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Isolation of endonuclease from mammalian cells by DNA - cellulose chromatography

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ABSTRACT

By means of DNA-cellulose chromatography an enzyme with endonucleolytic activity has been isolated from nuclear acidic protein fraction of mammalian cells. The main active fraction, eluted at 0.7 M NaCl, affects the velocity sedimentation of UV-irradiated and alkylated DNA, resulting in a decrease of the molecular weight. The fraction is completely inactive using native as well as heat-denatured DNA.

INTRODUCTION

The early event in the repair of UV-irradiated DNA is the removal of the damaged regions of the molecule. Molecular distorsions induced in DNA by different agents, such as UV-irradiations, mitomycin, nitrous acid and some alkylating compounds, are similarly excised by specific enzymes. Therefore the enzymes involved in the initial stage of different repair systems are able to recognize the regions of distorsion present in the primary and secondary structure of the DNA molecule. The enzymatic excision of UV-photoproducts was first reported by Strauss (1), by Moriguchi and Suzuki (2) and by Carrier and Setlow (3). Subsequently many others have provided experimental evidences indicating that an early acting enzyme, an endonuclease, causes the cleavage of a single phosphodiester bond next to each pyrimidine dimer, thus generating a single-stranded region. Endonuclease was partially purified from bacteria by Grossman et al. (4) and was further investigated by Takagi and coworkers (5) on its

specific role in dark repair. Friedberg and Goldtwait (6) have isolated an endonuclease II from E. coli, which degrades T4 or T4 DNA, treated with actinomycin, acriflavin, RNA polymerase or RNAase. The enzyme was purified approximately 350 folds (7), and some of its properties were defined: it does not demonstrate an absolute requirement for divalent cations, but is stimulated by addition of Mg^{++} or Mn^{++}, in contrast to other preparations which are not activated by Mn^{++}. Up to now the most purified preparation is presented by an endonuclease, responsible for the excision of thymine dimers from UV-irradiated DNA (8). Kaplan et al (8) have demonstrated that the enzyme has a molecular weight of 15,000 daltons and is activated by Mg^{++}, even if it is not magnesium-dependent. Mg^{++} cannot be replaced by Mn^{++}.

It is rather difficult to draw a parallel between microbial and mammalian cell repair systems (9), even if it is obvious that a similar enzymatic pattern may occur in mammals. Two nucleases, localized in the cell nuclei, were found in rabbit liver by Lindahl (10). One of these enzymes, called DNAase IV, is able to hydrolyze UV-irradiated poly (dA•dT) with a 70% efficiency compaired to the corresponding unirradiated polymer. With the DNA as substrate similar results were obtained and the difference in the rate of hydrolysis between unirradiated and irradiated DNA was less than two folds. This indicates that the cell nucleus contains, among other nucleases, an endonuclease which causes the specific nicking of UV-irradiated DNA.

The purpose of this paper is to describe the isolation and partial purification of an endonuclease from hamster's plasmocytoma (PPL1 from Southern Research Institute of Birmingham, Alabama).

From this tumor two different sublines, one resistant and the other sensitive to radiations and/or alkylating agents, were previously

isolated (11,12). In a following paper we will report on the behaviour of endonuclease in both tumor lines in order to ascertain the role of repair systems in sensitivity or resistance.

EXPERIMENTAL

Enzyme preparation. The tumors were aseptically excised and freed from necrotic areas, blood vessel and fibrous components. Pooled fragments were employed for the isolation of pure, nude nuclei following the stepwise sucrose gradient techique slightly modified from Blobbel and Potter (13).

Saline soluble components of the nuclei were removed by washing in 0,14 M NaCl 0,005 M Tris-HCl pH 7.5 followed by stirring; the centrifuged pellet was resuspended in 1 M NaCl and homogenized. The suspension was stirred slowly overnight and centrifuged in a Spinco L50 ultracentrifuge at 20,000 rpm for 1 hour. The clear supernatant was collected and dialyzed against cold distilled water. After dialysis the retained material was centrifuged at 20,000 x g for 30 min to eliminate the DNA and reassociated histones, and the supernatant was collected and concentrated by dialysis against sucrose. This fraction represents the total non-histone chromatin acidic protein (N.A.P.) which, as previously reported (14), contains the bulk of the endonucleolytic activity.

