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Purification and Some Properties of a Deoxyribonucleic Acid Endonuclease Endogenous to Rat Liver Chromatin†

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ABSTRACT: A deoxyribonucleic acid (DNA) endonucleolytic activity has been purified from a 0.3 M KCl extract of rat liver chromatin by a combination of selective precipitation and ion-exchange and gel filtration chromatography. The purified protein has a molecular weight of 35 000 as determined by Sephadex G-200 gel filtration and sodium dodecyl sulfate–acrylamide gel electrophoresis. The nuclease activity is stimulated by the addition of Mg^{2+} and thus may represent the Mg^{2+}-activated DNase endogenous to chromatin. The purified enzyme has the ability to make both single-strand nicks and double-strand cuts in DNA.

Nuclease digestion studies have proven invaluable in the elucidation of the nucleosome as the basic structural subunit of eukaryotic chromatin. Indeed, one of the first indications of such a chromatin organization was a periodic deoxyribonucleic acid (DNA)[1] digestion pattern observed during chromatin autodigestion mediated by endogenous DNases (Hewish & Burgoyne, 1973a,b). Since then nucleases exogenous to chromatin such as micrococcal nuclease and DNases I and II have been widely used to investigate both the structure of the nucleosome itself and the organization of nucleosomes into domains of higher order structure varying in their sensitivity to digestion [for a recent review see McGhee & Felsenfeld (1980)]. In addition, many workers have utilized the autodigestion of chromatin to examine these topics; this process has been shown to result in the liberation of nucleosome multimers and monomers (Keichline et al., 1976; Krueger, 1978; Suciu, 1979; Chikhirzhina, 1979) and may be able to differentiate between transcriptionally active and inactive chromatin (Paul & Duerksen, 1976a,b). The nature of the DNases responsible for such phenomena is less clear. However, nucleolytic activity has been ascribed to the nonhistone protein component of chromatin and nuclei (O'Connor, 1969; Urbanczyk & Studzinski, 1974; Vinter et al., 1974; Gainuillina et al., 1976; Lambert & Studzinski, 1979), and the purifications to varying degrees of several DNase activities endogenous to chromatin and nuclei have been described (Ishida et al., 1974; Cordis et al., 1975; McGuire et al., 1976; Fischman et al., 1979).

Here we report the purification of a DNA nucleolytic enzyme extracted from rat liver chromatin. The enzyme activity is stimulated by the addition of divalent magnesium cations but differs from those DNases previously described in its mechanism of action on DNA.

Materials and Methods

Frozen rat livers were obtained from Pel-Freeze, standards for $NaDodSO_4$–acrylamide gel electrophoresis was from Pharmacia Fine Chemicals, and standards for gel filtration were from Sigma Chemical Co. Agarose was purchased from SeaKem. All other chemicals were of reagent grade.

Isolation of Nuclei. In a standard preparation nuclei were purified from 20 rat livers as described previously (Wallace et al., 1977).

Isolation of pBR322 DNA. Plasmid DNA was prepared from *Escherichia coli* HB101 essentially by the method of Clewell & Helinski (1969).

Nuclease Assay. The conversion of superhelical pBR322 DNA to relaxed circular and linear forms was used to monitor the nuclease activity throughout the purification. An aliquot (10 μL) of the column fractions to be assayed was incubated in a reaction mixture of 20-μL total volume containing 0.16 μg pBR322 DNA at a final concentration of 0.1 M KCl, 30 mM Tris-HCl (pH 7.0), 2 mM $MgCl_2$, and 40 μM EDTA (derived from the plasmid storage buffer). The reaction was stopped after 15 min at 37 °C by the addition of $NaDodSO_4$ to 0.3%, and the products of digestion were analyzed by agarose gel electrophoresis as described below. One unit of enzyme activity was defined as that amount of enzyme required to convert 0.1 μg of superhelical DNA to other forms in 15 min at 37 °C.

