An Endonuclease Activity of Chicken Erythrocyte Nuclei and Mononucleosomes*

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Endogenous nuclease is present in the nuclear sap of chicken erythrocyte nuclei. This enzyme resembles the nuclease of mammalian nuclei in requirements for bivalent cations and in production of large chromatin fragments that gradually decrease in size, but differs in that the products do not go through the stage of discrete bands on gel electrophoresis. Endogenous nuclease and micrococcal nuclease are also detectable in mononucleosomes prepared from chicken erythrocytes with the aid of micrococcal nuclease. Both nucleases are extractable with 0.35 M NaCl, and both are inhibited by pTP. In the absence of Ca°++, the micrococcal nuclease is totally inactive, whereas the endogenous nuclease shows a low level of activity.

In the previous paper of this series (1), we investigated the action of three different single strand specific nucleases on chicken erythrocytes (chromatin). All three produced large, transient (lasting 2 to 4 h) fragments of chromatin that were soluble in 10 mM EDTA. None of these enzymes led to the stage of mononucleosomes or their multimers, and no discrete bands were observed on gel electrophoresis of chromatin digests.

Even though chicken erythrocyte nuclei are often used directly as a chromatin preparation or indirectly as a source for mononucleosomes, little attention has been paid to the possible existence of endogenous nuclease (1). In contrast, the endogenous nuclease of mammalian nuclei has been known for many years (2-5). DNA prepared from autolyzed mammalian nuclei formed discrete bands on gel electrophoresis which corresponded to nucleosomes and their multimers (2). Recently it was shown that mononucleosomes prepared from rat liver nuclei with the aid of micrococcal nuclease bound some of the micrococcal enzyme (6), which could then be eluted with 0.35 M NaCl.

Several questions arose with respect to chicken erythrocyte nuclei and the mononucleosomes prepared from them. Do these nuclei have endogenous nuclease with properties similar to those described for the mammalian enzyme? If so, do mononucleosomes prepared from chicken erythrocytes with micrococcal nuclease contain both bound micrococcal nuclease and the endogenous nuclease? The experiments presented below show that both enzymes are detectable in the nucleosomal extract and that they can be distinguished using PM2 DNA as substrate.

EXPERIMENTAL PROCEDURES

Chicken blood was collected at the Springville Laboratories of this institute and treated as described (1); we thank Dr. M. McGarry for it. Nuclei (chromatin) were prepared according to Penman (7). Oligo- and mononucleosomes were prepared by previously described methods (1) using the same conditions for hydrolysis, the same preparation of enzymes, and the same conditions for chromatography on a Bio-Gel A-15m column; we thank Dr. Sulkowski for micrococcal nuclease (8). To ensure that the sample used as mononucleosomes was free of higher multimers, only the chromatographic fraction corresponding to the mononucleosome peak and the adjacent fractions on the descending side of this peak were pooled; analysis by gel electrophoresis revealed a uniform population of mononucleosomes.

Routinely, three buffers were used. Buffer A contained 0.3 M sucrose in 10 mM Tris-HCl, 0.75 mM CaCl2, 1 mM MgCl2, and 1 mM phenylmethylsulfonyl fluoride, pH 7.8; Buffer B had the same composition except the pH was 8.5; Buffer C contained 210 mM KCl and 100 mM Na2HPO4, pH 7.4. Usually, 2 volumes of Buffer C were used/ volume of nuclei suspension; this solution (two-thirds of Buffer C), which contained 140 mM KCl and 66 mM Na2HPO4, approached isotonicity.

Endogenous nuclease activity was measured by the rate of release of EDTA-soluble nucleoprotein fragments. The standard reaction mixture included 200 l of nuclei suspension (A260 ~ 100 in 0.1 n NaOH) in Buffer B (optimal for endogenous nuclease) plus 10 l of the solution of nucleases. Samples were incubated at 37°C for 20 min, and the reaction was stopped by the addition of an equal volume of 20 mM EDTA, pH 7.5. The mixture was centrifuged at 5000 X g for 10 min, and A260 of the supernatant was recorded. One unit of enzyme activity = 1 A260/min.

