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Partitioning of zinc and copper within subnuclear nucleoprotein particles

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ABSTRACT

Nuclei from frozen calf thymus suspended in buffer were analyzed for metal content prior to and after repeated washing. After three such extractions about 0.1 μg Zn/mg DNA and 0.025 μg Cu/mg DNA remained tightly associated with chromatin. This level of metal was essentially unchanged with subsequent washings. Digestion of extracted nuclei with micrococcal nuclease yielded soluble nucleoprotein containing zinc and copper. Metal enriched regions of chromatin appeared to be preferentially solubilized by digestion, and the solubilized metal was only partially dializable either with or without EDTA. Metal profiles generated from gel (A-5m) chromatography analysis of chelated and non-chelated solubilized chromatin were distinctive in that copper was undetectable (by flame AA) while zinc was associated only with low molecular weight products when EDTA was used. In contrast, both metals were detected with higher molecular weight oligonucleosomes in the absence of chelating agents. Additionally, the two metals localized within nucleoprotein peaks and these metal-containing regions were only resolved by gel chromatography when EDTA was omitted throughout the procedure. A discrete Cu-rich species in a region of the profile suggests a subset of Cu-rich nucleoprotein complexes.

INTRODUCTION

Zinc and copper are among the naturally occurring metal constituents of the cell's nucleus and their associations with chromatin have been demonstrated by a variety of analytical techniques (1-5). Very little, however, is known about the precise location of these and other metals within subnuclear fractions or what function, if any, they play in the nucleus.

The subunit structure of chromatin consists of repeated nucleosome units connected by internucleosome nucleoprotein bridges. Treatment of chromatin with certain endonucleases yields nucleoprotein fractions and many of these fractions have been extensively studied (6-10). There is evidence which suggests that certain nuclear fractions are soluble in the presence of endogeneous (and exogeneous) divalent cations, and that

chelation of cations results in the selective loss of such fractions. For example the early fractionation of chromatin into template active "soluble" chromatin (11, 12) was based on differential solubility in the presence of Mg^{++}. More recently, an antigentically active fraction of reticulocyte chromatin was recovered because of its unique solubility in the presence of divalent cations (13). This fraction was rich in non-histone protein and contained DNA in lengths of about 30-45 base pairs (unpublished results).

In this paper, we address the question of whether divalent cations, such as zinc and copper, localize with nucleoprotein fractions following nuclease treatment. The partitioning of endogenous zinc and copper is measured in nuclei preparations prior to and after digestion with micrococcal nuclease, an endonuclease which preferentially hydrolyzes chromatin at internucleosomal sites.

MATERIALS AND METHODS

ISOLATION of NUCLEI. Nuclei were isolated from calf thymus by a modification of the procedure described by Jackson et al. (14). All operations were carried out at 0-5°C. Previously frozen calf thymus tissue (20g) was homogenized in 80 ml of 0.25 M sucrose, TKMC buffer (50 mM Tris, 3 mM KCl, 5 mM MgCl2, 0.2 mM CaCl2, pH 7.4), 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF dissolved previously in ethanol to a concentration of 0.1 M), using a Willem's Polytron Homogenizer at medium speed for 30 sec. The homogenate was then filtered through four layers of cheese cloth and a crude nuclear pellet recovered by centrifugation at 700xg for 5 min. The pellet was washed twice by resuspending in the above solution and centrifuging. Further purification was performed by resuspending in this solution but containing 2.0 M sucrose, and pelleting into a layer of buffered 2.3 M sucrose (without the Triton X-100) by centrifugation at 75,000xg for 1 hr. The purified nuclear pellet was either used immediately or overlayed with buffered 2.3 M sucrose and stored at -20°C.