DNA Gel Electrophoresis. Supercoiled, circular relaxed, and linear DNA molecules were resolved in 1% agarose gels containing 36 mM Tris base, 30 mM NaH_2PO_4, and 1 mM EDTA (pH 7.7; also the reservoir buffer). Samples were brought to 0.3% $NaDodSO_4$, 0.05% bromophenol blue, and 10% sucrose and heated to 55 °C for 15 min prior to elec-

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; QAE-Sephadex, quaternary aminoethyl-Sephadex; DNA, deoxyribonucleic acid; Sh, superhelical form of pBR322 DNA; Cr, circular relaxed form of pBR322 DNA; l, linear form (strand) of pBR322 DNA; c, circular strand of pBR322 DNA; DNase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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trophoresis at 40 V for 8 h. Electrophoresis of DNA under denaturing conditions was carried out in alkaline agarose (1%) gels by the method of McDonnel et al. (1977).

NaDodSO$_4$-Acrylamide Gel Electrophoresis. NaDodSO$_4$–acrylamide gel electrophoresis of proteins was by the method of Laemmli (1970) using a linear polyacrylamide gradient of 7.5–20%. Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Results

All operations during the purification of the enzyme were carried out at 4 °C.

Chromatin Isolation and Extraction. The isolated nuclei (see Materials and Methods) were lysed by glass–Teflon homogenization in 200 mL of 2.5 mM Tris-HCl (pH 7.0) and 2.5 mM EDTA, and chromatin was pelleted by centrifugation at 6000*g* for 20 min. Chromatin was resuspended in the above buffer and centrifuged at 1500*g* for 15 min to form a loose gel and a supernatant which was discarded. To the chromatin gel was slowly added solid KCl with vigorous stirring to a final concentration of 0.3 M. Precipitated chromatin was removed by centrifugation at 1500*g* for 10 min, followed by high-speed centrifugation at 100000*g* for 30 min. The supernatant was dialyzed overnight against 10 mM KCl and 10 mM KH_2PO_4 (pH 7.4) and precipitated protein removed by centrifugation at 6000*g* for 20 min. The supernatant after this procedure is termed fraction I.

Phosphocellulose Chromatography. The dialyzed chromatin extract (fraction I) was added to a phosphocellulose column (1.5 × 18 cm) which had been equilibrated with 10 mM KCl and 10 mM KH_2PO_4 (pH 7.4) and the column washed with 100 mL of the same buffer. The nuclease activity was recovered by washing the column with 200 mL of 10 mM KCl and 0.2 M KH_2PO_4 (pH 7.4). This procedure separates the nuclease activity from the bulk of nicking–closing enzyme (DNA topoisomerase 1) activity which is present in the chromatin extract and can be eluted from the phosphocellulose column at 0.4 M in a 0.2–0.7 KH_2PO_4 (pH 7.4) gradient in 10 mM KCl (G. C. Machray and J. Bonner, unpublished observations). The protein eluted from the column in 10 mM KCl and 0.2 M KH_2PO_4 (pH 7.4) was dialyzed extensively against 50 mM KCl and 50 mM Tris-HCl (pH 8.0) to give fraction II.

QAE-Sephadex Chromatography. Fraction II was loaded onto a QAE-Sephadex (1.5 × 30 cm) column preequilibrated with 50 mM KCl and 50 mM Tris-HCl (pH 8.0) and the column washed with 100 mL of the same buffer. Bound protein was eluted from the column by a linear gradient (200 mL) of 50–500 mM KCl in 50 mM Tris-HCl (pH 8.0). Protein elution was monitored by absorbance at 280 nm (Figure 1a) while enzyme activity was determined by the conversion of superhelical pBR322 DNA to relaxed and linear forms (Figure 1b) as described under Materials and Methods. The presence of partially relaxed intermediates as well as linear DNA in several fractions may be the result of residual nicking–closing enzyme activity. Fractions containing nuclease activity were pooled and concentrated by Amicon pressure filtration (PM 10) in a stirred cell to yield fraction III.

