PROPERTIES OF A DEOXYRIBONUCLEASE FROM A NUCLEAR EXTRACT OF PARACENTROTUS LIVIDUS EMBRYOS

Elio Parisi and Benita De Petrocellis
Laboratory of Molecular Embryology, Arco Felice, Naples-Italy

Revised September 13,1972

SUMMARY
Study of the deoxyribonuclease activity contained in the nuclei of sea urchin embryos shows that this is an alkaline endonuclease which preferentially hydrolyses native DNA. The sea urchin embryo DNAase requires divalent cations and is synergistically activated by the simultaneous presence of Ca^{++} and Mg^{++}. Degradation of heat-denatured DNA is strongly dependent on the nature of the divalent metals added. In the presence of Mg^{++}, single-stranded DNA is not attacked. Ca^{++} or Ca^{++} combined with Mg^{++} brings about degradation of heat-denatured DNA, although at a lower rate than that observed when using native DNA under the same conditions.

INTRODUCTION - Although most of the available information on deoxyribonucleases comes from bacterial (1-5) or viral (6-11) systems, some initial progress has been made in characterizing deoxyribonucleases from mammalian (12-14) and plant systems (15). In a previous paper we showed that in sea urchin embryos DNAase activity parallels DNA synthesis (16). Owing to the possible role that this enzyme may have in DNA replication, we have considered it of interest to study the properties of sea urchin DNAase. In this paper we describe the preliminary characterization of the enzyme present in the nuclei of P. lividus.

MATERIALS AND METHODS - Sea urchin were obtained from the Zoological Station, Naples. Salmon sperm DNA and snake venom were supplied by Sigma. Total yeast RNA, Total t-RNA of E.coli, M. lysodekticus DNA and polydeoxyadenylate-thymidilate were purchased from Miles Labs. Alkaline phosphatase was obtained from Worthington. Agarose 5m Biogel (200-400 mesh) was provided by Bio-Rad. T4-phage DNA was a gift of Dr. Tocchini-Valentini. DNAs from chick embryo muscle, P. lividus and S. granularis sperm were prepared by the method of Marmur (17).

All the experiments were performed on embryos at the blastula stage. Eggs were collected and fertilized according to the previously used method (18). Nuclei prepared according to Hinegardner (19) were collected by centrifugation at 1,000xg for 30 min. The supernatant was considered as cytoplasmic fraction. Nuclei, further purified by density gradient centrifugation (19), were suspended in two volumes of 25 mM Tris-HCl pH 8.0 containing 2 mM Mg^{++}, disrupted by sonication and centrifuged at 100,000xg for 1 h. The nucleolytic activity was tested on 100,000xg supernatant by measuring the conversion of DNA to acid soluble fragments. One unit of DNAase activity corresponded to an increase in

absorbance of 0.1 at 260 mμ in 10 min. under the conditions of the assay. Specific activity was expressed as units per mg of protein. Gel filtration analysis of DNA after digestion with DNAase was performed according to Birnboim (20), using Agarose 5m-Biogel chromatography. Protein determination was carried out by the procedure of Lowry (21). Thin layer chromatographies, on microcristalline cellulose plates (Merk), were developed with isopropanol/HCl/water (22).

