FRACTIONATION OF PLANT CHROMATIN WITH IMMOBILIZED RNA-POLYMERASE

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1. Introduction

The formation of lateral roots after cutting off the main root of a young seedling is preceded by a period of cellular activation, characterized by important modifications in cellular morphology and biochemistry [1]. One of the first events during this phase is a dramatic change in the structure of chromatin correlated with an increase of its template activity. Moreover, in this case, the dispersion of the chromatin into the nucleus is accompanied by an "unscheduled" DNA synthesis which does not take place in the S phase as (normally) expected [2].

In an attempt to elucidate the possible function of early synthesized DNA and to localise this DNA within the chromatin i.e. within its condensed and inactive part or within its extended and active form, we looked for a method for the fractionation of these constituents.

Usually, after shearing of chromatin, the components are separated according to physical parameters. This can be achieved by different ways: centrifugation [3]; ion exchange chromatography [4]; or isopycnic centrifugation in an appropriate medium [6,7]. In our hands, fractionation by centrifugation on a linear density gradient of glycerol (7.5–75%) gave satisfactory results buth this method is based rather on physical properties of nucleoproteins than on functional ones.

In the present report we describe a procedure which utilizes directly the affinity of RNA-polymerase for the available regions of chromatin.
First, sheared chromatin is incubated with matrix-bound RNA polymerase in a suitable buffer, then the unbound material is quantitatively eluted with the same medium, and finally the bound material is released with a buffer containing EDTA.

2. Materials and methods

2.1. *Reagents*

Affi-Gel 10 was obtained from Bio-Rad laboratories; *E. coli* RNA-Polymerase (EC 2.7.7.6) from Boehringer; [methyl-^{3}H] thymidine (40 Ci/mM) from C.E.A. (France). Other chemicals were analytical reagent grade. All solutions were sterilized by filtration through Millipore HA-45 filter just before use.

2.2. *Materials*

About 5 g of segments of the remaining part of roots after cutting were incubated in a medium containing 1.5% sucrose, 10 μg/ml chloramphenicol and 125 μCi [^{3}H] thymidine for one hour at 22°C. After washing with sterilized water, tissues were stored in liquid nitrogen.

2.3. *Preparation of chromatin*

Chromatin was prepared from whole tissues without detergent or saline washes in order to keep its structure as close as possible to its native form. The tissues were homogenised for 1 min at high speed witl a Waring Blendor in 20 ml of: Tris–HCl 50 mM, pH 8.0 (4°C); sucrose 0.25 M ; $MgCl_2$ 5 mM ; mercaptoethanol 5mM ; polyethylene glycol 1 mg/ml. After filtration, the homogenate was centrifuged at 4000 *g* for 30 min. The pellet of crude chromatin was resuspended in 2.5 ml Tris–HCl 1 mM, pH 7.6, EDTA, 1 mM and sheared for 3 min in a Vir Tis homogeniser set at 40 V. The sheared chromatin plus contaminants were then centrifuged at 10 000 *g* for 30 min; the supernatant was kept on ice while the pellet was processed in the same way as the pellet of crude chromatin.

The two supernatants were pooled and centrifuged on a cushion of 1 ml of glycerol 70% containing 2 mM $MgCl_2$ at 30 000 *g* for 12 h. The pellet and the glycerol phase, which also contained some chromatin, were mixed and resuspended in buffer A (see below).

2.4. *Affinity chromatography*

0.5 g of Affi-Gel 10 was mixed with 12.5 ml of buffer A containing 100 units of RNA polymerase and shaken for 24 h at 4°C(buffer A = Tris–HCl pH 7.8 (4°C) 10 mM ; $MgCl_2$ 1 mM ; dithiothreitol 0.5 mM; glycerol 5%).