Sepharose 6B Gel Filtration. The concentrated pool of active fractions from the QAE-Sephadex column (fraction III) was passed over a Sepharose 6B column (1.8 × 80 cm) at a flow rate of 20 mL/h in 50 mM KCl, 50 mM Tris-HCl (pH 7.0), and 5% glycerol. The protein elution and enzyme activity profiles are presented in parts a and b of Figure 2, respectively. Nuclease activity is again indicated by the conversion of superhelical DNA to relaxed circular and linear forms. Fractions containing activity were pooled and concentrated as for fraction III to yield fraction IV.

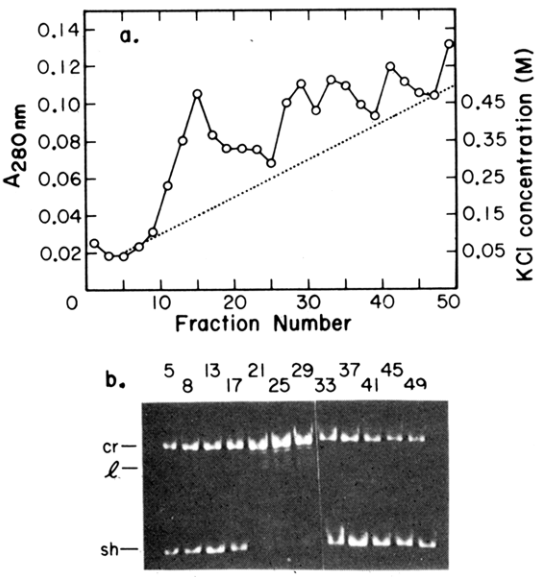

FIGURE 1: QAE-Sephadex chromatography. Fraction II was chromatographed on QAE-Sephadex as described in the text. The column was eluted with a linear KCl gradient of 50–500 mM in 50 mM Tris-HCl (pH 8.0) (dotted line), and fractions containing 4 mL were collected (a). The absorbance profile at 280 nm (O) is shown (a), and the indicated fractions were assayed for nuclease activity (b) as described under Materials and Methods.

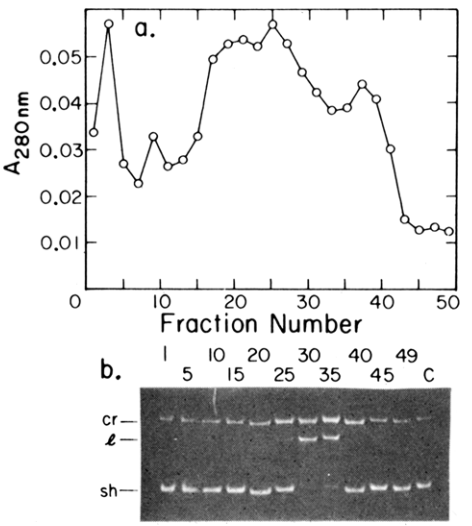

FIGURE 2: Sepharose 6B gel filtration. Fraction III was chromatographed on Sepharose 6B as described in the text. The absorbance at 280 nm (O) of fractions containing 2.5 mL was monitored (a), and the indicated fractions were assayed for nuclease activity (b) as described under Materials and Methods. Slot c contained column buffer in place of a column fraction.

Sephadex G-200 Gel Filtration. Fraction IV was chromatographed through a Sephadex G-200 column (3 × 36 cm) preequilibrated with 50 mM KCl, 50 mM Tris-HCl (pH 7.0), and 5% glycerol. The column was run under upward flow conditions at a rate of 20 mL/h. The nuclease activity after this step corresponds to a plateau of protein elution (fraction numbers 24–34) detected by absorbance at 280 nm as in shown in Figure 3.

