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PURIFICATION AND PROPERTIES OF A NUCLEAR DNA ENDONUCLEASE FROM HeLa S_3 CELLS

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Summary

An endonuclease that can act on calf thymus DNA and circular double-stranded phage PM2 DNA has been isolated from HeLa S_3 cell chromatin. Approximately 200-fold purification was achieved by a sequence of subcellular fractionation, differential NaCl solubility and chromatography on CM-Sephadex, DEAE-cellulose and hydroxyapatite, and isoelectric focusing. The pH optimum of the enzyme is 7.0 ± 0.5 and the isoelectric point is pH 5.1 ± 0.2. Divalent cations are necessary for its activity and the enzyme is heat inactivated at 60°C. The enzyme activity is sensitive to caffeine and sulfhydryl reacting compounds. The molecular weight, determined by gel filtration and SDS gel electrophoresis, is approx. 22 000.

Introduction

Nucleases have been implicated in such cellular functions as synthesis and repair of DNA [1—3], maintenance of growth [4—6], and the metabolism of nucleic acids [7,8]. A DNA endonuclease has been suggested as a controling factor in the initiation of DNA replication [9,10], and such enzymatic activity has actually been shown to prime DNA as a substrate for DNA polymerase in cell-free systems [11,12]. The stimulation in vitro of DNA polymerase action by DNAase activity could be a reflection of the physiological role of endonucleases with break a phosphodiester bond within the DNA in chromatin to provide an origin for each replicon.

Purification and characterization of a number of endonucleases isolated from mammalian tissues have recently been reported [13—16]. Four chromatin-

associated DNA endonucleases have been identified in HeLa cells [17]. Each of these has been shown to increase the priming activity of calf thymus DNA for *Escherichia coli* DNA polymerase. We now report the purification and characterization of one of these HeLa cell chromatin-associated DNA endonucleases which we have designated DNAase N22. The name, DNAase N22, was chosen to signify a nuclear protein with a molecular weight of 22 000.

Materials and Methods

Tissue culture. HeLa S_3 cells were cultivated in spinner culture or in monolayer culture at 37°C as previously described [18]. Weekly tests were made for mycoplasmal contamination [19].

Enzyme purification. Nuclei from exponentially growing HeLa cells were isolated and freed from cytoplasmic contamination by the detergent method of Berkowitz et al. [20]. The nuclear pellet was extracted three times with 0.14 M NaCl in buffer 1 (0.05 M Tris-HCl, pH 7.5, 1 mM Na_2EDTA, 1 mM dithiothreitol) at 5°C. Isolation of DNAases from the residue was carried out in buffers containing 1% Triton N-101 and at −20°C. The residue was homogenized in 1 M NaCl in buffer 1 and the resulting suspension was diluted to 0.14 M NaCl to precipitate histones plus DNA, and centrifuged 1000 $\times g$ for 20 min [17]. The supernatant fraction was dialyzed against buffer 2 (0.05 M potassium phosphate (pH 7.1), 1 mM Na_2EDTA, 1 mM dithiothreitol, 40% ethylene glycol) at −20°C [17] and then gently stirred for several hours with CM-Sephadex (Pharmacia); the preparation was then centrifuged at 100 $\times g$ for 10 min. The supernatant fraction, designated fraction I, was dialyzed against buffer 3 (0.05 M Tris-HCl, pH 7.1, 1 mM Na_2EDTA, 1 mM dithiothreitol, 40% ethylene glycol) and then stirred with DEAE-cellulose (Whatman, Inc.). several hours. The slurry was centrifuged at 1000 $\times g$ for 20 min and the supernatant fraction (fraction II) was discarded. Two more fractions (III and IV) were eluted from the DEAE-cellulose with 0.1 M NaCl and 0.2 M NaCl, respectively, in buffer 3. Fraction IV was dialyzed against buffer 3 containing 0.01 M potassium phosphate and applied to a hydroxyapatite (Bio-Rad) column (0.9 × 2.0 cm). DNAase activity was eluted from the column with a linear gradient of 0.01—0.3 M potassium phosphate (Fig. 1). The non-adsorbed fraction (V) was discarded. The fraction eluting at 0.08 M potassium phosphate, Fraction VI, was dialyzed against buffer 3 and applied to a 110 ml LKB electrofocusing column prepared using using a 0—60% sucrose gradient and ampholines with a pH range of 3.5—10.0. Electrophoresis was carried out at 5°C at 600 V for 24 h and an additional 24 h at 1000 V. The fraction eluting at pH 5.1 ± 0.2 was called DNAase N22 (Fig. 2). This fraction was extensively dialyzed against buffer 2 and characterized.