NUCLEASE DIGESTION and FRACTIONATION of CHROMATIN. Nuclease digestion and chromatin fractionation procedures were modeled after established methods (14-16). Nuclei were washed by suspending in 20 ml of 10 mM Tris, 0.25 M NaCl, 50 mM $NaHSO_3$, 1 mM PMSF, pH 7.5, polytroning at slow speed for 15 sec, and centrifuging at 700xg for 5 min. The pellet was rewashed in the same solution and resuspended in digestion buffer

(0.25 M sucrose, 15 mM Tris, 15 mM NaCl, 60 mM KCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, pH 8.0 adjusted with solid cacodylic acid) by polytroning as above. [Two washes were essential for removal of soluble nucleoproteins, RNA, and low molecular weight nuclear products which would otherwise co-chromatograph on BIO-GEL A-5m]. The washed nuclear suspension was treated with micrococcal nuclease (Sigma Chemical Co., St. Louis, MO, 0.059 units of activity per unit of absorbance at 260 nm) for 22 min in a 37° water bath shaker with mild agitation. The digestion was terminated by adjusting the EDTA concentration to 15 mM transferring the sample to an ice bath. The chromatin digest was then dialyzed for 3 hours against 10 mM Tris, 1 mM PMSF, 1 mM EDTA, pH 7.5, after which the insoluble chromatin was removed by pelleting at 12,000xg for 15 min. An aliquot of the soluble chromatin supernatant was layered onto an 85 x 2.5 cm BIO-GEL A-5m column, 100 - 200 mesh (BIO-RAD Laboratories) and eluted with dialysis buffer. In experiments where EDTA was omitted, digestion was terminated by transferring to an ice bath, and the elution buffer was the same except for the omission of EDTA.

QUANTITATION of METALS and PREPARATION of REAGENTS and GLASSWARE. For flame atomic absorption (flame A.A.) analyses, a Perkin Elmer 360 atomic absorption spectrophotometer with Perkin Elmer Intensitron hollow cathode lamps and acetylene-air was used. Zinc was quantitated using a wavelength of 213.9 nm with a slit width of 0.7 nm and 15 ma lamp current. Copper was measured at 324.8 nm with a slit width of 0.7 nm and 10 ma lamp current.

For flameless atomic absorption (flameless A.A.) analyses, a Perkin Elmer 5000 graphite furnace atomic absorption spectrophotometer (equipped with a HGA 500 programmer and automatic sampler) was used with a background correcter Zinc was measured at 213.9 nm with a 0.6 nm low slit width and 15 ma lamp current. Copper was measured at 324.7 nm with a 0.7 nm low slit width and 15 ma lamp current.

An evaluation of the accuracy and precision of both copper and zinc graphite furnace measurements was made by use of quality control check samples and standard reference material from the National Bureau of Standards. Line regression analyses of three replicate "blind" measurements performed on each sample reflected a correlation coefficient of 1 for zinc and 0.999 for copper. In Figs. 4-6 each point represents an average of at least two measurements which varied only slightly within a given run (standard deviations <10%). The location of metal peaks within

profiles was reproduceable from run to run, although there was variability in the absolute value of the metal peaks from different column runs.

All determinations were made directly on column fractions except for zinc by flameless AA, where tenfold dilutions were made. The method of standard additions was used to rule out matrix interference.

Dialysis and elution buffers (10 mM Tris, 1 mM PMSF, pH 7.5) were prepared with deionized water which had been subsequently treated with Chelex 100. Glassware was cleaned by immersion for at least 2 hours in 8 N nitric acid followed by extensive rinsing in deionized, Chelexed water. No detergents or metal utensils were used in either preparation of reagents or cleaning of glassware.

RESULTS and DISCUSSION

METAL CONTENT of ISOLATED NUCLEI. Calf thymus nuclei were suspended in buffer used in nucleosome isolation (10 mM Tris, 0.25 M NaCl, 50 mM $NaHSO_3$, 1mM PMSF, pH 7.5) and were analyzed for metal content prior to and after repeated washings. Approximately 50% of the total nuclear zinc was readily removed by simply washing the intact nuclei; the remaining metal adhered to chromatin in the ratio of 0.10-0.15 µg Zn/mg DNA, despite repeated extractions in buffer (Fig. 1). When fresh rat liver tissue was used, purified but unextracted nuclei contained approximately 0.5 µg Zn/mg DNA, and this concentration of nuclear zinc decreased to approximately 0.3 µg Zn/mg DNA after repeated extractions in nucleosome buffer. On the other hand, nuclei isolated from frozen rat liver contained lower levels of zinc, and the unextractable metal was in the same concentration ranges as was the unextractable nuclear zinc in frozen calf thymus. There is, therefore, a portion of nuclear zinc which is released by freezing tissue; nevertheless, a level of about 0.1 µg Zn/mg DNA remains tightly associated with the chromatin of both calf thymus and rat liver. We operationally define this metal as "tightly bound"; i.e., it is not

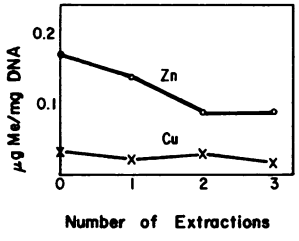

Figure 1. Metal content of isolated nuclei after repreated extractions with nucleosome isolation buffer (10 mM Tris, 0.25 M NaCl, 50 mM $NaHSO_3$, 1 mM PMSF, pH 7.5).

released by one cycle of freezing-thawing of tissues or by repeated washing of nuclei in buffer.

