THE CALCIUM DEPENDENT ENDONUCLEASE ACTIVITY OF ISOLATED NUCLEAR PREPARATIONS. RELATIONSHIPS BETWEEN ITS OCCURRENCE AND THE OCCURRENCE OF OTHER CLASSES OF ENZYMES FOUND IN NUCLEAR PREPARATIONS.

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Summary

Nuclear preparations have been examined for endonuclease activity. An endonuclease has been identified which requires both calcium and magnesium ions for activity and which is responsible for the calcium primed DNA synthesis observed in certain nuclear preparations. This enzyme increases during the later stages of liver regeneration. A magnesium requiring endonuclease has also been identified in certain nuclear preparations.

Introduction

Previous reports from this laboratory[1,2] have described the activation of DNA synthesis in isolated mammalian cell nuclei in the presence of calcium ions. This activation coincides with a fall in the molecular weight of the nuclear DNA and thus suggests the action of an endonuclease.

This report describes further studies on the endonuclease class of activities associated with isolated liver nuclei from mice, normal rats and rats with regenerating livers. An endonuclease has been extracted and studied which has properties to account for the calcium ion dependent activation of *in vitro* DNA synthesis.

Methods

Nuclear Preparations

Unless otherwise stated the nuclear preparation procedure was the buffer A method previously published[1,2] with the following modifications. The

*Abbreviations. EGTA : ethylene glycol bis(2 aminoethylether)-N-N' tetra acetic acid.

HEPES : N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid.

TEMED : N,N,N'N' tetramethyl ethylene diamine.

buffered 2.4 M sucrose was replaced by buffered 2.1 M sucrose. This increased the reliability of the preparation. Nuclei prepared in buffer D were isolated by the method previously described[3].

DNA Substrate Preparation

^{32}P-labelled Escherichia coli DNA was isolated from E. coli B cells grown in DIFCO No. 2 broth for 7 hours at 37° with the addition of ½ mc ^{32}Pi. Cells were harvested by low speed centrifugation and were lysed, and nucleic acid extracted, essentially by the detergent-salt method of Kay, Simmons & Dounce[4]. Nucleic acid was precipitated by ethanol and further purified by phenol extraction, treatment with a mixture of T_1 and pancreatic ribonuclease and a further phenol extraction. Finally the DNA was spooled out with ethanol, washed with absolute ethanol and left overnight at 4° in 70% ethanol. The DNA was dried and dissolved in buffer A or 0.05 M tris HCl, pH 7.4, 0.15 M KCl.

A Preferentially Endonuclease-Sensitive Assay

Radioactive DNA was embedded in polyacrylamide gel by the method of Melgar & Goldthwait[5] with the inclusion of 0.05% TEMED in the stock acrylamide solution. The polymerised gel was fragmented by passage through a 28 gauge syringe needle, washed extensively with buffer A and finally suspended in this buffer.

Assays of endonucleases were performed in 1 ml centrifuge tubes. 50 µl of gel suspension (containing approximately 6.5 µg of DNA and 40,000 counts/min) and 100 µl of enzyme solution were mixed and incubated at 37°. The reaction was stopped by the addition of 50 µl 50 mM EDTA, 0.05 M phosphate, pH 7.5, and the mixture was centrifuged at low speed. A 100 µl sample of the supernatant was removed, dried and counted for radioactivity.

Reaction Product Characterisation

Chromatography was carried out on DEAE cellulose paper (Whatman DE81) by the method of Kelly, Atkinson, Huberman and Kornberg[6]. After chromatography, 1 cm strips were cut and counted for radioactivity by liquid scintillation.

Nuclear DNA was estimated by Burton's reaction[7].

Nuclear DNA synthesis and DNA polymerase activity was assayed by the methods previously described[1,2,8].

Results

Preliminary studies of mouse nuclear nucleases using the DEAE cellulose analysis of products indicated the presence of a mixture of exo- and endonucleases but indicated that the release of ^{32}P DNA from an acrylamide gel

*Buffer A contained 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris HCl, pH 7.4.

*Buffer D contained 35 mM KCl, 0.2 mM NaCl, 0.5 mM spermine, 10 mM glycine, 10 mM HEPES, pH 7.4.