DNAase activity. Endonuclease activity of the various fractions was assayed by the ability of the enzyme preparation to increase the priming efficiency of calf thymus DNA (Worthington) for DNA polymerase [21]. One unit of DNAase activity was defined as the amount of enzyme in the assay system which increased the priming activity of DNA by 100% in 3 h.

To establish that the nuclease activity measured in these studies was truly endonucleolytic, the fractions containing enzyme activity were also examined

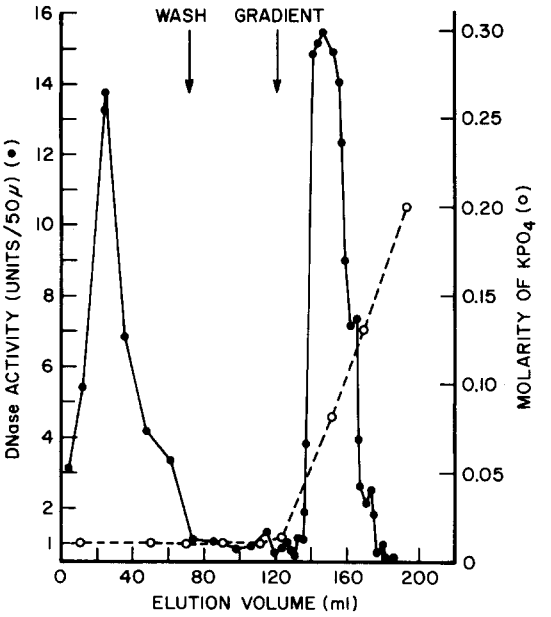

Fig. 1. Hydroxyapatite elution profile of fraction VI. The activity eluting from DEAE-cellulose with 0.2 M NaCl (fraction IV) was dialyzed overnight against 0.01 M potassium phosphate to a 0.9 × 2 cm hydroxyapatite column previously equilibrated with 0.01 M potassium phosphate in buffer 2 (pH 7.1). The enzyme activity was eluted off the column by a continuous phosphate gradient from 0.01—0.30 M potassium phosphate. •———•, DNAase activity; ○———○, concentration of potassium phosphate.

in an assay in which circular double-stranded PM2 DNA was used as a substrate. Various fractions were incubated with PM2 DNA (Boehringer Mannheim, Indianapolis, IN) in a reaction mixture (65 μl) containing 5 mM $MgCl_2$, 10 mM Tris/maleate buffer, pH 7.5 at 37°C for 2 h. The reaction was stopped by the

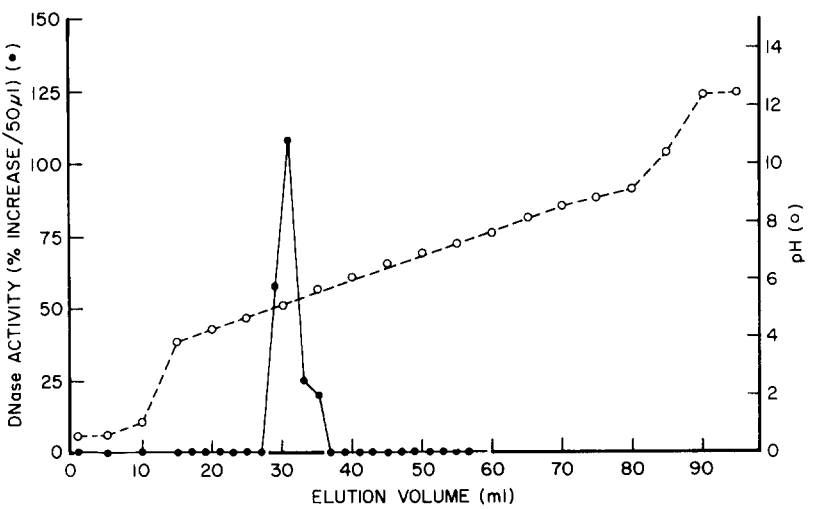

Fig. 2. Isoelectric focusing of fraction VI. Fraction VI (300 units) in buffer 2 was applied to a 110 ml LKB electrofocusing column at 5°C and run at 600 V for 24 h. The column was then run for an additional 24 h at 1000 V. •———•, DNAase activity; ○———○, pH gradient.