RESULTS - Deoxyribonuclease activity present in sea urchin embryos is almost equally distributed between the cytoplasmic and nuclear fractions. The specific activity of the nuclear enzyme is, however, 6 times higher than that in the cytoplasm. DNAase activity as a function of pH is shown in Fig. 1A. Maximal activity is obtained in Tris-HCl buffer at pH 7.8 and at a temperature of 30°C. Fig. 1B shows a relationship between the release of DNA acid-soluble fragments and protein concentration during 30 min. of incubation with salmon sperm DNA as substrate. Electrophoretic analyses on polyacrylamide/DNA gel (23) show that one band of DNAase activity is detectable at a protein concentration used in the standard assay (0.1-0.2mg). A second, much less intense band of activity is visible when the protein concentration is raised to 1-2mg/gel. Table I shows that only native DNA is attacked, while no apparent degradation is observed with denatured DNA, yeast total RNA or E.coli t-RNA. Regardless of the substrate used, A-T-rich DNAs are degraded more rapidly than G-C-rich DNAs, with the exception of T4-phage DNA, which is probably more resistant because of the presence of glycosidic groups on the hydroxymethylcytosine. DNA hydrolysis requires divalent cations and is markedly inhibited by NaCl, phosphate, LiCl, and by a relatively high concentration of Tris buffer. Inhibition is also observed in the presence of $(NH_4)_2SO_4$. Table II shows that native DNA hydrolysis is strictly dependent on the presence of added divalent cations. Optimal concentration for Mg^{++} is $5x10^{-3}M$. Mn^{++} at optimal concentration ($1x10^{-3}M$) is less effective than Mg^{++}, while optimal Ca^{++} concentration ($7x10^{-4}M$) causes an activity value twice as high as that produced in the presence of Mg^{++} alone. When both Ca^{++} and Mg^{++} are present at optimal concentration, the activity is about two times higher than the sum of the activities measured in the presence of either cation alone. This synergistic effect seems a peculiarity of the couple Ca^{++}-Mg^{++}, since Mn^{++} with Mg^{++} or Mn^{++} with Ca^{++} does not show any stimulatory property. On the contrary, the activity measured in the presence of Mn^{++} combined with Ca^{++} or Mg^{++} is the same as that measured in the presence of Mn^{++} alone. Probably Mn^{++} forms a complex with the enzyme that is much more stable than the complex formed by Mg^{++} or Ca^{++}.

In order to distinguish between endo-and exonucleolytic degradation, the products released during enzymatic digestion of DNA were analyzed by gel fil-

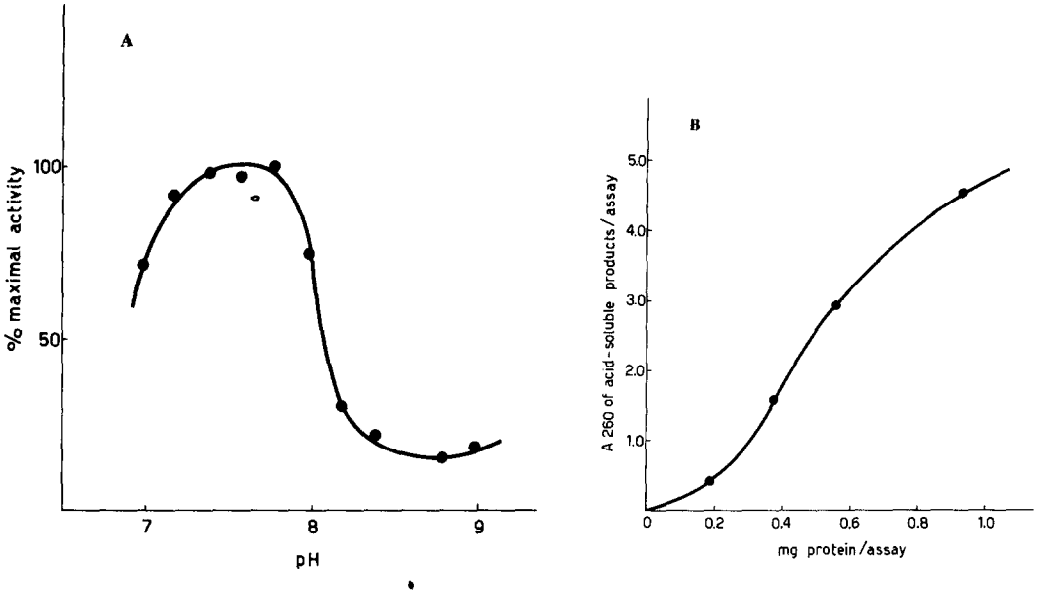

Fig. 1 - Effect of A) pH, and B) increasing concentration of protein on degradation of DNA by nuclear DNAase. Each incubation mixture contained in 1 ml 400 µg of salmon sperm DNA, 25 µmoles of Tris-HCl pH 8.0, 5 µmoles of $Mg\ SO_4$ and 0.2 mg of protein. After 30 min. the reaction was stopped by cooling in an icebath. 100 µl of 50% $HCl\ O_4$ were added, and after 5 min. the precipitate was removed by centrifugation for 5 min. at 12,000xg. The absorbancy of the supernatant was measured at 260 mµ, using as a blank a mixture in which the reaction was stopped soon after the addition of the enzyme.

