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Mammalian chromatin was exposed to either Deoxyribonuclease I or Micrococcal Nuclease digestion as a function of time of incubation and enzyme concentration. Endonuclease enzymatic reactions were stopped with EDTA. Samples were run in 1.5% agarose gels and the oligonucleosomal electrophoretic migration patterns compared. Endonuclease experiments were carried out with rat liver chromatin pre-incubated in the presence or absence of 200 μM βNAD^+. A solution of 1.0 mM benzamide was used to stop enzymatic modification. The electrophoretic observations demonstrated a faster and increased degradation of chromatin when proteins were poly(ADP-ribosyl)ated prior to digestion. These results support the hypothesis that the covalent poly(ADP-ribosyl)ation of chromatin proteins, particularly histones, induces a more relaxed structure, rendering chromatin more sensitive to endonuclease digestion.

CHANGES IN MAMMALIAN CHROMATIN STRUCTURE AS A FUNCTION OF PROTEIN-POLY(ADP-RIBOSYL)ATION BY ENDONUCLEASE DIGESTION

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LIST OF ABBREVIATIONS

ADP-ribose	adenosine diphosphate ribose
Arg	arginine
AT	adenine-thymine
AU	adenine-uracyl
bp	base pairs
βNAD^{+}	β-Nicotinamide adenine dinucleotide
°C	degree celsius
Ca^{+2}	calcium ion
CME's	Ca^{+2}/Mg^{+2} dependent endonuclease
dH_2O	deionized water
DNase I	deoxyribonuclease I
DNA	deoxyribonucleic acid
EDTA	ethylene-diamine-tetracetic acid
EGTA	ethylene glycol bis(2-aminoethylether)-N-N' tetra acetic acid
Lys	lysine
Mg^{+2}	magnesium ion
$MgCl_2$	magnesium chloride
Mn^{+2}	manganese ion
MNase I	Micrococcal Nuclease
nm	nanometer

PARG	poly(adenosine diphosphate ribose) glycohydrolase
PARP-1	poly(adenosine diphosphate ribose) polymerase-1
RLC	rat liver chromatin
RNA	ribonucleic acid
rpm	revolutions per minute
room T	room temperature
TB	Tris-Boric Acid
TBE	Tris-Boric Acid-EDTA
μM	micromolar
mM	millimolar
w/v	weight/volume
3'-OH	3' hydroxyl

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INTRODUCTION

The major difference between eukaryotic and prokaryotic cells is the presence of a nucleus, a separate compartment which contains the genetic material (DNA) isolated from the rest of the cell. Due to its great size (6×10^9 nucleotide pairs for humans), the DNA must be organized in some way to fit inside the nucleus and, at the same time, be protected from degradation or be accessible to the different proteins and factors involved in all the nuclear events occurring during the cell cycle. The way the eukaryotic cell does this is by packing the chromatin into different levels, ranging from loose to tightly condensed forms.

CHROMATIN

Chromatin is defined as a filamentous complex of DNA, histones, and other proteins, constituting the eukaryotic chromosome (Nelson, 2000). The basic repeating unit of packing the chromatin is called a nucleosome. The first evidence of the presence of a small substructure repeat that formed the chromatin came from studies by Hewish and Burgoyne (Hewish, 1973a), where they used an endogenous rat liver, Ca^{+2}/Mg^{+2} endonuclease that degraded chromatin, giving a series of bands that increased in molecular weight and that were multiples of the smallest band. Later, Kornberg proposed the model of the nucleosome. He defined it as a repeating unit of 2 each main type of histone and about 200 base pairs (bp) of DNA (Kornberg, 1974a). Noll *et al.* presented evidence that at least 87% of the chromatin could be converted to monomers of

about 205 bp (Noll, 1974), and fragments of higher molecular weight that were integral multiples of the monomer: 405 (dimer), 605 (trimer), etc. This evidence confirmed Kornberg's chromatin structure model.

Histones are basic proteins with a high content of the positively charged amino acids lysine (Lys) and arginine (Arg) that stabilize the DNA by interacting with the negative phosphate backbone, making the folding and compaction of DNA possible. There are at least five main types of histones in eukaryotes: H2A, H2B, H3, H4, and H1 (H5 in chicken erythrocytes). Two copies of each H2A, H2B, H3, and H4, make the octamer core of the nucleosome. These histones are very well conserved through evolution, which indicates the importance of their role for chromatin structure. Histones H3 and H4 are the most conserved of all core histones. They form a very stable tetramer (Kornberg, 1974b) that makes the center of the core. On the other hand, histones H2A and H2B make two separate dimers (Kelly, 1973) that lay at the periphery of the core on each face of the H3-H4 tetramer.