Molecular Weight and Purity of the DNase. The approximate molecular weight of the DNase as determined by comparison with standard run on the above Sephadex G-200 column under identical conditions (Figure 3) was 35000. When 1 mL of the fraction containing the most enzyme ac-

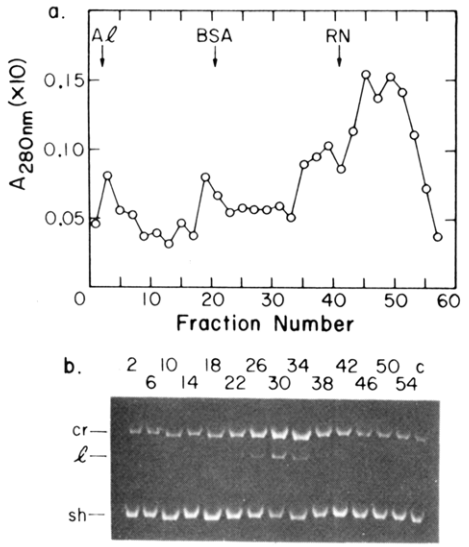

FIGURE 3: Sephadex G-200 gel filtration. Fraction IV was chromatographed on Sephadex G-200 as described in the text. The absorbance at 280 nm (O) of fractions containing 2.5 mL was monitored (a), and the indicated fractions were assayed for nuclease activity (b) as described under Materials and Methods. Slot c contained column buffer in place of a column fraction. Arrows indicate the positions of marker proteins chromatographed on the Sephadex G-200 column under identical conditions: Al, aldolase, molecular weight 160 000; BSA, bovine serum albumin, molecular weight 68 000; RN, RNase, molecular weight 14 000.

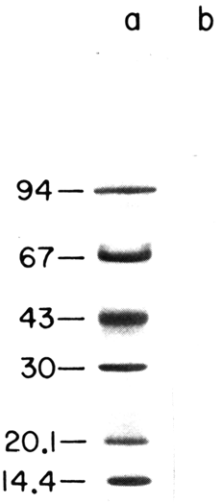

FIGURE 4: $NaDodSO_4$–acrylamide gel electrophoresis of purified nuclease. A sample (b) prepared as described in the text was analyzed by $NaDodSO_4$–acrylamide gel electrophoresis as described under Materials and Methods. Standards (a) run in the same gel are, in order of decreasing molecular weight, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin (molecular weight × 10^{-3} is shown on left).

tivity (fraction 30) of this column was dialyzed, lyophilized, and analyzed by $NaDodSO_4$–acrylamide gel electrophoresis, one major band of molecular weight 35 000 was visualized (Figure 4). This correspondence between molecular weights as determined by $NaDodSO_4$–acrylamide gel electrophoresis and gel exclusion chromatography suggests that the enzyme may consist of a single polypeptide of this molecular weight.

The conversion of superhelical DNA molecules to circular relaxed and linear forms has been used previously to assay for DNAse activity (Vinter et al., 1977; Fischman et al., 1979). Using this assay we have been able to quantitate nuclease activity during the later stages of purification; we have found, however, that the quantitation of enzyme activity is subject

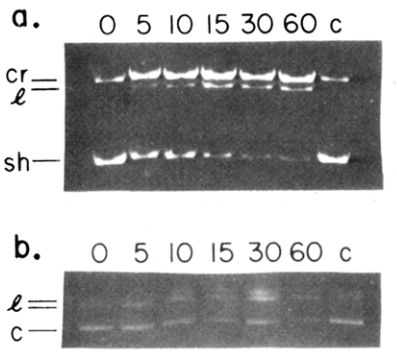

FIGURE 5: Time course of nuclease digestion of pBR322 DNA. 0.5 unit of purified nuclease was used to digest pBR322 DNA in a standard reaction mixture (see Materials and Methods) for the indicated time (shown above in minutes). In slots marked c buffer replaced enzyme and the incubation time was 60 min. Products of digestion were analyzed under nondenaturing (a) and denaturing (b) conditions (see Materials and Methods).

to interference by various factors during the early stages of purification (e.g., after selective precipitation and phosphocellulose chromatography). One such factor is the presence of nicking–closing enzyme which also utilizes superhelical DNA as a substrate. A further complication is the presence of proteins which precipitate DNA. Other assay systems we have attempted have been found to be subject to error for these reasons. We therefore do not report data on the fold purification or yield of the nuclease activity at this time.