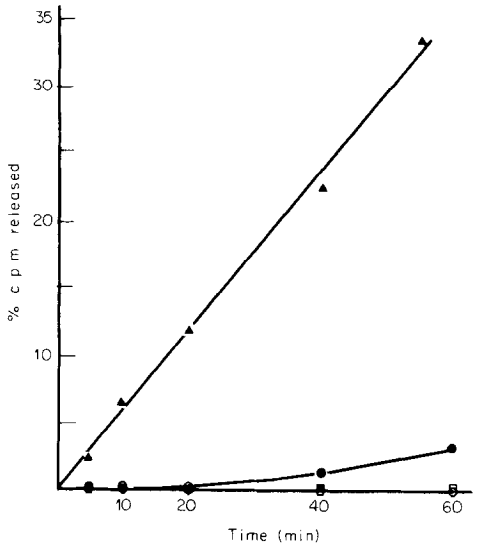

Figure 1

Liberation of ^{32}P E. coli DNA from an acrylamide gel matrix by endonuclease contained in mouse liver nuclei. Nuclei were prepared by the buffer A procedure as described for nuclease assays in Methods and incubated with ^{32}P DNA-acrylamide gel for the times indicated. The reaction was stopped with EDTA and supernatant radioactivity measured. 7.98×10^6 nuclei were added/assay. O - O, No $CaCl_2$ or $MgCl_2$; □ - □, + 1 mM $CaCl_2$; ● - ●, + 10 mM $MgCl_2$; ▲ - ▲, + 10 mM $MgCl_2$ + 1 mM $CaCl_2$.

suspension was almost entirely due to endonuclease activity.

The Ca-Mg Endonuclease

The endonuclease-class activities associated with whole nuclei from normal mouse liver were highly dependent on both calcium and magnesium (Fig. 1). When liver cells were fractionated in to crude nuclear, light particulate, and soluble fractions, in the presence of EDTA and EGTA, calcium-ion-stimulated-endonuclease could only be reliably detected in the nuclear fraction. The bulk of cellular nucleases in the light-particulate and soluble fractions were primarily activated by magnesium ions.

The calcium-dependent endonuclease was extracted from nuclei by 0.14 M KCl, 0.1 M phosphate buffer, pH 7.4. The enzyme was found to be considerably stabilised by the presence of either 20% ethylene glycol or 1 mM $CaCl_2$ but its stability still remains a problem. Sephadex G200 chromatography was carried out in 0.02 M tris HCl,

pH 7.4, 20% ethylene glycol, 5 mM $MgCl_2$. The calcium-dependent activity eluted as a single peak, the mobility of which relative to marker proteins, indicated a molecular weight of approximately 40,000. If the chromatography is carried out in the absence of magnesium, however, (plus 1 mM EDTA) the molecular weight is approximately 20,000 and the enzyme loses much of its calcium dependence.

The extracts of mouse liver nuclei, containing Ca-Mg endonuclease activity, activated calf thymus DNA with respect to the high molecular weight ascites DNA polymerase as prepared by Wallace et al.[3]. In regenerating rat liver, the quantity of nuclear-associated-Ca-Mg endonuclease decreases slightly until approximately 24 hours after partial hepatectomy, then rises and is approximately 6 times the resting activity by 72 hours (Fig. 2).

The activity of the total soluble cytoplasmic endonucleases does not change during these times.

Nuclear Preparations that have been Demonstrated to Contain Relatively High Levels of the Ca-Mg Endonuclease

Preparations from normal mouse liver, normal and regenerating rat liver, rat thymus, rabbit thymus and calf thymus.

Nuclear Preparations that do not Contain Detectable Ca-Mg Endonuclease

Preparations from rabbit bone marrow.

The Nuclear Mg Endonuclease

When nuclei from rat liver were prepared by the buffer D procedure instead of the buffer A procedure they were found to contain approximately 7x as much total endonuclease-class activity as buffer A nuclei. However, this activity was distinct from the endonuclease in buffer A nuclei in that it required only magnesium ions. Calcium ions stimulated its activity but were not absolutely necessary for it (Table I). Moreover, this nuclease is virtually inactive on the nuclear DNA in situ, which is in marked contrast to the Ca-Mg nuclease which is highly active on nuclear DNA in situ.