Approximately 146 base pairs (Prunnel, 1979) of DNA wind tightly around the histone core, in a 1.75 left handed turn, forming what is termed the *nucleosomal core particle*. Between the nucleosome core particles there is an additional fragment of DNA of 50-60 bp. This is the linker DNA that keeps nucleosomes together in the same fiber, and this DNA is the most accessible and susceptible to enzymatic digestion. The fiber structure formed by the nucleosomes and the linker DNA is the first level of compaction of chromatin, and is called the "beads-on-a-string" fiber (Olins, 1974) or the 10 nm fiber. Studies from different laboratories show a linker histone, H1, which attaches to the linker

DNA, on the same side where the DNA enters and leaves the nucleosome stabilizing nucleosomal structure (Thoma, 1979; Noll, 1977). These studies also demonstrated that H1 is necessary for the formation of a more condensed structure of the DNA: the solenoidal (30 nm) or the 300-angstroms form (Finch, 1976). In this report, chromatin was submitted to changes in ionic strength that provoked dramatic structural change. At high ionic strengths, in chromatin containing H1, an increased condensation of the fiber was observed, giving a zigzag-like structure, while in the H1-depleted chromatin the same degree of compaction was not achieved. At low ionic strength, the presence of H1 seemed to be necessary for condensation. H1 depleted nucleosomes were less stable than those containing H1, and only the "beads-on-a-string" structure was present (Thoma, 1979).

As mentioned before, chromatin must go through different levels of packing in order to fit inside the nucleus. After the 10 nm fiber, there is the 30 nm fiber, which comprises 6 nucleosomes per turn, giving a compaction of the DNA of approximately 30 to 40 fold (Gasser, 1987). But other higher levels of folding must occur to compress chromatin to the highest condensed form: the metaphase chromosome, which has a compaction of about 10,000 X (Gasser, 1987). The less condensed forms of chromatin are known as euchromatin, which is considered to be transcriptionally active chromatin because it is where most of the active genes are found. On the other hand, those areas where chromatin is more condensed are called heterochromatin. This is the "silenced" or transcriptionally inactive chromatin because it is mostly, but not exclusively, present in those areas where there are silent genes, like centromeres and telomeres. How chromatin

folds from the 30 nm euchromatic fiber into the different condensed forms of heterochromatin is not yet well understood. It is suggested that the fiber folds into loops that are kept together and anchored to a non-histone protein scaffold (Gasser, 1987). Also, interactions between modified histones and linker histone variants with other non-histone proteins, and the ionicity of the environment (Horn, 2002), could dictate further degrees of condensation up to the metaphase chromosome, and stabilize them depending on the stage of the cell cycle at a particular moment.

These transitions between the condensed and relaxed forms of chromatin are necessary for the different nuclear processes to occur, allowing the access of factors, proteins, and endonucleases involved, and at the same time, blocking their interaction with chromatin according to the cell's needs.

ENDONUCLEASES

An endonuclease is an enzyme that cleaves a nucleic acid (DNA or RNA) at specific internal sites in the nucleotide base sequence. Therefore, most nuclear processes require endonucleases at some point. Because of their function on cleaving nucleic acids, they can be very useful to study the structure, interactions and modifications that either DNA or RNA undergo. Two of the most used endonucleases to study DNA structure, especially at the nucleosomal level, are Deoxyribonuclease I and Micrococcal Nuclease.