On the Mechanism of Action of the DNase. The purified nuclease is established as an endonuclease by its ability to cleave closed-circular superhelical pBR322 DNA. One of the products of this cleavage is linear DNA which can be visualized after treatment with relatively small amounts of enzyme (e.g., Figure 3, fractions 22 and 42). This is further illustrated in Figure 5 by a time course of digestion of DNA at one enzyme concentration (0.5 unit). The products of digestion are analyzed under both native and denaturing conditions (parts a and b of Figure 5, respectively). At the earliest time of digestion (5 min), linear molecules can be seen (Figure 5). This result might be expected of nucleases which make a double-strand cut in DNA. However, at longer times of digestion an increase in the amount of circular relaxed DNA is also observed. These molecules cannot result from a double-strand cut in superhelical DNA, and therefore the nuclease cannot exclusively make double-strand cuts in DNA.

Circular superhelical DNA can also be linearized by an accumulation of random single-strand nicks. In this case essentially all superhelical molecules should be converted to the circular relaxed form prior to the appearance of linear molecules (since only one nick is required to convert superhelical DNA to circular relaxed while two adjacent nicks on opposite DNA strands are required to result in the formation of linear DNA). Such a result has been observed in control experiments using DNase 1 (data not shown). In contrast, the nuclease purified here can convert some superhelical molecules to the linear form while others remain superhelical (Figure 5a). In addition, when the products of digestion over the same time period are analyzed under denaturing conditions to reveal single-stranded circular and linear DNA, no nicking of DNA can be detected (Figure 5b). Thus, the nuclease does not convert superhelical to linear DNA exclusively by the accumulation of single-strand nicks.

Two further mechanisms can be postulated for the action of the purified nuclease. Single strand specific nucleases such as S1 from *Aspergillus* have the ability to convert superhelical DNA to a linear form (Beard et al., 1973). Superhelical DNA

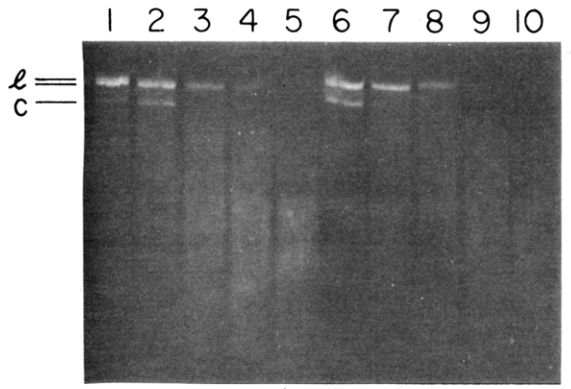

FIGURE 6: Nuclease digestion of native and denatured pBR322 DNA. Native (slots 1–5) and denatured (slots 6–10) pBR322 DNA was digested for 0, 5, 10, 20, and 30 min (slots 1–5 and 6–10, respectively) with 1 unit of enzyme. Assay mixtures were as described under Materials and Methods with the exception that 0.32 μg of DNA in a total volume of 40 μL was used per sample. Nicked circular relaxed λpBR322 DNA at a concentration of 16 μg/mL was denatured by boiling at 100 °C for 5 min, followed by rapid cooling on iced water, immediate nuclease digestion, and analysis under denaturing conditions as described under Materials and Methods.

