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Note

Fractionation of nucleic acids from mammalian cells in culture by polylysine–Kieselguhr column chromatography

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Polylysine-coated Kieselguhr (PLK) columns have been used to fractionate nucleic acid mixtures isolated from bacteria[1–6] and also from yeast[7,8]. In this paper, the application of PLK chromatography to the separation of the total nucleic acids from lysates of cultured mammalian cells is reported.

PROCEDURES

Cell lines and culture method

Chinese hamster fibroblasts (CH-23) and mouse lymphoma (P388 F-36) continuous cell lines were used. The former was isolated by Tjio and Puck[9] and was cloned in this laboratory[10]. The P388 F cells were isolated by Fischer and Sartorelli[11]. Cultures were propagated in 5- or 30-oz. Pyrex tissue-culture bottles, containing 10 or 70 ml, respectively, of Ham's F12 meduim[12] supplemented with 10% foetal calf serum (Flow Labs.), 50,000 U/l penicillin (I.C.I.), 50 mg/l streptomycin (Glaxo) and 2 mg/l amphotericin B (Squibb). Subcultures of confluent monolayer cultures, in the case of CH-23 cells only, were obtained by trypsinization and by transferring to new bottles with fresh medium, under a 5% carbon dioxide in air atmosphere. P388 F-36 cells, grown in suspension, were subcultured by diluting a confluent culture with fresh medium.

Labelling of the DNA was achieved by growing the cells in medium containing tritiated thymidine (sp. act. 25 Ci/mmol, The Radiochemical Centre, Amersham) at a conc. of 0.2 μCi/ml of medium, for 4–6 cell generations. Tritiated deoxyuridine (sp. act. 4.25 Ci/mmole, The Radiochemical Centre, Amersham) at 0.1 or 0.2 μCi/ml for 4–6 cell generations, was used for RNA labelling.

Preparation of cell lysates

$0.3–1.7 \times 10^8$ cells were trypsinized, washed 3 times with 10 ml of physiological saline and centrifuged at $2600 \times g$ for 5 min. The cell pellet was then dispersed in 2–3 ml of physiological saline and the cells lysed with 3 cycles of freezing in acetone–dry ice mixture, thawing and homogenizing (50–80 strokes using a glass hand homogenizer). The degree of lysis was followed by microscopic inspection of the cell suspension. Lysis of P388 F-36 cells was accomplished after 4–5 cycles of freezing and thawing and 150 strokes using the same homogenizer.

The cell lysates were usually used immediately after their preparation, although preservation at −20° for at most one week gave the same results.

Preparation of polylysine–Kieselguhr columns

PLK columns were prepared as described by Ayad and Blamire[1]. The cell lysates were applied on 1.5-g PLK columns in which the fractionating layer consisted of 1.5 g of washed Kieselguhr treated with 3 mg of poly-L-lysine. In order not to block flow through the column, 0.5 cm of the top layer of the column was dispersed gently.

Elution was achieved by using a linear gradient of 0.4–4 *M* NaCl containing 0.02 *M* KH_2PO_4, pH 6.7, according to the method described before[13].

The extinction at 260 nm of the effluent was continually measured using an LKB Uvicord absorption spectrophotometer and 32 drop-fractions were collected using an LKB fraction collector linked to the Uvicord system.

Treatment of fractions

Immediately after fractionation, each fraction was treated as follows: (a) The optical density was re-estimated at 260 nm using a Unicam SP500 spectrophotometer. (b) The refractive index was estimated using a Bellingham and Stanley refractometer and the corresponding NaCl molarity was found from a standard curve. (c) Radioactivity was assayed by precipitating the nucleic acids present in 3 drops of each fraction on Whatman paper, grade 3 discs, with 10% ice-cold trichloroacetic acid (TCA) (25 min). The discs were washed with 10% ice-cold TCA (25 min), cold 95% ethanol (25 min, twice) and ether (15 min, twice) and then dried under an IR lamp. The discs were placed in 10 ml of scintillator (10 g of 2.5-diphenylozazone, 1 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene in 2.5 l of xylene), and counted in a Packard "Tricarb" liquid scintillation spectrometer, using three channels with a gain of 55% and window setting of 50–1000. (d) Protein was estimated by the Lowry assay[14] using 1.5×10^9 unlabelled cells. (e) RNA was estimated by the orcinol reaction[15] using 1.5×10^9 unlabelled cells.