Products of digestion were electrophoresed either as nucleoprotein on 0.7% agarose gels (0.5 X 16 cm) or, after deproteinization (1), as DNA on 1.4% agarose gels (0.5 X 18 cm). Staining with ethidium bromide and subsequent photography remained as described (1). PM2 virus and its DNA were prepared as before (1).

RESULTS

The starting point of this study was the observation that, after autolysis of the nuclei suspension in Buffer A (Fig. 1A, I to 3), nucleoprotein electrophoresed on agarose gels forms tails but no detectable discrete bands. When the concentrated enzyme from native nuclei (pool of Fractions 90 to 92, Fig. 2) is added to the reaction mixture, the rate of the reaction increases (Fig. 1A, 4), but discrete bands are still absent. Fig. 1B shows that DNA prepared from the autolyzed nucleoprotein is composed of very large fragments which "tail" slowly but do not form discrete bands.

Fig. 2 shows that molecular sieving of the nuclear extract in isotonic two-thirds of Buffer C through a column of Bio-Gel A-0.5m (dashed line) clearly separates large chromatin fragments from proteins soluble in the nuclear sap and isotonic buffer. The nuclease activity is detected in tubes 90 to 92. If nuclei (or mononucleosomes) are extracted with 0.35 M NaCl (Fig. 2, solid line), the chromatin peak drastically decreases.
Buffer A were autolyzed at 37°C. The reaction was terminated by the addition of 100 μl of 20 mM EDTA, pH 7.5. Products of digestion (25 μl) were electrophoresed as nucleoprotein fragments on 0.7% agarose gels (A1, 0 min; A2, 60 min; A3, 90 min) and, after dissociation of proteins, as DNA on 1.4% agarose and 0.1% sodium dodecyl sulfate gels (B1, 0 min; B2, 60 min; B3, 90 min). Fifty microliters of nuclei sample were electrophoresed as nucleoprotein fragments on 0.7% agarose gels (pooled tubes 90 to 92, Fig. 2) extracted from nuclei with 0.35 M NaCl and incubated by the addition of 100 μl of nuclei suspension (BI, 15 ml) extracted by adding 7 ml of 0.21 M KCl and 0.1 M Na2HPO4, pH 7.4, to 3.5 ml of loosely packed nuclei in Buffer A and incubating for 60 min at 37°C. The reaction was stopped by the addition of 100 μl of 20 mM EDTA, pH 7.5, and 25 μl of the sample were electrophoresed as nucleoprotein fragments on 0.7% agarose (A4, 60 min).

Since the experiments illustrated in Fig. 3A show a very fast formation of EDTA-insoluble material. Characteristically, the increase of micrococcal nuclease leads not to a plateau, but to a significant decrease of soluble material, presumably because particulate EDTA-insoluble materials are formed faster. Omitting Ca2+ from the medium (curve b) does not prevent the rapid phase of the reaction; calcium introduced with the substrate is sufficient to activate small amounts of enzyme, and the amount of EDTA-insoluble material is 20% lower than that observed in a complete system. Once this limit is reached, further increase in enzyme does not decrease EDTA-soluble material significantly, and a dashed line). This nuclease differs from the known endogenous mammalian nuclease, since the products of digestion of chromatin with chicken endogenous nuclease do not form discrete bands on gel electrophoresis (Fig. 1). In most of the previous experiments in which chicken erythrocytes were used as substrate, the low level of activity caused by the endogenous nuclease was justifiably disregarded. This weak activity, however, may have been a source of serious error when either the specificities or the kinetics of an introduced enzyme were investigated.

The activity of the endogenous enzyme was next evaluated using intact nuclei as substrate. The nuclei, of course, contained diluted endogenous nuclease as well as calcium and magnesium. Three different sources of added enzyme were used: 0.35 M NaCl extract of intact nuclei (Fig. 3A), 0.35 M NaCl extract of mononucleosomes (Fig. 3B), and a solution of homologous (12) micrococcal nuclease (Fig. 3C).