Deoxyribonuclease I (DNase I) was isolated for the first time by Kunitz in 1948 from bovine pancreas (Kunitz, 1948). The endonuclease has a molecular weight of 31,000 Dalton and an optimum working pH of 7.8. It is activated by divalent cations like Mg^{+2}, Ca^{+2}, and Mn^{+2}, and can be inhibited by chelating agents like EDTA. The enzyme does not seem to have sequence specificity for cleavage, but appears to have preference for areas of exposed DNA, like it occurs in the double helix minor groove (Drew, 1987). It was first believed that DNase I cut the DNA in multiples of 10 nucleotides, in accordance with the packing of DNA around the nucleosome (Noll, 1974; Drew, 1987). Later it was determined that the cleavage sites could be located at intervals of 10.4 bases instead of 10; this finding established the periodicity of the double helix around the core (Lutter, 1979; Prunell, 1979). More recent studies have shown that digestion by DNase I is dependent on the protection provided by core and linker histones, and by DNA-protein interactions that occur in higher order chromatin, which condenses the DNA at an extent that the enzyme cannot access any cleavage site (Staynov, 2000). According to these studies, DNase I cuts nucleosomal DNA in a single-stranded mode, depending on which of the strands is more exposed (Staynov, 2000; Cousins, 2004). DNase I splits the phosphodiester linkages with an average stagger of 4 nucleotides to produce 5'-phosphate terminated polynucleotides with free 3'-OH group (Cousins, 2004). Also, as result of these studies it seems that in high molecular weight chromatin and polynucleosomes, the linker DNA is inside of the fiber, well protected from nuclease attack by the increased condensed state of higher order structures (Staynov 1998, 2000). This statement is supported after patterns of digestion of the DNase I in short oligonucleosomes and linker

histone-depleted DNA, showed bands of 10 base repeats and a background or "smear" above 140 nucleotides that disappears gradually with increased time of DNase I digestion, suggesting that the enzyme also degrades linker DNA when it is more open, as it happens in low molecular weight and linker histone free DNA (Staynov, 1998, 2000).

A key protagonist in the elucidation of the nucleosome structure and basic organization of chromatin is the endonuclease Micrococcal Nuclease (MNase) [EC 3.1.31.1], discovered in 1956 by Cunningham in *Staphylococcus aureus* cultures (Cunningham, 1956). The enzyme showed unusual characteristics not found in other deoxyribonucleases at that moment, which made it suitable to be exploited in studies related to chromatin structure. These characteristics were its high thermostability that allowed an easier isolation and purification, and its specificity (Cunningham, 1956). The enzyme is composed of 159 amino acid residues and has a molecular mass of 16,800 Dalton (Telford and Stewart, 1989). In addition to its deoxyribonuclease activity, the enzyme can also digest RNA (Alexander, 1961), although a higher affinity for DNA is observed (Cuatrecasas, 1967). Cleavage occurs preferentially at AT and AU rich regions (Cuatrecasas, 1967). According to some studies, MNase has an absolute requirement of Ca^{+2} for activation (Cunningham, 1956; Alexander, 1961; Heins, 1967), although strontium also induced the DNase activity (Cuatrecasas 1967). Magnesium also stimulated DNase activity but only when Ca^{+2} was also present (Cuatrecasas, 1967). Activity can be inhibited by chelating agents EDTA (Heins, 1967) or Ca^{+2} specific EGTA (Telford, 1989). The optimum pH found on Cunningham experiments is of 8.6, but other studies show maximum activity for DNA and RNA digestion at pH of 9.2 (Heins, 1967)

and 10.3 for calf thymus DNA that has been previously heated at 100 °C (Alexander, 1961). It hydrolyzes the 5'-phosphodiester bond of deoxyribonucleotides to produce oligonucleosomal fragments and mononucleotides with terminal 3'-phosphate and 5'-hydroxyl group (Cunningham, 1956). MNase appears to have both endonuclease and exonuclease activities (Alexander, 1961). At first, the endonuclease cuts in a single-stranded way, cutting the second strand next. Exonuclease activity can be seen at the last steps of the digestion to give the final oligonucleosomal and mononucleotide fragments (Cuatrecasas, 1967). The most important characteristic of Micrococcal Nuclease is its specificity to cleave the linker DNA regions, between nucleosomes (Noll and Kornberg, 1977). It is this property that has made the enzyme so useful in all kind of studies related to structure, modifications, interactions and processes of chromatin. MNase attacks the linker DNA initially producing fragments of approximately 200 bp and continues digesting the DNA up to the nucleosome core (Noll and Kornberg, 1977). This mode of action is very similar to a Ca^{+2}/Mg^{+2} dependent endonuclease found in rat liver chromatin (Hewish and Burgoyne, 1973 b) that was used before MNase to study chromatin and that together led to the discovery of the basic structural unit of chromatin, the nucleosome, and the localization of histone H1, which set the bases for our current understanding on chromatin compaction and the processes where it is involved, i.e., transcription, replication, DNA repair, etc. (Noll, 1974; Noll, 1977; Telford, 1989).

The modulation of chromatin structure necessary for nuclear processes to occur is possible in great deal as consequence of different post-translational modifications of the proteins interacting with chromatin.