Table I. Effect of different substrates on the activity of the nuclear DNAase.

Substrate	Relative activity	G-C contents moles %
Salmon sperm native DNA (control)	100	41.2
Salmon sperm heat-denatured DNA	7	
S. granularis sperm native DNA	96	
P. lividus sperm native DNA	120	35
Chick embryo muscle native DNA	81	42
Micrococcus lysodekticus native DNA	33	71
T4-phage native DNA	28	34
Poly d (A-T) native	370	0
Yeast total RNA	0	
E.coli t-RNA	0	

Each standard incubation mixture contained 400 µg/ml of the indicated substrate. Each substrate solution before being tested was dialyzed against 10^{-3}M EDTA in 0.015M NaCl plus 0.0015M trisodium citrate and then against several changes of distilled water.

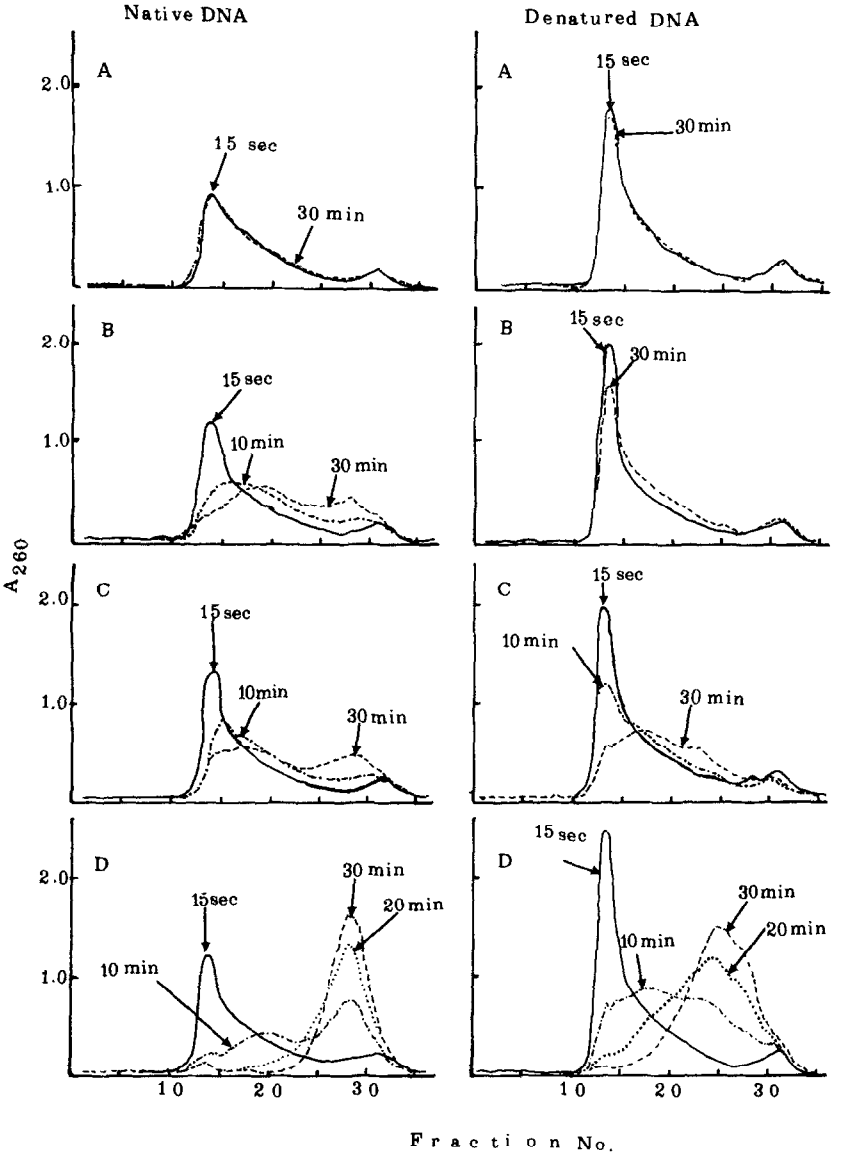

Fig. 2 - Elution profile from Agarose 5m Biogel column of DNA from P. lividus treated with nuclear DNAase in the presence and absence of divalent cations. Incubation mixture was as described in the legend to Fig. 1. Reaction was stopped by adding 1ml of 0.015M NaCl containing 0.0015M trisodium citrate and heating for 10 min. at 100°C. Samples were then rapidly cooled in an ice-water bath. A) No metal additions; B) + 5mM Mg^{++}; C) + 0.7mM Ca^{++}; D) + 5mM Mg^{++} and 0.7mM Ca^{++}. Standard mononucleotides were eluted from fraction 30 to 40.

tration. The elution profiles reported in Fig. 2 show that DNA is degraded by the action of an endonuclease. Indeed, DNA is first converted into high molecular weight polynucleotides and then into progressively lower molecular weight

products as the digestion proceeds. Moreover, Fig. 2 shows that some differences exist according to whether native or heat-denatured DNA is used as substrate. In the absence of divalent cations, neither native nor denatured DNA is attacked. Native DNA only is degraded in the presence of $5x10^{-3}$M Mg^{++}. With $7x10^{-4}$M Ca^{++}, native DNA is degraded in the same way as with Mg^{++}, and a slight endonucleolytic attack of the single stranded DNA is also observed. With $5x10^{-3}$M Mg^{++} and $7x10^{-4}$M Ca^{++}, both native and heat-denatured DNA are attacked, and after 30 min. almost all the substrate is converted into a peak of low molecular weight products. Further analysis of the digestion products by thin layer chromatography shows that they are low molecular weight oligonucleotides. Indeed, in order to be converted into the four deoxyribonucleosides, the DNAase digestion products require further enzymatic hydrolysis with both alkaline phosphatase and snake venom.