Fig. 3A shows that in a complete system (curve a) the reaction rate is proportional to the amount of enzyme. Omitting Ca2+ from the reaction mixture (curve b) drastically decreases the rate which, however, still increases with increased amounts of enzyme. The most surprising result was the finding that endogenous nuclease is strongly inhibited by pTp (curve c). In many laboratories, including ours, inhibition with pTp was considered specific for micrococcal nuclease. Since the experiments illustrated in Fig. 3A were performed without exposing either the substrate or the enzyme to micrococcal nuclease at any time, this opinion is no longer tenable (see "Discussion").

Fig. 3C (curve a) shows a very fast formation of EDTA-soluble material. Characteristically, the increase of micrococcal nuclease leads not to a plateau, but to a significant decrease of soluble material, presumably because particulate EDTA-insoluble materials are formed faster. Omitting Ca2+ from the medium (curve b) does not prevent the rapid phase of the reaction; calcium introduced with the substrate is sufficient to activate small amounts of enzyme, and the amount of EDTA-soluble material is 20% lower than that observed in a complete system. Once this limit is reached, further increase in enzyme does not decrease EDTA-soluble material significantly, and a
plateau appears. As expected, inhibition of micrococcal nuclease with pTp is quite strong (curve c); the rate corresponds to the previously observed 95% inhibition (9). In agreement with the previous observations, the inhibition can be overcome with an excess of enzyme, as is evidenced by the break in curve c. Interpretation of the results obtained with the extract of mononucleosomes (Fig. 3B) is more difficult. The results represented by curves a and b conform to an expected intermediate between the results of 3A and 3C. Curve a shows a plateau, presumably as a result of the combined action of the two nucleases. The sigmoid shape of curve b (Ca²⁺ omitted) resembles previously described curves observed with micrococcal nuclease (9) in the presence of very low concentrations of calcium; therefore, curve b is still conceivable as an intermediate between the results of 3A and 3C. The sigmoid curve c, however, is not an intermediate between curves c of 3A and 3C. One possible explanation for this apparent discrepancy is that the nucleosomal extract contains components (other than nucleases) that preferentially bind pTp and, thus, reverse the inhibition of either of the two nucleases. The results of Fig. 3 convincingly show that endogenous nuclease is inhibited by pTp and requires calcium for optimal activity. They also show that mononucleosomes possess nucleolytic activity, but do not permit a definitive conclusion as to the dual origin of this activity.

The activity of the endogenous nuclease was next evaluated using PM2 DNA as substrate. No quantitative methods can be used with this substrate, but the system is much more sensitive and the qualitative results obtained can be interpreted semiquantitatively. Fig. 4 shows an experiment similar in concept to that of Fig. 3. The endogenous nuclease digests PM2 DNA in a characteristic manner (Fig. 4A); form I rapidly disappears, while form II accumulates and is slowly changed to full length form III and smaller fragments (tails, Fig. 4A). With micrococcal nuclease alone the pattern is different (Fig. 4C); form I lasts much longer; form II does not accumulate as rapidly, and the formation of form III and of the tails occurs simultaneously. With the enzyme eluted from mononucleosomes, the intermediate pattern is observed (Fig. 4B), confirming the previous conclusion that both endonucleases are present in mononucleosomes, from which they are extractable with 0.35 M NaCl.

The most convincing evidence for duality, however, is presented in Fig. 5. In reactions involving PM2 DNA, it is possible to use EDTA to chelate all bivalent cations carried by the viral DNA and still use DNA as the substrate. Fig. 5 represents experiments in which enzyme and substrate have been treated with EDTA. The results convincingly show that the extract from mononucleosomes contains nuclease activity which is typical for the endogenous nuclease (compare Fig. 5A with Fig. 4A and 4B). In contrast, when micrococcal nuclease and substrate are deprived of Ca²⁺ (Fig. 5B), no visible degradation occurs. The reaction observed in Fig. 5A cannot be caused by the remaining micrococcal nuclease and is, therefore, due to the endogenous nuclease. None of the data presented here can either rule out or confirm the possibility that the observed endogenous nuclease activity is due to a single enzyme.