exhibits a partially single stranded nature (Dean & Lebowitz, 1971; Beerman & Lebowitz, 1973); S1 nuclease recognizes these single-stranded regions and nicks the superhelical DNA to a transient circular relaxed molecule. S1 nuclease then specifically cleaves the other strand of the intermediate form opposite the nick it has just created, forming a linear molecule. To determine if the nuclease described in this work might act by such a mechanism, we have examined its activity on native and denatured (single-stranded) DNA (Figure 6). The nuclease shows no difference in activity on a single- or double-stranded DNA substrate over the time period and enzyme concentration assayed. In addition, no stable linear intermediate is observed throughout the time course of digestion, and thus the nuclease is unlikely to be a single strand specific nuclease like S1. We conclude that the nuclease has the ability to make both single-strand nicks and double-strand cuts in DNA. Such a mechanism is consistent with the early formation of linear molecules and the increased amount of circular relaxed form observed during the time course of digestion shown in Figure 5 and also with the observed similarity in the rate of digestion of native and denatured DNA shown in Figure 6. In this respect the nuclease resembles DNase II which also has the ability to make single- and double-strand cuts in DNA (Bernardi, 1971).

Divalent Cation Activation of DNase Activity. Throughout the purification of the nuclease, column fractions were routinely assayed in the presence of 2 mM Mg^{2+} (see Materials and Methods). The action of the nuclease in the range of 1–10 mM Mg^{2+} is shown in Figure 7 (slots 3–6). The enzyme is activated by as little as 1 mM Mg^{2+}, and little further activation is seen at higher concentrations. The enzyme is inactive in 2 mM EDTA (slot 1) and also shows no activity at 40 mM EDTA (slot 2; present as a result of storage of plasmid DNA in 0.2 mM EDTA). The effect of addition of 1 mM Ca^{2+} is shown in slot 7, which demonstrates that Ca^{2+} is not as effective as Mg^{2+} at the same concentration in the activation of the nuclease. Mixing experiments (results not shown) showed no further enhancement of nuclease activity when Ca^{2+} ions were added at a given concentration of Mg^{2+}. Thus, it is unlikely that this nuclease is the Ca^{2+},Mg^{2+}-activated enzyme described by Hewish & Burgoyne (1973b). However, it may represent the Mg^{2+}-activated nucleolytic activity described in extracts of nuclei by the same authors.

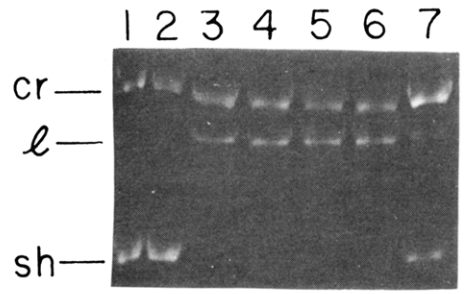

FIGURE 7: Stimulation of nuclease activity by divalent cations. Standard reaction mixtures (see Materials and Methods) contained additions at the following final concentrations: slot 1, 2 mM EDTA; slot 2, no addition; slots 3–6, $MgCl_2$ at 1, 2, 5, and 10 mM, respectively; slot 7, 1 mM $CaCl_2$. Time of incubation was 15 min. The products of digestion were analyzed under nondenaturing conditions as described under Materials and Methods.

Discussion

We have purified a DNA endonuclease of molecular weight 35 000 from a 0.3 M KCl extract of rat liver chromatin. The nuclease is activated by the addition of Mg^{2+} ions; Ca^{2+} ions at the same concentration result in a lesser activation. Double- and single-stranded DNA's are attacked with equivalent facility, and the enzyme can make both single-strand nicks and double-strand cuts on pBR322 DNA. A DNA endonuclease of 36 000 molecular weight whose activity is stimulated by Mg^{2+} and not by Ca^{2+} has been purified from mouse 3T3 cell chromatin (McGuire et al., 1976). This nuclease can cleave both single-stranded and double-stranded DNA but differs from the enzyme we have purified in that the 3T3 cell chromatin nuclease cleaves single-stranded DNA at around 100 times the rate of cleavage of double-stranded DNA, only a limited number of single-strand nicks being introduced into the duplex DNA molecule. The purifications of several other chromatin-endogenous endonucleases, whose properties can be readily distinguished from those of the rat liver chromatin endonuclease studied in this work, have been described. Endonuclease N22 from HeLa cell chromatin (Fischman et al., 1979) has a molecular weight of 22 000 and attacks superhelical PM2 DNA probably by a single-strand nicking mechanism, as does the chromatin-endogenous nuclease described by Vinter et al. (1977). An endonuclease (molecular weight 27 000) which makes single-strand cuts on native DNA has been purified from rat liver nuclei (Ishida et al., 1974). This enzyme does not attack denatured DNA, and its activity is maximally stimulated only in the presence of mixtures of Ca^{2+} and Mg^{2+} ions (1 and 5 mM, respectively), omission of Ca^{2+} resulting in a 86% reduction of activity. This enzyme may constitute the Ca^{2+},Mg^{2+}-activated nuclease detected in crude extracts of chromatin by Hewish & Burgoyne (1974b); the endonuclease we describe more closely resembles the Mg^{2+}-activated nuclease detected in the same and similar (O'Connor, 1969) extracts.