The fraction was submitted to chromatography on a DNA-cellulose column. A solution of calf thymus DNA (1-2 mg/ml) either native, heat-denatured or exhaustively irradiated with UV-light was mixed with clean dry cellulose (Munktell 410) to give a thick paste (~ 1g per 3 ml).

Alkylated DNA (alkylation yield with Dimethylsulphate: 2%) was alternatively employed (15). DNA-cellulose chromatography was performed according to Litman (16). The columns were eluted with increasing,

not buffered, NaCl concentrations from 0.2 M to 2 M.

Enzyme Assay: The nuclease activity was measured by incubating the enzyme preparation with H^3 labelled native or UV-irradiated calf thymus DNA (Radiochemical Centre, Amersham; specific activity 56 μCi/2 mg). The reaction mixture (volume: 1 ml) was: $MgCl_2$ 0.001 M and Tris-HCl 0.001 M at pH 7.5 plus 100 μg of DNA. After incubation at 37°C for 30 min an equal volume of saturated phenol was added to the samples and DNA was extracted very gently. The phenol was exhaustively removed by centrifugation and ether extraction. The nicking activity of the enzyme on native, heat-denatured, UV-irradiated and alkylated DNA was evaluated on the basis of the hyperchromicity at 260 mμ and the decrease of molecular weight in zonal sedimentation. In the latter case, 500 μl of the incubation mixture (containing approximately 50-60 μg of DNA) were layered on the top of a 5%-20% linear sucrose gradient and centrifuged in the SW 40 Ti rotor with the Spinco L 50 ultracentrifuge for 16 hours at 27,000 rpm at 4°C. 34 fractions were collected from each tube and the acid-precipitable (5% TCA) radioactivity was measured in a liquid scintillation counter.

RESULTS

From the measurement of the activity of the effluent fractions from the DNA-cellulose column results that the bulk of the active fraction is eluted with 0.7 M NaCl. This fraction, when stored frozen, is stable for several months, while it looses all the activity in a few hours, when stored at 4°C. Binding of endonuclease occurred only when DNA was specifically modified by UV-irradiation or alkylation, whereas the enzyme could not be recovered from the column when native DNA or heat-denatured DNA was employed. The fact that the same DNA-cellulose column may be used for repeated enzyme isolation with an identical yield, provides an element of stability and specificity

for this procedure. Using Tris-HCl buffer, the endonuclease activity reaches a maximum after an incubation time of 30 minutes at pH 7.5; the enzyme requires the addition of divalent cations (Mg^{++}).

It is known that single and/or double strand breaks show an increase of absorption at 260 mμ. As reported in Fig. 1 only in the case of UV-irradiated or alkylated DNA, the addition of the enzyme determines a significant increase in the optical density, whereas the photometric properties of native and heat-denatured DNA after the addition of the enzyme are only slightly changed and not evaluable.