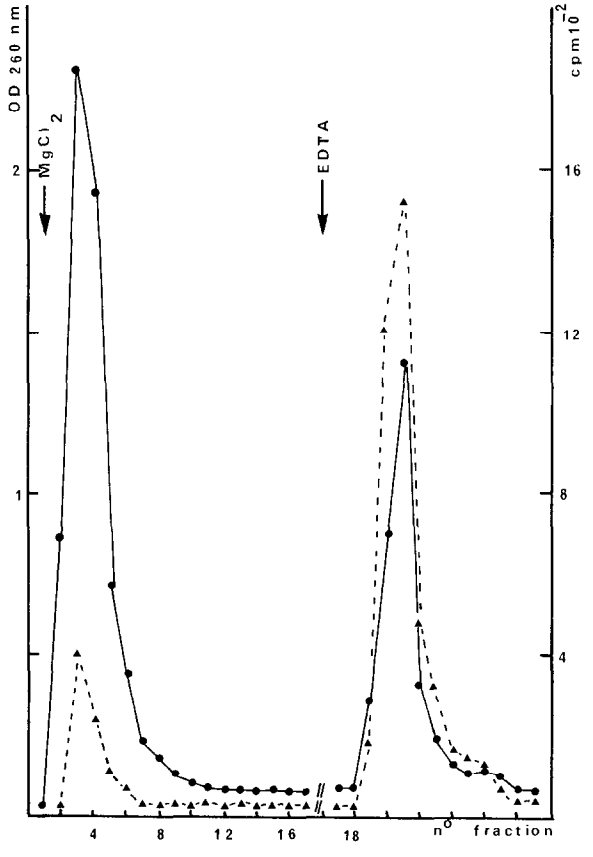

Fig.1. Affinity chromatrography of chromatin from 'activated' tissues, labelled with [^{3}H] thymidine on matrix-bound RNA-polymerase. First peak : material eluted with buffer A ; second peak : material eluted with buffer B. Using the same conditions, deproteinized DNA loaded on the column is eluted in fractions 18–20. (●——●) Optical density at 260 nm. (▲- - -▲) Radioactivity.

The slurry was poured into a column (1 cm in diameter, 5 ml gel bed) and the coupling reaction stopped by washes (three bed volumes) with 1 M ethanolamine buffered at pH 8.0 ; the gel was washed with buffer A containing 1 M NaCl until no absorption at 260 nm could be detected. The column was then equilibrated with several volumes of buffer A.

One ml of chromatin (about 6–10 A_{260}) was loaded on the column still at 4°C. The column was then transfered to 24°C for 20 min. Preliminary tests (to be reported elsewhere) showed that these conditions allowed deproteinized DNA to bind to the chromatographic support.

Then, unbound material was eluted with buffer A, this step was followed by elution with buffer B in order to release material bound to RNA-polymerase (Buffer B = Tris–HCl pH 7.8 (4°C) 5 mM ; EDTA 1 mM; KCl 0.3 M ; dithiothreitol 0.5 mM).

During elutions, the flow rate was 0.4 ml/min. fractions of 1 ml were collected, their absorbance at 260 nm measured and aliquots were processed for the

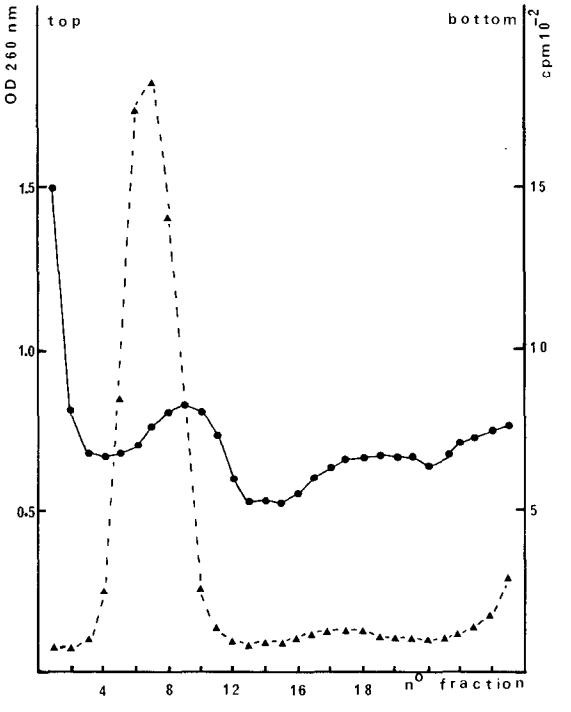

Fig.2. Linear density gradient centrifugation (glycerol, 7.5–75%) of the same chromatin as in fig.1. (●——●) Optical density at 260 nm. (▲- - -▲) Radioactivity.