DISCUSSION - The results reported here show that at least 50% of the deoxyribonuclease activity found in sea urchin embryos at the blastula stage is associated with the nuclear fraction. The activity found in the cytoplasmic fraction is due, at least partly, to the presence of DNAase in mitochondria (24). Fractionation of the homogenate on disc electrophoresis and assay "in loco" show only one band of activity when using the same protein concentration as in the solution assay. Increasing the amount of protein 10 times shows only the presence of an additional minor component. Although the presence of exonuclease cannot be entirely excluded, gel filtration analysis shows that the enzyme acts mainly as an endonuclease. Optimum alkaline pH and requirement for divalent cations of the enzyme from sea urchin embryos are similar to those of DNAase I. Sea urchin DNAase does not digest heat-denatured DNA in the presence of Mg^{++} alone. However, when Mg^{++} is used in combination with Ca^{++}, denatured DNA is degraded but at a maximum rate one-half that for native DNA.

An explanation of the synergistic effect observed with Ca^{++} and Mg^{++} in sea urchin DNAase would require extensive purification of the enzyme. The same effect obtained in purified pancreatic DNAase (25) has been explained in two different ways: 1) the presence of two nucleases, one Mg^{++}-and the other Ca^{++}-dependent (26); 2) the degradation induced by Mg^{++} of one strand, whereas that of both strands is obtained in the presence of Ca^{++} and Mg^{++} (27).

Endonucleolytic activity in nuclei of sea urchin embryos (a system with a high level of DNA synthesis) strengthens the possibility that DNAases participate in the process of DNA synthesis in vivo (28). It has been shown that oligodeoxynucleotides increase the template activity of DNA for E.coli DNA polymerase (29). More recently, Loeb (30) has reported that a purified preparation of DNA polymerase from sea urchin embryos shows higher activity on nicked DNA. For sea urchin DNAase, the ability to produce only a limited number of

Table II. Effect of divalent cations on nuclear DNAase activity.