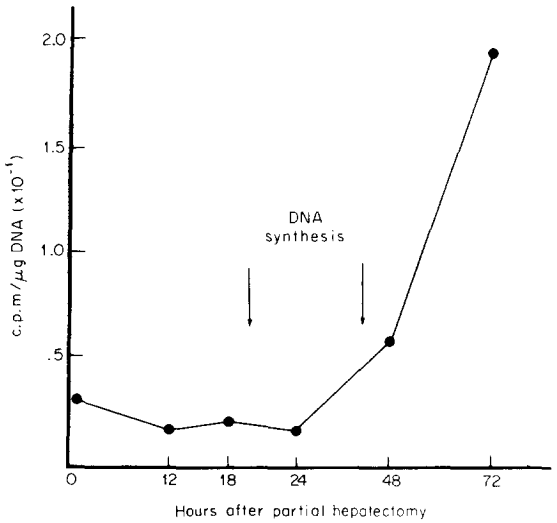

Figure 2

Changes in the nuclear concentration of Ca-Mg endonuclease during liver regeneration. Nuclei were prepared from regenerating rat livers as in Methods and assayed for Ca-Mg endonuclease by the DNA-acrylamide gel method. Enzyme activity is expressed as counts/min. released per 75 minutes relative to the nuclear DNA added per assay measured by Burton's reaction[7].

Discussion

A calcium-dependent endonuclease was found, in appreciable amounts, associated with nuclei isolated in buffer A. This enzyme has a near absolute requirement for traces of calcium ions. It can be extracted from nuclei and will activate isolated DNA toward DNA polymerase action.

The level of Ca-Mg-requiring endonuclease in nuclei isolated from regenerating rat liver is initially lower than the level in nuclei from normal liver, but then rises to much greater than normal. The rise occurs after the time of maximal DNA synthesis and corresponds roughly to the rise in activity of the high molecular weight DNA polymerase[9], but the two activities are definitely not associated as the resting nuclei from which the Ca-Mg endonuclease is routinely extracted lack the high molecular weight polymerase[3].

The function of the Ca-Mg endonuclease in DNA replication can not be determined from these results and there is no obvious correlation of the changes in its activity with DNA synthesis during liver regeneration.

TABLE I

The Mg-Endonuclease

Metal requirements of endonuclease associated with nuclei isolated in buffer D. Nuclei were isolated from 24 hour regenerating rat liver in buffer D[3]. An extract was prepared by suspension of the nuclei in, and centrifugation through, 0.34 M sucrose in buffer A. Endonuclease activity was assayed with DNA acrylamide gel as described in Methods.

	Release of Radioactivity cpm/min/ml extract
Buffer A extract of buffer D nuclei + 10 mM $MgCl_2$	258.0
Buffer A extract of buffer D nuclei + 10 mM $MgCl_2$ + 1 mM $CaCl_2$	350.0
Buffer A extract of buffer D nuclei + no $MgCl_2$ or $CaCl_2$	12.4

An enzyme has previously been observed[10] which rises during liver regeneration[11] and which may be identical to the Ca-Mg endonuclease. A very similar enzyme has also been identified in sea-urchin eggs[12].

The endonuclease-class discovered in association with buffer D nuclei is more like DNAase I with regard to its divalent cation requirements than the Ca-Mg endonuclease. This Mg-dependent endonuclease does not degrade the nucleoprotein DNA of the nuclei from which it is extracted.

Once again, a change in the ionic environment of the nucleus from buffer A to buffer D has had the effect of exchanging one complement of enzymes for another. In this case, the change from buffer D to buffer A in the isolation procedure caused the nuclei to change the nucleases bound to them. Previously Wallace *et al.*[3] showed that a change from buffer D to buffer A caused the loss of the high molecular weight DNA polymerase bound

to the nuclei *in vitro*. It is tempting to speculate that these effects of changing the ionic environment *in vitro* may be related to changes which occur *in vivo* during the cell cycle as has been previously suggested by Wallace *et al.*[3].

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