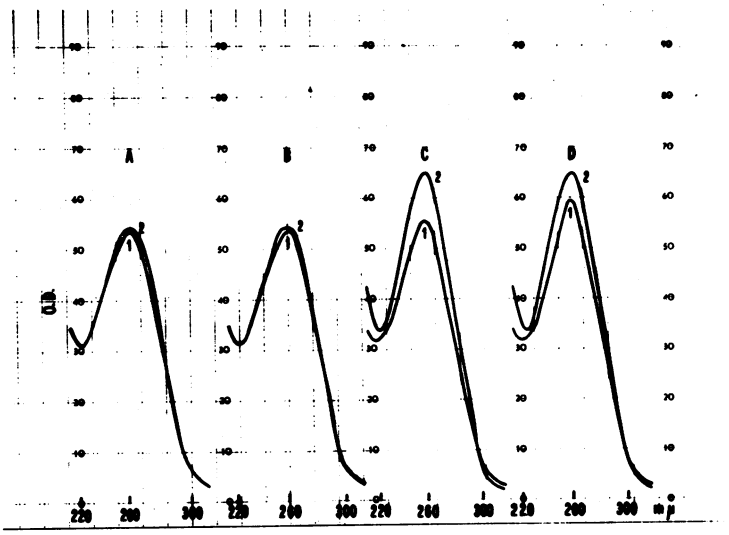

Fig. 1 - Specificity of endonucleolytic activity measured as increase of the optical density of the substrate. 1A: native DNA; 2A: native DNA plus enzyme; 1B: heat-denatured DNA; 2B: heat-denatured DNA plus enzyme; 1C: UV-irradiated DNA; 2C: UV-irradiated DNA plus enzyme; 1D: alkylated DNA; 2D: alkylated DNA plus enzyme.

A further demonstration of the specific action of the endonuclease preparation on UV-irradiated or alkylated DNA was obtained by measuring the decrease of molecular weight on the basis of Studier's (17) relation between the rate of molecular weight and the distance sedimented in sucrose gradients. Fig. 2 indicates that the sedimen-

tation properties of native and heat-denatured DNA are not modified after incubation with enzyme. The ability of the enzyme to nick the

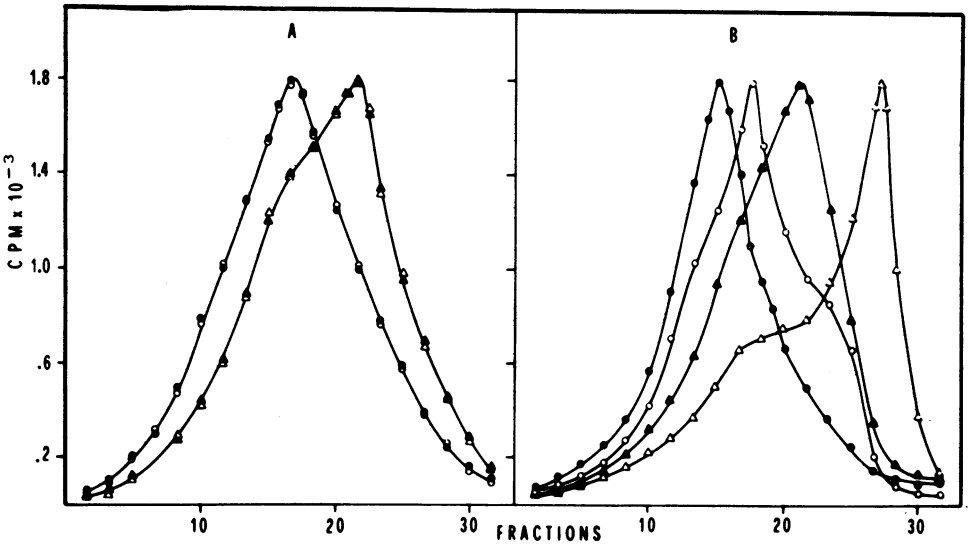

Fig. 2 - Velocity sedimentation analysis of H^3 calf thymus native DNA (A ●: alone; A ○: plus enzyme), heat-denatured DNA (A ▲: alone; A △: plus enzyme), UV-irradiated DNA (B ●: alone; B ○: plus enzyme), and alkylated DNA (B ▲: alone; B △: plus enzyme), in neutral sucrose gradient. The reaction mixture was divided in two equal parts. One was used for analysis in an alkaline sucrose gradient (Fig. 3), while the other was layered on the top of 5%-20% linear sucrose gradient buffered at pH 7.0 (KH_2PO_4-K_2HPO_4 0.02 M). The direction of sedimentation is from right to left.

irradiated or alkylated DNA molecule is well documented by the marked shift of the distance sedimented as shown in Fig. 2.