Chromatin structure is dynamic, undergoing continuous transition between the condensed and relaxed forms. These transitions are possible due to chemical modifications of some proteins, especially histones. The reversibility of these modifications suggests their involvement in the modulation of chromatin structure necessary for gene expression. These modifications occur mostly at the N-terminal tails of histones by the addition of a moiety to the free group of some amino acids. Among the most studied of these post-translational modifications are acetylation, methylation, phosphorylation, and poly(ADP-ribosyl)ation.

Acetylation consists in the transfer of an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-amino group of lysine residues in core histones (Ausio, 2001). Linker histone H1 and core histone H3 are preferred targets for phosphorylation, especially in serine and threonine residues (Ausio, 2001; Wolffe, 2000). Methyl groups are added to specific lysine and arginine residues in histones H3, H4, and H2B mostly (Ausio, 2001). Poly(ADP-ribosyl)ation of proteins on the other hand is different to the modifications mentioned above and involves the addition of a much bigger moiety, therefore, it is expected for this modification to influence at a higher extent the structural state of chromatin.

POLY(ADP-RIBOSE) METABOLISM

Poly(ADP-ribosyl)ation consists in the addition to the acceptor protein, of a polymer of ADP-ribose units that result from the utilization of βNAD^+ as a substrate. The main enzyme that catalyzes this reaction is PARP-1, or poly(adenosine diphosphate

ribose) polymerase-1 (EC 2.4.2.30), an enzyme present in most eukaryotic nuclei, tightly bound to chromatin and highly conserved through evolution.

The synthesis of the polymer occurs in three steps: initiation, elongation, and branching. The initiation step is a mono(ADP-ribosyl)ation, where an ADP-ribose unit is covalently attached to the acceptor protein through an ester linkage between C1 of the ribose and the carboxylate free group of a glutamic acid, or less frequently, aspartic acid of the acceptor protein. Elongation consists in the attachment of additional ADP-ribose units through a 1"→2' ribose-ribose linkage to form a linear polymer of approximately 200 units in vitro (Alvarez-Gonzalez and Jacobson, 1987). Finally, branching is the side's extension from the linear polymer, where there is an average of 6 branches per each molecule of 200 units (Alvarez-Gonzalez and Jacobson, 1987). The constitutive formation of this polymer is relatively low, but in the presence of DNA strand breaks the reaction can be activated (D'Amours, 1999). The final product of this three-step reaction is a highly negative charged polymer which can add a considerable molecular mass to the modified protein. Nevertheless, the half-life of the poly(ADP-ribose) chain is very short, and it seems to be dependent on the size of the polymer: the larger it is, the faster is degraded (Alvarez-Gonzalez and Althaus, 1989). The reason for this rapid turnover is the decrease in catalytic activity of the poly(ADP-ribose) polymerase as a result of automodification, which detaches it from the DNA due to the repulsion between the negative charges on the phosphate backbone in DNA and the polyanionic polymer on the enzyme (Zahradka, 1982; Ferro, 1982). The polymers attached to the acceptor protein are then more easily and rapidly degraded by a glycohydrolase present in the cell nucleus,

which has both exo- and endonuclease activity, and cuts the glycosidic bonds of the polymer, up to the last unit. This enzyme is poly(ADP-ribose) glycohydrolase or PARG . The last step for the complete reversibility in the modification of the protein is the removal of the last ADP-ribose moiety which is catalyzed by an ADP-ribosyl protein lyase.

Many nuclear proteins can be targets for poly(ADP-ribosyl)ation. Extensive studies have shown that the primary protein acceptor is the poly(ADP-ribose) polymerase itself (Jump, 1980; Ferro, 1982) followed by histone proteins, responsible for folding and compaction of chromatin. Poirier *et al.* (Poirier, et al., 1982) used electron microscopy and ultracentrifugation to show that poly(ADP-ribosyl)ation was responsible for the relaxation of chromatin even under conditions of high ionic strength, when native chromatin tends to condense into the solenoidal form (Thoma, 1979). Few years later, De Murcia *et al.* (De Murcia, et al., 1986) decided to study the catabolism of the ADP-ribose polymer and its effect on chromatin structure. As it was expected, degradation of the polymer returned chromatin to a condensed state. The work of Poirier and De Murcia demonstrated that relaxation of chromatin was consequence of hyper(ADP-ribosyl)ated histone H1, and the reversed reaction a result of the glycohydrolase (PARG) catalytic activity on the ADP-ribose polymer. Also, they were able to show that it was H1 the main histone acceptor of poly(ADP-ribose) followed by core histone H2B.