addition of 10 μl 0.1 M EDTA. The DNA samples were mixed with 20 ml 42% sucrose and 0.01% bromophenol blue and applied to 0.7% agarose gels (6 × 100 mm). Electrophoresis was carried out at room temperature at 50 V for 3.5 h in a buffer containing 40 mM Tris-HCl, (pH 8.0) 5 mM sodium acetate, and 1 mM EDTA [22]. Gels were stained in 0.5 μg/ml ethidium bromide and fluorescence of the DNA detected using a C-61 mineralight transilluminator (Ultra-Violet Products, CA).

Molecular sieving. DNAase N22 was applied to a Sephadex G-75 column (0.7 × 45 cm). Using blue dextran (Pharmacia), cytochrome *C*(ICN), hemoglobin (Miles) and α-chymotrypsin (Sigma) as markers, the molecular weight of the enzyme was estimated [23].

SDS gel electrophoresis. DNAase N22 in 8% sucrose and 0.002% bromophenol blue was applied to gels (100 × 3 mm, containing 7.5% acrylamide, 0.2% bisacrylamide and 1% SDS). Electrophoresis was carried out at 2mA/tube in a 0.1 M sodium phosphate buffer containing 0.1% SDS until the tracking dye migrated to 8 cm. The gels were fixed for 2 h in 12.5% trichloroacetic acid, stained overnight in 0.05% Coomassie blue in 12.5% trichloroacetic acid, and then destained in 10% trichloroacetic acid. The molecular weight of DNAase N22 was estimated from the linear relationship between electrophoretic mobility and the logarithm of the molecular weights of the protein standards. The standards were ovalbumin (Pharmacia), chymotrypsinogen A (Pharmacia) and ribonuclease A (Pharmacia).

Protein determinations. The protein content of the enzyme preparations was measured by the method of Lowry et al. [24].

Results

The specific activity of DNAase N22 increased 188-fold during the purification (Table I). The exact increase in specific activity over the starting material is impossible to assess since cell homogenates and nuclei contain other potent DNAases with activities which are measured by the methods used to detect DNAase N22, so the 188-fold increase in specific activity of the enzyme shown in Table I is probably a minimal estimate.

The following physicochemical criteria indicate that pure protein was isolated. Only one protein band was seen on SDS gels when DNAase N22 was examined by electrophoresis. The mobility of marker proteins on three similar gels showed that the molecular weight of this single protein band was 22 000.

TABLE I

PURIFICATION SUMMARY FOR DNAase N22

Sample	Total activity (units)	Total protein (mg)	Specific activity (units/mg)
Nuclei	21 055	35.33	918
I/M NaCl extract	15 651	6.13	2 553
Fraction I	7 208	1.20	6 007
Fraction IV	5 967	0.31	19 248
Fraction VII	3 086	0.018	171 444

TABLE II

DIVALENT CATION DEPENDENCE ON DNAase N22

DNAase N22 was dialyzed into buffer 2 containing varying concentrations of either Mg^{2+}, Mn^{2+} or Ca^{2+} and the activity of the enzyme was determined.

Cation	Unit of activity
No ions	0.0
1 mM Mg^{2+}	17.1
5 mM Mg^{2+}	1108.4
10 mM Mg^{2+}	1677.3
20 mM Mg^{2+}	131.0
1 mM Mn^{2+}	600.8
5 mM Mn^{2+}	409.8
10 mM Mn^{2+}	74.7
1 mM Ca^{2+}	0.0
10 mM Ca^{2+}	16.8
50 mM Ca^{2+}	0.0
10 mM Mg^{2+} + 1 mM Ca^{2+}	1604.0

DNAase N22 eluted from a Sephadex G-75 column as a single homogenous peak of activity, and the molecular weight determined by this method (21 500) was in close agreement with the value determined by gel electrophoresis.

DNAase N22 is active in the pH range of 5.5—8.5 with an optimal pH of 7.0 ± 0.2. Divalent cations are necessary for activity. The optimum concentration for Mg^{2+} was found to be 10 mM. Mn^{2+} partially substituted for Mg^{2+}; Ca^{2+} did not activate DNAase N22 (Table II). When Ca^{2+} and Mg^{2+} were used together in the reaction mixture, there was no increase in activity over that which Mg^{2+} produced alone. Therefore, DNAase N22 is not the enzyme described by Hewish and Burgoyne [25]. The monovalent cations, Na^+ or K^+, did not substitute for the divalent cations; however, when 20 mM K^+ was used in conjunction with 10 mM Mg^{2+} there was a 50% increase in activity above that produced with Mg^{2+} alone (Fig. 3).