Addition	Concentration M	Relative Activity %
Mg^{++}	$4x10^{-3}$	9
	$5x10^{-3}$	100
	$8x10^{-3}$	24
Ca^{++}	$3x10^{-4}$	77
	$5x10^{-4}$	140
	$7x10^{-4}$	200
	$1x10^{-3}$	77
Mg^{++} + Ca^{++}	$5x10^{-3}$ + $7x10^{-4}$	500
Mn^{++}	$5x10^{-4}$	38
	$8x10^{-4}$	49
	$1x10^{-3}$	50
	$2x10^{-3}$	29
Mn^{++} + Mg^{++}	$8x10^{-4}$ + $5x10^{-3}$	51
Mn^{++} + Ca^{++}	$8x10^{-4}$ + $7x10^{-4}$	55

breaks in the DNA molecules probably arises from a combination of suitable conditions of temperature, ionic strength and Mg^{++} and Ca^{++} concentrations.

REFERENCES

1. Lehman,I.R.,Roussos,G.G.,and Pratt,E.A.,J.Biol.Chem.,237(1962)819.
2. Studier,F.W.,J.Mol.Biol.,11(1965)373.
3. Meselson,M.,and Yuan,R.,Nature,217(1968)1110.
4. Friedberg,E.C.,and Goldthwait,D.A.,Proc.Natl.Acad.Sci.,62(1969)934.
5. Oishi,M.,Proc.Natl.Acad.Sci.,64(1969)1292.
6. Oleson,A.E.,and Koerner,J.F.,J.Biol.Chem.,239(1964)2935.
7. Short,E.C.,and Koerner,J.F.,Proc.Natl.Acad.Sci.,54(1965)595.
8. Hurwitz,J.,Beker,A.,et al., J.Cell Phys.suppl.1,70(1967)181.
9. Sadowski,P.D.,and Hurwitz,J.,J.Biol.Chem.,244(1969)6182.
10. Sadowski,P.D.,and Hurwitz,J.,J.Biol.Chem.,244(1969)6192.
11. Center,M.S.,Studier,F.W.,and Richardson,C.C.,Proc.Natl.Acad.Sci.,65(1970)242.
12. O'Connor,P.J.,Biochem.Biophys.Res.Commun.,35(1969)805.
13. O'Connor,P.J.,Biochim.Biophys.Acta,185(1969)153.
14. Howk,R.,Wang,T.Y.,Eur.J.Biochem.,13(1970)455.
15. Mikulski,A.J.,and Laskowski,M.,J.Biol.Chem.,245(1970)5026.
16. De Petrocellis,B.,and Parisi,E.,Exptl.Cell Res.,in press.
17. Marmur,J.,J.Mol.Biol.,3(1961)208.
18. Grippo,P.,Parisi,E.,et al.,J.Mol.Biol.,36(1968)195.
19. Hinegardner,R.T.,J.Cell Biol.,15(1962)503.
20. Birnboim,H.,Biochem.Biophys.Acta,119(1966)198.

21. Lowry,O.H.,et al.,J.Biol.Chem.,193(1951)265.
22. Wyatt,G.R.,Biochem.J.,48(1951)584.
23. Boyd,J.B.,and Mitchell,H.K.,Anal.Biochem.,13(1965)28.
24. De Petrocellis,B.,and Parisi,E., in preparation.
25. Wiberg,J.S.,Arch.Biochem. and Biophys.,73(1958)337.
26. Hacha,R.,and Fredericq,E.,Bull.Soc.Chim.Belges,72(1963)580.
27. Bollum,F.J.,J.Biol.Chem.,240(1965)2599.
28. Haskell,E.H.,and Davern,C.I.,Proc.Natl.Acad.Sci.,64(1969)1065.
29. Goulian,M.,Proc.Natl.Acad.Sci.,61(1968)284.
30. Loeb,L.,J.Biol.Chem.,244(1969)1672.