RESULTS

The elution profiles of labelled CH-23 cell lysates are shown in Figs. 1–3. The protein content of each fraction is shown in Fig. 4. Similar results to those shown in Figs. 1–3 were obtained by using different amounts of labelled cells. Using unlabelled cells, it was found that at least 2×10^7 cells had to be lysed in order to elute a detectable amount of UV absorbing material. Under the same chromatographic conditions, the elution profiles did not alter, *i.e.* the molarity of NaCl which RNA species and DNA were eluted, even with the small quantities of cells.

Fractionation of 1.7×10^8 CH-23 cells (Fig. 1) gave a recovery of 134 μg of DNA, which corresponds to a 65% recovery in comparison with the recovery of DNA isolated by the method of Berns and Thomas[15], which was taken as 100% recovery. With this second method the amount of DNA per cell was calculated to be 1.22×10^{-6} μg.

The molarities of buffered saline in which tRNA, rRNA and DNA are eluted are 0.70–0.80, 1.00–1.10 and 1.75–1.90, respectively. High amounts of labelled RNA were found to be eluted together with DNA at 1.75–1.90 *M* NaCl.

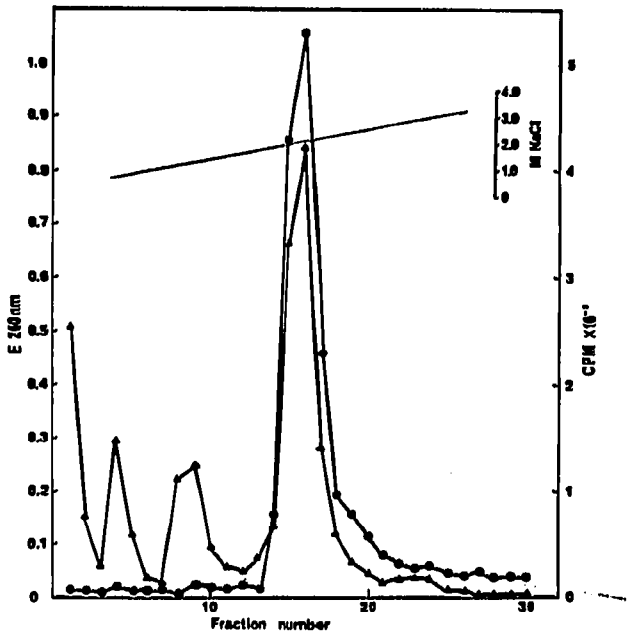

Fig. 1. Elution profile of a crude CH-23 cell lysate (1.7×10^8 cells) on a 1.5-g PLK column, using a linear gradient of 0.4–4.0 *M* buffered saline. The cells were labelled with [^{3}H]thymidine (0.2 μCi/ml) for 6 generations. 32-drop fractions (1.8 ml) were collected. ▲, E_{260nm}; ●, radioactivity.

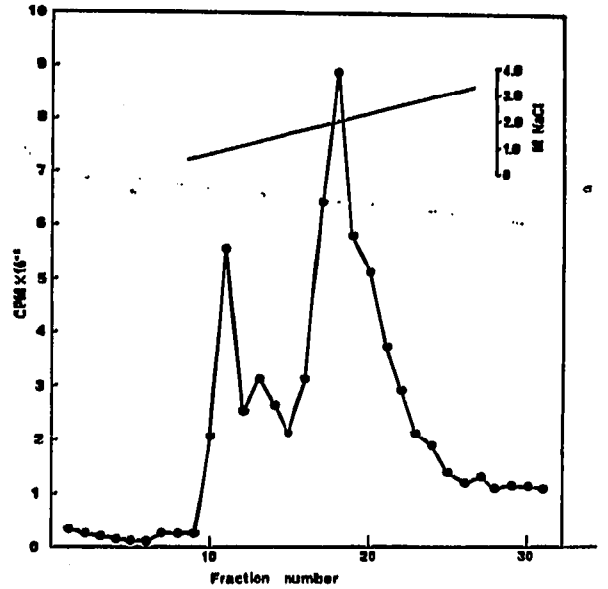

Fig. 2. Elution profile of a crude CH-23 cell lysate (0.3×10^8 cells) on a 1.5-g PLK column, using a linear gradient of 0.4–4.0 *M* buffered saline. The cells were labelled with [^{3}H]thymidine (0.2 μCi/ml) and [^{3}H]uridine (0.2 μCi/ml) for 6 generations. 32-drop fractions (1.8 ml) were collected. Absorbance at 260 nm was not clearly detected due to the very small amount of cells.