Additional work gave further evidence that supported Poirier and De Murcia findings. The appearance of relaxed regions of chromatin after poly(ADP-ribosyl)ation by the endogenous poly(ADP-ribose) polymerase was observed at high concentrations of

βNAD^+, showing hyper(ADP-ribosyl)ation of histone H1 once more (Aubin, 1983). Huletsky *et al* (Huletsky, 1989) compared the effect of poly(ADP-ribosyl)ation on H1-depleted and native chromatin and once again H1 resulted the main histone acceptor in native chromatin, while in H1-depleted were the core histones, especially H2B. Also, when trying to return the poly(ADP-ribosyl)ated H1-depleted chromatin to the condensed form by the addition of exogenous histone H1, it was not possible, opposite to poly(ADP-ribosyl)ated native chromatin. It seemed possible that the polymers on the modified core histones could be blocking the site where H1 binds in DNA (Huletsky, 1989). This result in particular showed a very strong influence of poly(ADP-ribosyl)ation on chromatin structure modulation. On the other hand, work from the Althaus lab *et al* (Panzeter, 1992) showed that noncovalent interactions between histones and polymers of ADP-ribose exist, H1 being the major polymer–binding protein and then the core histones. Interesting to mention is the fact that only the carboxyl terminal domain of H1 bound to the polymer, which happens to be the same domain highly involved in the formation of higher-order structures of chromatin. All the histones showed preference for the branched polymers, then long linear, and finally short linear. Histones seemed to protect these polymers from degradation by poly(ADP-ribose) glycohydrolase, being the linear chains the easiest ones to cut. These noncovalent interactions lead Althaus to propose a histone shuttling mechanism that could be a mechanism for the transient detachment and re-association of histones from DNA during nuclear processes.

RESEARCH PROSPECTUS

With the evidence provided by De Murcia, Poirier, Althaus and other labs, it can be proposed that poly(ADP-ribosyl)ation modulates chromatin structure, and that this may regulate DNA-protein interactions which facilitate the assembly of factor complexes involved in DNA metabolism and the accessibility of the different enzymes to chromatin.

Different to the molecular biology approach of electron microscopy used by the investigators mentioned above, my work, as described here, biochemically shows the influence of poly(ADP-ribosyl)ation on chromatin structure modulation by means of its susceptibility to DNase I and MNase endonuclease digestion, as observed on the degradation patterns produced on agarose gel electrophoresis.

MATERIALS AND METHODS

Establishment of a chromatin nuclease digestion assay

Naked calf thymus DNA (Catalog No.D-1501, Sigma, St. Louis, MO) was dissolved in a solution of 20 mM $MgCl_2$ and 0.5X TB (Tris-Boric Acid) buffer. The DNA was subjected to digestion by Deoxyribonuclease I (DNase I, Catalog No. LS 006330, Worthington Biochemical Corporation, Freehold, NJ) at room temperature for 1, 5, 15, and 30 minutes. A control was also run, where no DNase was added. The digestion reactions were stopped at the respective times with gel loading buffer Type I (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose in water*) containing 2 mM (final concentration) EDTA. Fifty microliters from the samples (100 µL final volume) were run in a 1% agarose gel (w/v in 0.5X TBE buffer), in a Bio-Rad DNA Sub Cell electrophoresis chamber, with 0.5X TBE electrophoretic running buffer at a constant voltage of 200 volts (Bio-Rad Power Pac 3000). The gel was stained overnight with a 0.5 µg/mL ethidium bromide solution, using a Junior Orbit Shaker (Lab-Line Instruments, Inc., Melrose Park, ILL.), at constant 100 rpm. The digestion patterns were observed at 312 nm UV Fisher Biotech/Fisher Scientific Variable Intensity Transilluminator. Photographs of the gels were taken using Polaroid 667 Instant Black & White Film and a CAMAG Reprostar II camera at 302 nm UV light.

The same conditions and protocol were followed using Micrococcal Nuclease (MNase, Catalog No. N-3755, Sigma, St. Louis, MO) instead of DNase I.