Previous analyses of unfrozen mouse liver nuclei revealed that concentrations of copper approached that of zinc, although copper concentrations were found to be more variable than were zinc concentrations (2). Nuclei isolated from frozen calf thymus were found to contain only about one fourth to one fifth as much copper as zinc. Moreover, this level of copper (approximately 0.025 μg Cu/mg DNA) remained tightly bound to chromatin through three extractions in nucleosome buffer (Fig. 1). Such a low concentration of copper in washed calf thymus nuclei made subsequent attempts at subnuclear localization of the metal difficult, and in many cases concentrations of copper were below instrumental detection limits.

RELEASE of ZINC from EXTRACTED NUCLEI DURING DIGESTION. After two extractions in nuclei wash buffer, nuclear pellets were resuspended in digestion buffer (0.25 M sucrose, 15 mM Tris, 15 mM NaCl, 60 mM KCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, pH 8.0) and were treated with micrococcal nuclease (.059 units (Sigma) per A_{260} unit) at 37°C. At the end of timed incubations, as shown in Fig. 2a, digestion was terminated by transfer to an ice bath without addition of any chelating reagent. Insoluble chromatin was pelleted by centrifuging at 12,000xg for 15 min,

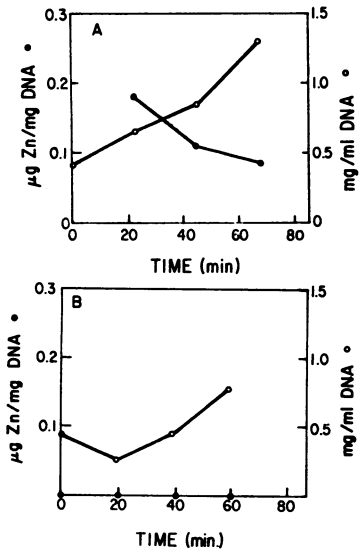

Figure 2a, b. Release of zinc and DNA from chromatin during digestion (A) with micrococcal nuclease and (B) without micrococcal nuclease.

and the resulting supernatant (soluble chromatin) was analyzed for metal and DNA prior to dialysis.

Upon nuclease digestion, zinc was rapidly released into the soluble chromatin fraction, and no further release of zinc into the soluble chromatin fraction occurred after the 22 minute digestion time noted in Fig. 2. Accordingly, the maximum ratio of Zn/DNA occurs at the earliest digestion time of this experiment (Fig. 2a), although solubilization of chromatin continues with digestion. The control experiment (Fig. 2b, without nuclease) showed no partitioning of zinc into solubilized chromatin, even though some chromatin solubilization (presumably from endogenous enzymes) was apparent. In order to minimize endogeneous enzymatic activity, subsequent digestions were performed for 22 minutes.

REMOVAL of ZINC by DIALYSIS AFTER DIGESTIONS. Zinc that is solubilized by micrococcal nuclease is partially dialyzable with or without EDTA, while zinc bound to undigested chromatin is nondialyzable under identical conditions. The time course for metal removal from both soluble and insoluble chromatin following a 22 min digestion, is given in Fig. 3A and B. Dialyses against 100 volumes of either Tris-EDTA or Tris without EDTA shows that more Zn/DNA remains in the soluble chromatin when EDTA is used, while more Zn/DNA remains in insoluble chromatin when EDTA is omitted. Concentrations of zinc in both chelated and non-chelated

