been used successfully in several other laboratories without appreciable difficulty. Screening new recombinant plasmids by size is useful in initially characterizing new plasmids, in looking for plasmids containing inserts of a particular size, and perhaps in cases where no specific selection marker is available for detecting recombinants. Analysis of supercoiled DNA may give a better estimate of length than linear DNA for high molecular weight molecules (23). Plasmid DNA in this partially purified form is sensitive to digestion by those restriction enzymes which we have tested. This property of the extracts should simplify analysis of recominant DNA molecules.

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