Alkaline sucrose gradients were also used in order to ascertain whether in neutral gradients a portion of the DNA was complexed to protein components of the extract other than endonuclease. Even if the resolution was less sharp for the presence of many peaks (Fig.3), the breaks produced are presumably due to the disruption of alkali-labile bonds and the enzymatic event recorded is overlapped by such alkali-labile state.

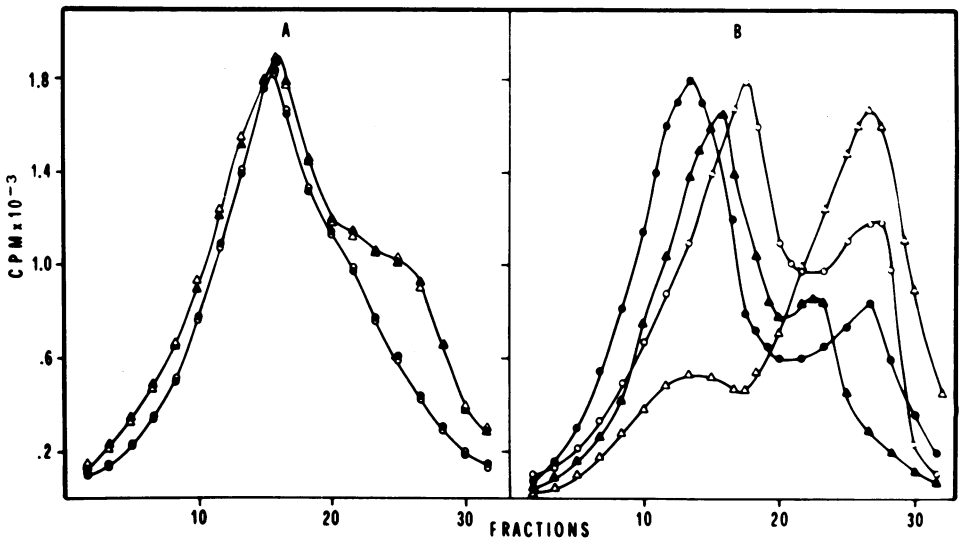

Fig. 3 - Velocity sedimentation analysis of H^3 calf thymus native DNA (A ● : alone; A o : plus enzyme), heat-denatured DNA (A ▲: alone; A △: plus enzyme), UV-irradiated DNA (B ● : alone; B o : plus enzyme) and alkylated DNA (B ▲: alone; B △: plus enzyme) in alkaline sucrose gradient. The reaction mixtures were stopped at $0^{o}C$ and immediately layered on the top of 5%-20% linear sucrose gradient adjusted to pH 12.4 with K_2HPO_4 0.02 M. The direction of sedimentation is from right to left.

DISCUSSION

In contrast to more conventional isolation schemes, the DNA-cellulose chromatography has the advantage of being a rapid and effective procedure for the purification of an endonuclease from mammalian cells.

Additional experiments employing other tumors, Hela cell cultures or human cultured fibroblasts, have shown that from all these cells it is possible to isolate an endonuclease with similar properties (unpublished). Moreover, the comparative use of native or specifically disrupted DNA represents the first step towards the definition of the specificity of this enzyme. In fact, many of the enzymes which function on DNA inside the cell nucleus, bind tightly to DNA at physiological ionic strength and are reversibly released from

the DNA at higher salt concentration. This method described herein is very suitable for isolating enzymes which are able to recognize conformational changes of the DNA molecule and accordingly are specifically bound to the DNA.

It is evident that the 0.7 M NaCl fraction contains an enzyme which causes the initial incision of the single DNA strand perhaps next to the area of the molecule which has been distorted by UV-irradiation or alkylation. It is worthwhile emphasizing that the enzyme does not affect the molecular properties of native or heat-denatured DNA and therefore one can presume that the specificity of the enzyme resides in the ability to recognize the conformational changes caused by the alterations of the primary and/or secondary structure of DNA. The reported results raise questions concerning the relationship between structural damage of DNA and activity of the enzyme. To answere these questions, it will be necessary to obtain a more purified enzyme preparation.

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