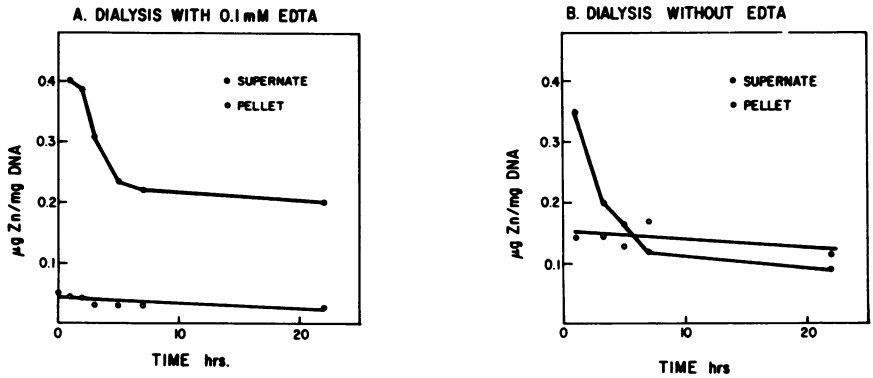

Figure 3a, b. Removal of zinc from digested chromatin during dialysis (A) against chelating buffer (0.1 mM EDTA, 10 mM Tris, 1 mM PMSF, pH 7.5) and (B) against non-chelating buffer (10 mM Tris, 1 mM PMSF, pH 7.5).

insoluble chromatin remained fairly constant throughout dialysis, while metal concentrations in the soluble chromatin fraction decreased sharply before appearing to reach a steady state after about 8 hours. Soluble chromatin fractions subsequently analyzed by gel chromatography were subjected to a 3 hour dialysis.

A-5m COLUMN METAL PROFILES. Chromatin was digested with micrococcal nuclease (35%) solubilized by dialysis against Tris-EDTA and the supernatant loaded onto a gel filtration column. The absorbance (A_{254}) profile of an A-5m column effluent (shown in Fig. 4A) is typical of nuclease digested chelated chromatin and agrees with profiles previously published (15). We identified mono- and oligonucleosomal peaks in this column run by electrophoretic analysis of both their protein and DNA constituents (data not presented). We focus in the present paper on the metal composition of the peaks in this profile. When EDTA is used in nucleosome isolation procedures, zinc is readily detected with low molecular weight products, eluting with the C peak (Fig. 4A). Under these conditions, no metal can be detected with the oligonucleosome fractions (peak A) or with mononucleosome fractions (peak B). Peak C, which contains undetectable protein or RNA (< 10 μg/ml), primarily contains non-dialyzable oligonucleotides. Zinc is likely bound to these nucleotides and/or to EDTA which also elutes with the C peak. Copper could not be detected (by flame AA) in dialyzed digested chromatin when chelating agents were used.

Omitting chelating agents from solubilization procedures and from column buffers yields an elution profile in which a new peak (B2) appears (Fig. 4B). It is of special interest to note that trace levels of zinc can now be detected in association with the leading edge of this B2 peak, and that a well resolved zinc peak coincides with the A peak (Fig. 4B).

The profiles in Fig. 4A and 4B were obtained from 22 min digestions at 37°C where the unit activity of enzyme to DNA concentration was the same (.059 units of enzyme/A_{260} units of DNA), although the chromatin concentration was increased. In a similar high concentration digestion copper as well as zinc concentrations were assessed by flame AA of the gel chromatography fractions shown in Fig. 5. In this particular experiment, the soluble chromatin fraction contained 35% of the total A_{260} units; 10 ml of the dialyzed supernatant, containing 0.23 μg Zn and 0.027 μg Cu per mg of DNA, was used to generate the profile in Fig. 5.