DNAase N22 lost 50% of its activity after 10 min at 50°C. Its stability was increased by incorporation of ethylene glycol (final concentration 40%) in the buffer solution containing the enzyme, and handling and storing the enzyme solution at —20°C.

DNAase N22 had the ability to hydrolyze phosphodiester bonds of double-stranded circular phage PM2 DNA when it was used as substrate (Fig. 4). Treatment of PM2 DNA with this enzyme for 2 h resulted in the conversion of form I DNA (superhelical [22]) for form II DNA (due to at least one single-strand scission/molecule [22]) and to a small amount of form III DNA (unit length form [22]). Partially purified enzyme preparations from an intermediate step in the purification of DNAase N22, fraction I, used at 1/6 the activity of DNAase N22, produced some nicked DNA as shown by an increased amount of form II DNA on agarose gels (Fig. 4). The ability of DNAase N22 to nick circular PM2 DNA indicates that this enzyme contains endonuclease activity. Exonuclease activity of the preparation was determined according to the method of Lindahl et al. [26] using poly(dA-dT) as substrate. DNAase N22 activity is clearly different from two known exonucleases by the fact that its ability to activate DNA for DNA polymerase, relative to its ability to produce

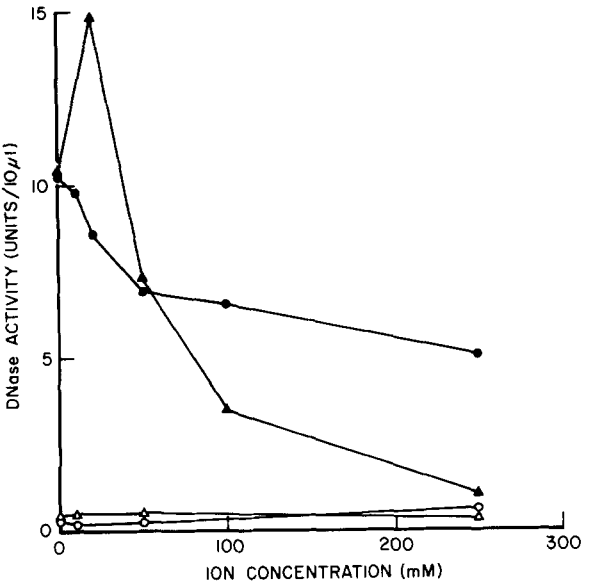

Fig. 3. The effect of monovalent cations on DNAase N22 activity. 17 units DNAase N22 were added to 40 μl 0.05 M buffer 3 containing appropriate amounts of monovalent cation under study. In one study, Mg^{2+} was omitted from the reaction mix which contained either Na^+ (○———○) or K^+ (△———△). In the second study the complete reaction mix containing 10 mM Mg^{2+} was used, with either Na^+ (●———●) or K^+ (▲———▲).