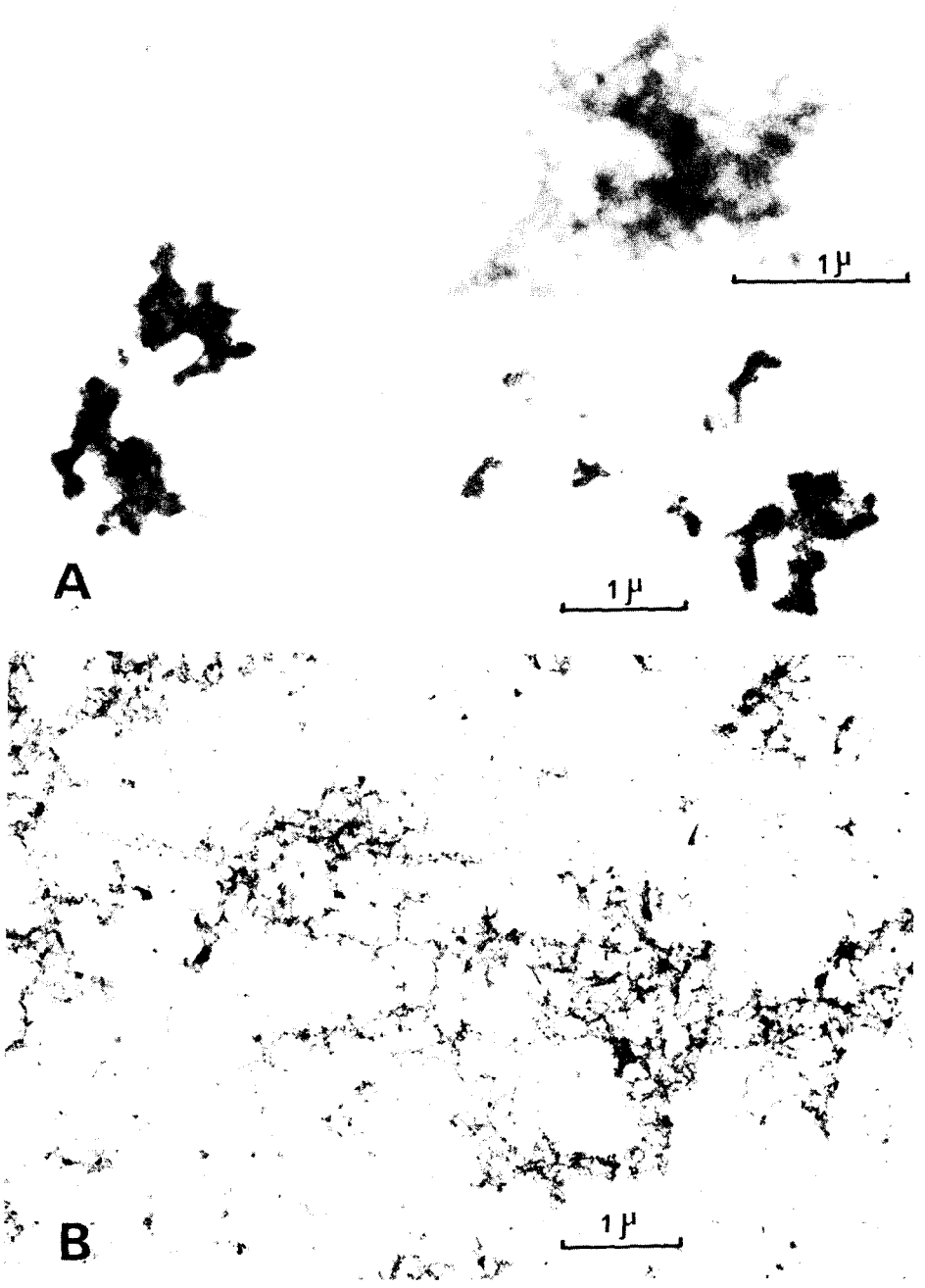

Fig.3. Electron micrographs of fractions of chromatin obtained by affinity chromatography. (A) material eluted with buffer A (peak 1 in the fig.1). Upper right : effect of treatment with EDTA. (B) material eluted with buffer B (peak 2 in fig.1).

counting of their trichloroacetic acid-precipitable radioactivity by liquid scintillation.

2.5. *Electron microscopy*

The samples of chromatin were diluted with a solution of cytochrome *c* (1 μg/ml) and drops were placed on a sheet of 'Parafilm'. Carbon coated specimen grids were gently brought in contact with the surface of the drop and allowed to dry. The grids were stained for 30 sec in uranyl acetate (2.5% in 50% ethanol), rinsed with 50% ethanol and dried before observation with a Hitachi HU II A electron microscope.

3. Results and discussion

In a typical experiment represented in fig.1, sheared chromatin from tissues labelled with thymidine was fractionated by stepwise elution from matrix-bound RNA-polymerase. As expected, two components were released from the column. The main peak, eluted with $MgCl_2$, represents material with no affinity for RNA-polymerase and little radioactivity is associated with it; the second, eluted with EDTA, represents material having affinity for the enzyme and almost 80% of the radioactivity was in this material.

In another experiment (fig.2), the chromatin was centrifuged in a linear gradient of glycerol. Almost all the radioactivity was found to be associated with a light fraction i.e. with a material showing the highest template activity [5].

Moreover, thermal denaturation, performed with EDTA 1 mM as solvent revealed a *T*m of 70°C for unbound chromatin and 64.5°C for the retained material (data not shown).

The question whether the first peak (material without affinity for the matrix-bound polymerase) is a packed form whereas the second peak is an extended form of chromatin can be answered by electron microscopy. It is clear from fig.3 that the second fraction is in the extended fibrous state while the first peak consists of aggregated material in which no filaments can be detected even after EDTA treatment. Other experiments involving gradient centrifugation of the two materials suggest some heterogeneity. Nevertheless, the structural differences between the two main fractions, as judged by electron microscopy, give us a further indication of the efficiency of the separation.

A further problem concerns the stability of the chromatographic support. The increased stability of matrix bound enzymes is now well established. In our hands, up to 10 experiments could be performed with the same column without loss of retention.

Despite some restrictions relating to the utilisation of EDTA which can lead to a random redistribution of proteins [8] and to the non-specificity of the enzyme, this method should be useful for the analysis of these deoxyribonucleoproteins called 'chromatin' and for the investigation of the relationship between its structure and function.

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