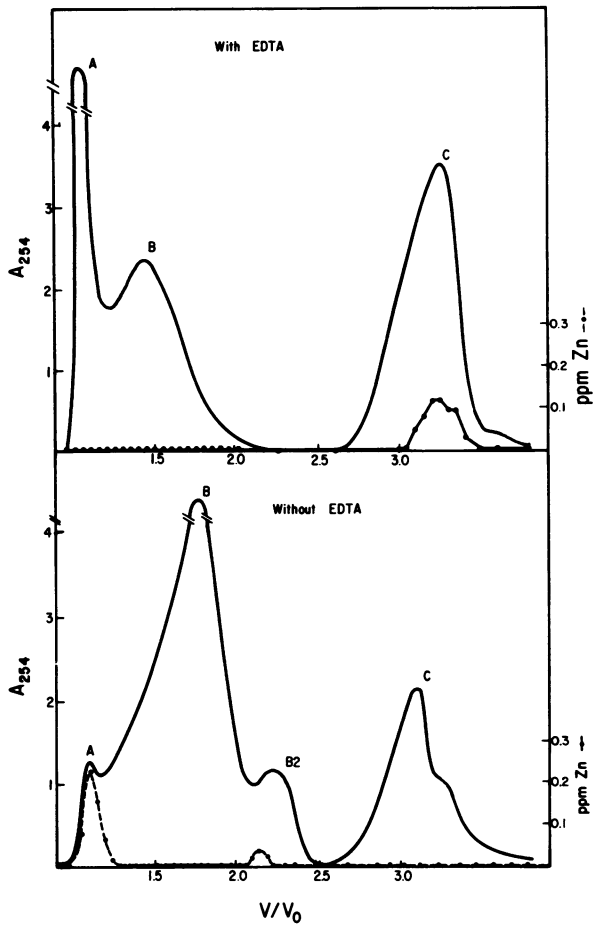

Figure 4a, b. A5 m Metal profile of chromatin digested and solubilized, (A) with EDTA, or (B) without EDTA. (Metal analysis by flame A.A.).

In this experiment, recoveries of metal following gel chromatography were calculated to be 98% for zinc and 85% for copper; 56% of the zinc and essentially all of the copper (detected by flame AA) were associated with the A peak. However, the limit of copper detection by flame AA is not sufficient to draw any solid conclusions about its distribution among chromatin peaks. Both metals can be measured with better resolution by graphite furnace flameless AA.

The elution profile in Fig. 6 is from a 16 hr digestion at 4°C

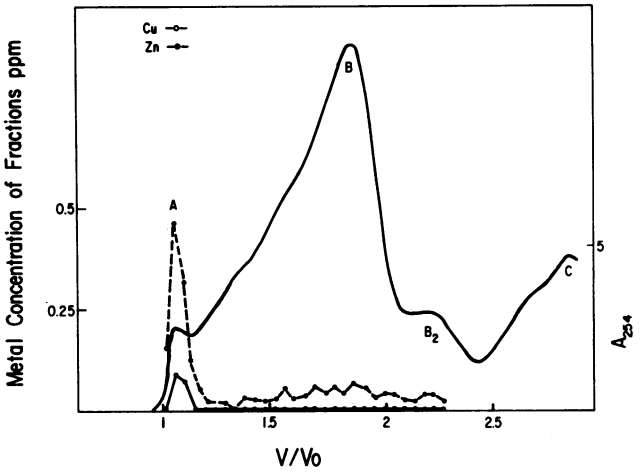

Figure 5. A-5m Metal profile of concentrated chromatin (16.2 mg/ml DNA), digested at 37°C for 22 min. (Metal analysis by flame A.A.)

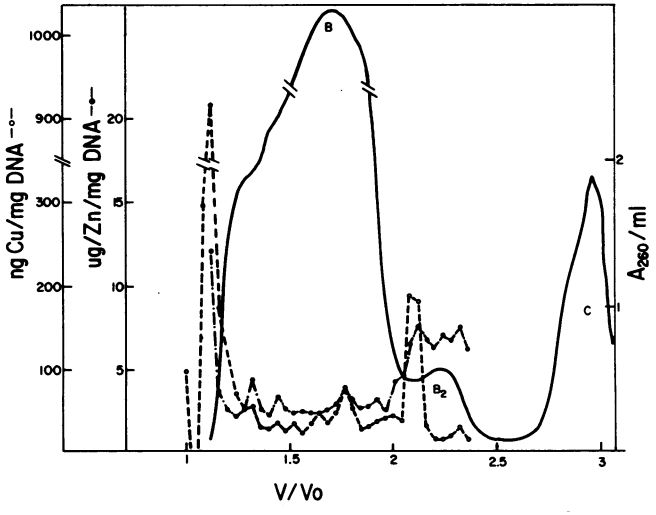

Figure 6. A-5m Metal profile of chromatin (13.0 mg/ml DNA) digested at 4°C for 16 hrs. (Metal analysis by flameless A.A.)