**DISCUSSION**

It is clear from these results that both endogenous and micrococcal nucleases are detectable in mononucleosomes prepared from chicken erythrocytes. The appearance of micrococcal nuclease activity is obviously an artifact caused by

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Fig. 4. Digestion of PM2 DNA. 0.5 μg of PM2 DNA in 8.1 mM Tris-Cl, 0.4 mM EDTA, and 2.1 mM MgCl₂ (2.4 mM CaCl₂ was used in place of MgCl₂ in digestion with micrococcal nuclease), pH 9.1, was incubated at 37°C with: 1.66 units/ml of endonuclease from nuclei (A) (A₁, 2 min; A₂, 10 min; A₃, 20 min; A₄, 40 min; A₅, 60 min; A₆, 75 min); 3.70 units/ml of endonuclease from mononucleosome (B) (B₁, 90 min control without added enzyme; B₂, 30 s; B₃, 10 min; B₄, 40 min; B₅, 90 min) or 0.005 unit/ml of micrococcal nuclease (C) (C₁, 15 s; C₂, 15 s; C₃, 2 min; C₄, 40 min; C₅, 60 min). Number 1 in all three series is the control (90-min incubation without enzyme). Reactions were terminated by the addition of sufficient ice-cold 20 mM EDTA solution (pH 7.5) to obtain a final concentration of 7 mM EDTA. A 35-μl sample was electrophoresed on 0.7% agarose gel. Endonuclease was extracted from nuclei and mononucleosomes with 0.35 M NaCl.

Fig. 5. Digestion of PM2 DNA after Ca²⁺ has been removed from enzyme and substrate solutions. Removal of Ca²⁺ from PM2 DNA: 40 μl of 2 mM EDTA, pH 8.0, was added to 21 μl of PM2 DNA (140 μg/ml) in 10 mM Tris and 0.1 mM EDTA, pH 8.0; after 15 min, 12 μl of 20 mM MgCl₂ was added (final concentration of nonchelated MgCl₂ ≈ 2.1 mM). Removal of Ca²⁺ from enzyme: 40 μl of 2 mM EDTA, pH 8.0, was added to 50 μl of enzyme in 0.02 M Tris-HCl, pH 7.4; after 15 min, 15 μl of 20 mM MgCl₂ was added (final concentration of nonchelated MgCl₂ ≈ 2.1 mM). 0.4 μg of PM2 DNA was incubated at 37°C with 43 units/ml of nuclease from mononucleosome (A) (A₁, 75 min control without added enzyme; A₂, 30 s; A₃, 15 min; A₄, 15 min; A₅, 60 min; A₆, 75 min) or with 0.005 unit/ml of micrococcal nuclease (B) (B₁, 90 min control without added enzyme; B₂, 2 min; B₃, 15 min; B₄, 40 min; B₅, 60 min; B₆, 90 min). Reactions were terminated by the addition of ice-cold 20 mM EDTA, pH 7.5 (final concentration of EDTA = 7 mM). A 35-μl sample was electrophoresed on 0.7% agarose gel. Endonuclease was extracted from mononucleosomes with 0.35 M NaCl.
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the method of preparation. The results of our experiments show the presence of endogenous nuclease in both the nuclear sap and the monoauucleosomes, but they do not distinguish between three possible alternatives. 1) The presence of nuclease in the sap results from liberation of the originally nucleosomal enzyme; 2) mononucleosomal enzyme originates in the sap and binds to nucleosomes in the hypotonic solution used during preparation (as does micrococcal nuclease); and 3) endogenous nuclease is distributed between sap and particulate structures of chromatin in vivo.

Many years ago, independently and almost simultaneously, in Anfinsen's (10) and in our (11) laboratories, it was found that in the presence of Ca^{2+}, pTp inhibits micrococcal nuclease. In both laboratories, it was tacitly assumed that pTp is specific for micrococcal nuclease. This belief was strongly supported by the x-ray diffraction studies of Cotton and his colleagues (for a review, see Ref. 12), showing a close fit between the active center of the enzyme and the calcium salt of the inhibitor. We now find that endogenous nuclease of chicken nuclei is also inhibited by pTp in a manner similar to that of micrococcal nuclease. No attempt to purify the endogenous nuclease has been made as yet, but it is already possible to speculate that studies of the purified enzyme will show the active center to be sterically similar to that of micrococcal nuclease.

REFERENCES