In addition to those nucleases specifically extracted from chromatin and nuclei, the purifications of other cellular DNases have been detailed (Wang & Furth, 1977; Lavin et al., 1976; Cordis et al., 1975; Kuebler & Goldwait, 1977). While these enzymes do require divalent cations to varying degrees, their molecular weights and the mechanisms by which they cleave DNA differ from those of the endonuclease we have extracted and purified from rat liver chromatin.

DNA endonucleases have been implicated in such functions as the initiation of DNA replication (Blair et al., 1971; Ionnow, 1973) and repair of DNA (Cleaver, 1974). The ability of the endonuclease we have purified to make both single-strand nicks

and double-strand cuts in native DNA is interesting in this respect and also may indicate involvement in other processes. It has been suggested that the separation of long linear eukaryotic chromosomes, which may be topologically interlocked, might require the mediation of enzymes able to cleave and rejoin duplex DNA (Hsieh & Brutlag, 1980). The nuclease described in this work could represent the nucleolytic subunit or part of a multienzyme complex able to perform such a function. Further investigation into the conditions and mode of action of this endonuclease will clarify its role within the cell nucleus.

Acknowledgments

We thank Dr. J. E. Wiktorowicz for many valuable discussions throughout the course of this work and Carlotta A. Glackin for the preparation of pBR322 DNA.

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Purification of a 9S DNA Polymerase α Species from Calf Thymus†

Frank Grosse and Gerhard Krauss*

ABSTRACT: A DNA polymerase α species from calf thymus has been purified 12 000-fold to near homogeneity. The enzyme sediments under high salt conditions in the preparative ultracentrifuge as a homogeneous band at 9 S. The specific activity is 50 000–70 000 units/mg of protein. Polypeptides of 148 000, 59 000, 55 000, and 48 000 daltons are detectable. The molecular weight as estimated from gradient gel electrophoresis is about 500 000. The 9S DNA polymerase is free from terminal nucleotidyl transferase activity and does not exhibit endonuclease or exonuclease activity. It is inhibited by low concentrations of salt, aphidicolin, and *N*-ethylmaleimide.

The purification and characterization of DNA polymerases have proven to be a difficult task. The subunit structure of DNA polymerase III holoenzyme—the main replicative enzyme in *Escherichia coli*—has been established only in the last few years [for a review, see Kornberg (1980)]. Whereas the polymerizing activity of the core subunit of this enzyme has been discovered rather early (Otto et al. 1973), little is known up to now about the function of the other subunits.

In mammals, DNA polymerase α is responsible for the

† From the Zentrum Biochemie, Abteilung Biophysikalische Chemie, Medizinische Hochschule Hannover, Karl Wiechert Allee 9, D 3000 Hannover 61, West Germany. *Received February 3, 1981.* This work was supported by a grant from the Deutsche Forschungsgemeinschaft to G.K. and from the Fonds der Chemischen Industrie to F.G.

0006-2960/81/0420-5470$01.25/0