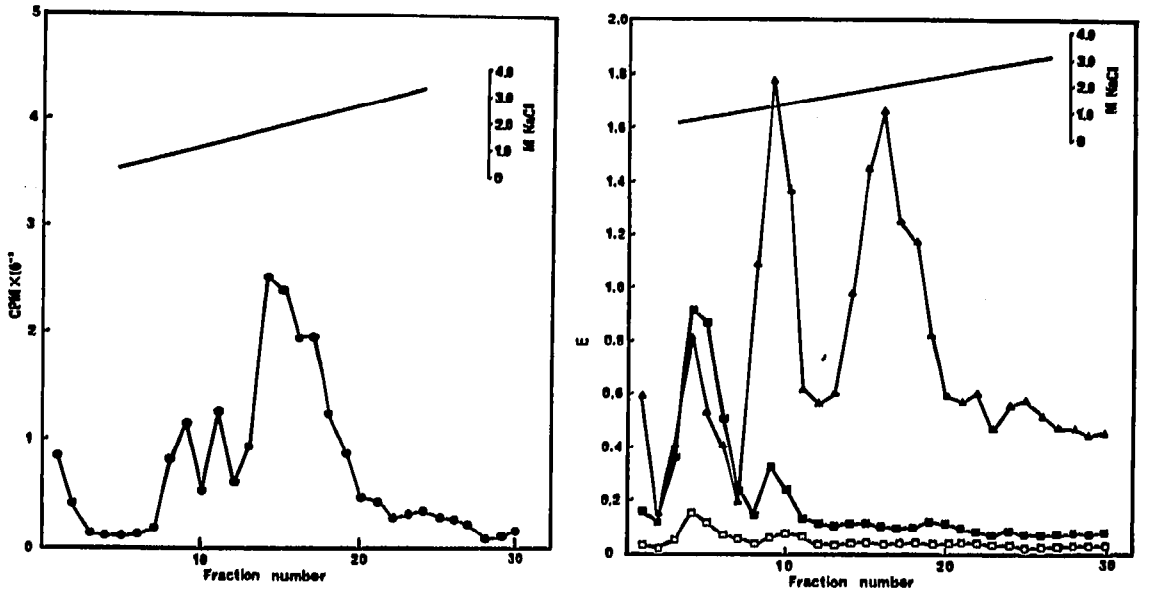

Fig. 3. As in Fig. 2 but the cells were labelled only with [^{3}H]uridine (0.1 μCi/ml) for 6 generations

Fig. 4. Elution profile of a crude P388F-36 cell lysate (9×10^8 cells) on a 1.5-g PLK column using the same gradient as in Fig. 1. The cells were unlabelled. 32-drop fractions were collected and the Lowry and orcinol reactions were carried out with 0.5 and 0.2 ml, respectively, of every fraction. ▲, E_{260nm} (nucleic acids); ■, E_{700nm} (proteins); □, E_{660nm} (RNA).

Nucleotides and other small molecules are eluted first. Protein appears a little later, together with tRNA at 0.70–0.80 *M* NaCl. A smaller amount of protein is eluted together with rRNA at 1.00–1.10 *M* NaCl, but no detectable amount of protein (Lowry assay) could be found in the DNA and following fractions (Fig. 4).

DISCUSSION

The advantages of PLK chromatography for nucleic acids have been discussed previously[6].

The method reported here, for the fractionation of total nucleic acids from small quantities of mammalian cells, might be useful as a tool for the direct and immediate preparation of nucleic acids, without the interference of chemicals (*e.g.* detergents, phenol etc.) which are used in other methods[16–18].

By knowing the molarity of NaCl at which the complex of nucleic acid with polylysine is dissociated, even unlabelled small quantities of total nucleic acids, cell lysates or impure nucleic acid samples, *e.g.* DNA preparations containing nucleotides or protein, can be fractionated successfully.

Protein, free or associated with DNA, does not seem to be a problem, if only DNA is intended to be isolated by this method.

In Figs. 2 and 3, the main macromolecular radioactive material appears to be eluted together with the DNA (3rd peak). As in these experiments [^{3}H]UdR is used as a special RNA precursor, this phenomenon could be explained on the basis

that newly synthesized RNA is associated with DNA and under the conditions used cannot be dissociated. The other two RNA peaks (first and second) always carry less radioactivity than the 3rd peak and this could be attributed to a relatively short life time. RNA could not be detected in the 3rd peak when the orcinol method[15] was employed (Fig. 4) although the test is positive for the first and second peaks. Thus, the 3rd peak carries a small amount of newly synthesized and highly radioactive RNA assiciated with DNA during the elution process.

The results presented here are reproducible and independent of the amount of nucleic acids applied on the columns; therefore the method might be applied to comparative studies, *e.g.* on the incorporation of RNA and DNA precursors, DNA and RNA synthesis, nucleic acid–protein interactions[19] or nucleic acids uptake by mammalian cells in culture.

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