under conditions found to be comparable to those of concentrated DNA digestions at 37°C. (Digestion at 4°for 16 hr solubilizes approximately two thirds as much chromatin as the 22 min 37°C digestion employed earlier.) Metals were analyzed by the graphite furnace technique and expressed as μg metal/mg DNA. Suggested metal peaks now seem to parallel mono- and perhaps oligo-nucleosome peaks in addition to well resolved metal peaks which coincide with peak A (Fig. 6). The striking feature of the data in Fig. 6, however, is the appearance of a very specific Copper-rich species near the leading edge of the B2 peak. Again, zinc is elevated in this region but it appears to be binding less specifically to a particular nucleoprotein than is copper. The sharp copper peak was reproduced on two additional columns, one of which was from a P-150 (BIO-RAD) column run of sample re-isolated from an A-5m column

PARTITIONING of METALS with NUCLEOPROTEINS. In the absence of EDTA, about 1.5 μg Zn/mg DNA remains with the precipitable chromatin even after extensive dialysis. Of the total zinc released into the solubilized fraction upon digestion, over 50% of the metal (following 3 hr dialysis) is associated with precipitable nucleoprotein and a small amount is localized with B2 nucleoprotein. This B2 nucleoprotein fraction is being further characterized and results will be presented in a subsequent publication. Preliminary data suggests that the peak represents a part of the internucleosome linker region. If zinc and other metals are attached to these regions, then one would expect the higher molecular weight nucleoprotein of peak A (oligonucleosomes connected by linker regions) to be enriched in metal. Indeed, this is what is observed; however, other types of nucleoprotein-metal-complexes may be associated with the A peak.

When the digestion mixture is dialyzed against buffer containing EDTA, approximately two thirds of the zinc which resides in the nonchelated precipitable chromatin partitions into solubilized material (Fig. 3). None of this solubilized metal can be detected with high molecular weight nucleoprotein (Fig. 4A), which indicates effective chelation. In this chelated condition, the B2 peak doesn't appear in the elution profile, suggesting that divalent cations, such as zinc and copper, are needed to maintain the structural integrity of the B2 fractions. Preliminary experiments indicate that the addition of Ca^{++} and Mg^{++} to digested chromatin that had been treated with EDTA results

in a gel filtration profile similar to experiments where EDTA was omitted. This possibly indicates reconstitution of the B2 peak.

SUMMARY AND CONCLUSIONS

Digestion of calf thymus nuclei with micrococcal nuclease yielded soluble nucleoproteins containing zinc and copper. Metal enriched regions of chromatin, especially in the case of zinc, appeared to be preferentially solubilized by digestion, and the solubilized metal was only partially dialyzable either with or without EDTA. Zinc bound to insoluble chromatin is non-dialyzable under similar conditions. Analysis by gel chromatography revealed that metal profiles for chelated and non-chelated solubilized chromatin are distinctive: zinc is associated with low molecular weight-digestion products when chelating agents are used, and in this case, the B2 peak is not seen in the A-5m chromatography profile. In contrast, when chelating agents are omitted, both zinc and copper are detected with higher molecular weight oligonucleosomes and there is localization of metal in that region of the A-5m column effluent where a clearly resolved new peak (B2) appears. Of particular interest is what appears to be a discrete Cu-rich species in the B2 region of this profile, which suggests a subset of Cu-rich nucleoprotein complexes.

Alternatively, the finding of 0.025 μg/mg DNA of tightly bound metal would roughly correspond to one copper atom per pentanucleosome, and one could speculate that copper might play a role in higher levels of chromatin organization. In support of this possibility are earlier studies, showing the location of copper in both condensed and non-condensed chromatin (1, 2). In both in-vivo and in-vitro experiments, the state of chromatin condensation was critical and the chromatin binding selectivity of copper appeared to arise from protein or protein-DNA associations.

Likewise, the localization of zinc in chromatin is entirely consistent with known requirements for this metal in a number of nuclear processes. There is an undisputed link between zinc and enzymatic activity in both DNA dependent and RNA dependent DNA polymerases; these enzymes are zinc containing and are tightly bound metalloproteins (17-19). Furthermore, zinc is essential in the cell cycle, (20) and has been associated with spindle formation (21) and chromosome structure (4). In fact, zinc is known to participate in most of the steps of cell division and of nucleic acid metabolism (20).

Since micrococcal nuclease preferentially excises nucleosomes from transcriptionally active regions of chromatin, divalent metals which localize with this region could become useful probes for further studies of non-histone proteins, DNA and possibly other constituents of active chromatin.

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