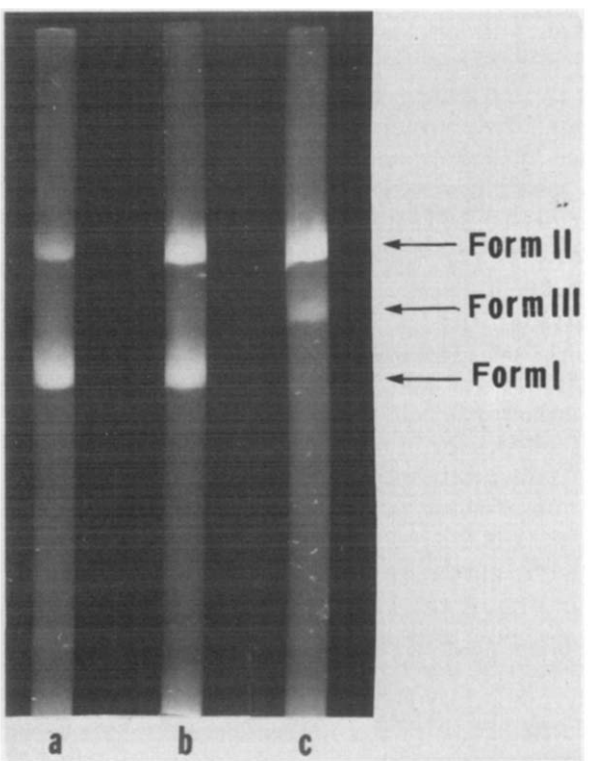

Fig. 4. Action of HeLa cell DNAase N22 on PM2 DNA. PM2 DNA (0.5 μg) was applied to 0.7% agarose gels. (a) Untreated; (b) treated with 2.5 μg of fraction I, and (c) treated with 0.5 μg of DNAase N22 (fraction VII). Electrophoresis was carried out at 50 V for 3.5 h. Gels were stained with 0.5 μg/ml ethidium bromide. The comparison of the amounts of form I (superhelical), form II (at lease one single-strand scission), and form III (unit length) DNA in each of these gels indicates that treatment with fraction I increased the amount of form II DNA, and fraction VII completely converted form I into forms II and III.

acid-soluble fragments from a macromolecule, was far greater than that of the exonucleases. This indicates that DNAase N22 is primarily an endonuclease.

The preparation was tested for other enzyme activities. It has no alkaline phosphatase activity when assayed as described by Lowry [27] using the synthetic substrate *p*-nitrophenol phosphate. Similarly RNAase activity was not detected when assayed using the homopolymers poly(A) or poly(C) (Miles).

Sulfhydryl-reacting compounds have been known to inhibit DNAase activity [26—28]. 50% inhibition of DNAase N22 activity was produced by 50 mM *N*-ethyl maleimide and 840 μM *p*-chloromercurobenzoate. Caffeine, a compound which is not a sulfhydryl-reacting agent, has been shown to inhibit phosphodiesterases [29] and the repair of ultraviolet light damage to DNA [30,31]. The addition of 5 mM caffeine to the assay system produced a 50% decrease in the activity of DNAase N22.

Discussion

The enzyme described here is clearly different in a number of respects from previously described mammalian DNAases. DNAase N22 has a molecular weight of 22 000. It is, therefore, significantly smaller than DNAase I, which has a molecular weight of 31 000 [32,33]. In addition, DNAase N22 activity is sensitive to sulfhydryl-reacting agents while DNAase I is relatively resistant [34]. DNAase II is a mammalian endonuclease that releases 3′-phosphate groups from native DNA [35,36] in contrast to DNAase N22 which probably releases 3′-OH groups, as shown by its ability to increase priming activity of calf thymus DNA for the action of DNA polymerase. DNAases III and IV are mammalian nuclear exonucleases [26,28]. DNAase V, an endonuclease isolated from calf thymus, is a larger molecule with a molecular weight of 53 000, and an isoelectric point at pH 10.3 [15].

The properties of DNAase N22 indicate that this enzyme is a non-histone chromosomal protein. The enzyme does not adsorb to carboxymethyl-Sephadex, but does bind strongly to DEAE-cellulose and has an isoelectric point of pH 5.1 ± 0.2. This implies that the enzyme is an acidic protein. Other DNAase activities have been shown to be associated with the acidic or non-histone chromosomal proteins of eukaryotic cells [37,38].

Certain low molecular weight classes of non-histone proteins have been implicated in the stimulation of cell proliferation [39,40] and the DNAase described in this report may be one of these proteins. An important event in cell replication is the initiation of DNA synthesis, a process requiring two fundamental steps. One of these is the partial unwinding of the DNA template. An endonuclease-catalyzed nick in one of the two DNA strands could release the topological restraints on the molecule and permit unwinding of the double helix [9]. The second important step is the binding of DNA polymerase to DNA. Nicks containing 3′-OH termini, introduced into duplex DNA by an endonuclease, have been shown to be active points for replication in some systems [41]. The present work indicates that N22 is an endonuclease and nicks DNA. The activity of N22 in the primer activation assay suggests that this enzyme generates 3′-OH primer ends for DNA polymerase action. It is possible, therefore, that N22 may be involved in one or both of the initiation steps

described above. Results of preliminary experiments indicate that the activity of DNAase N22 is higher during the S phase than during other phases of the cell cycle [42]. Work is now in progress to determine whether DNAase N22, with its endonucleolytic activity, can initiate DNA replication under physiological conditions, or whether it has a role in repair of damage to DNA.

Acknowledgements

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