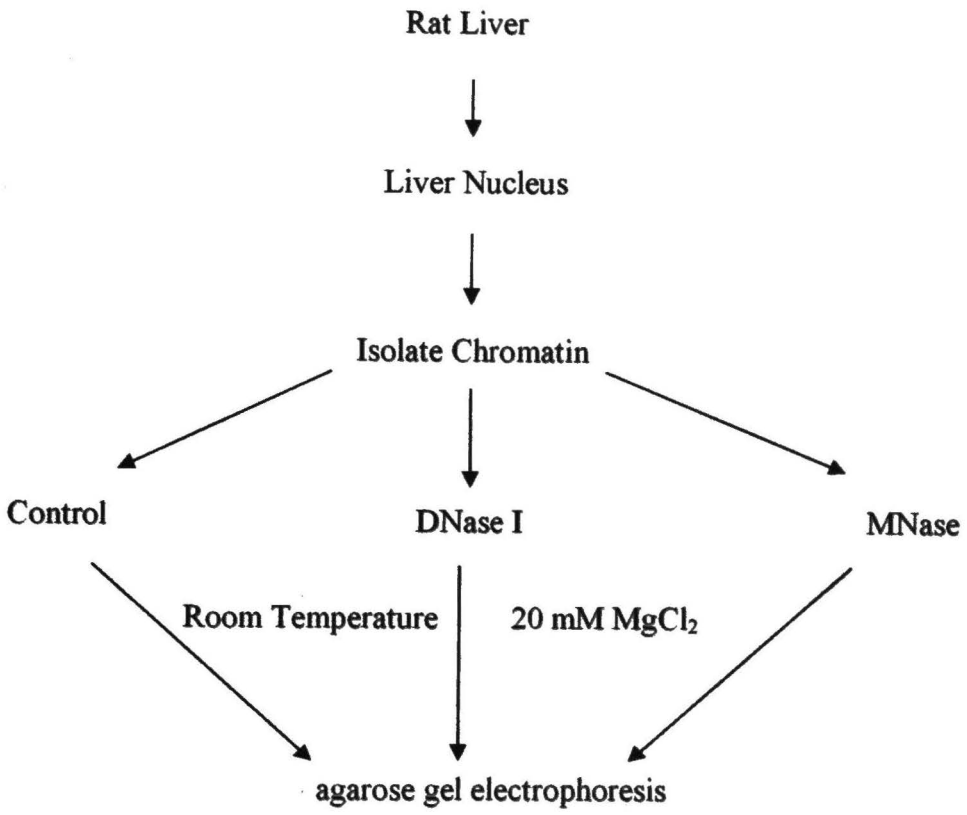
Agarose Gel Electrophoresis

The proper amount of agarose was weighed and dissolved in 0.5 X TBE (Tris-Boric Acid-EDTA) buffer, to give either a 1 % or a 1.5% (w/v) final concentrations. A 5X stock solution of buffer was previously prepared by dissolving 54 g Tris Base , 27.5 g Boric Acid, and 20 mL of 0.5 M EDTA (ethylene-diamine-tetracetic acid) at pH 8.0, to 1 L with deionized/distilled H_2O. From this stock, the 0.5X TBE buffer was prepared by dissolving in dH_2O, and used also as electrophoretic running buffer. The agarose was dissolved in the 0.5X TBE buffer by heating, and immediately poured in the gel tray. The electrophoretic chamber was filled with the 0.5 X TBE running buffer to cover the gel. Samples were loaded in their respective wells, the electrophoresis chamber was covered, and electrodes connected to the current generator to allow moving of the samples from the cathode (- charge) to the anode (+ charge). Constant voltage was set at 200 volts and allowed to run until the front loading dye reached approximately one inch before the end of the gel. Finally, the gel was removed and transferred to a tray with a 0.5 μg/mL ethidium bromide (in 0.5 X TBE), and stained overnight with gentle shaking.

Protocol for Enzymatic Degradation of DNA

Tube Number	Time of Incubation (minutes)	Time of Reaction (seconds)	Stop Reaction
1	0	0	0
2	1	90 ↑	2 min 30 sec ↓
3	5	60 ↑	6 min ↓
4	15	30 ↑	15 min 30 sec ↓
5	30	0 ↑	30 min ↓

Experimental Design for Chromatin Degradation



The same experiments were performed independently, varying the parameters of temperature and ionic strength, from room temperature to 37°C, and from 20 mM $MgCl_2$ to 75 mM $MgCl_2$, respectively.

Endonuclease Digestion of Poly(ADP-ribosyl)ated Chromatin

Rat liver chromatin was incubated at room temperature with a 200 μM solution of βNAD+ (β-Nicotinamide adenine dinucleotide, Catalog No. N-1511, Sigma, St. Louis, MO) (De Murcia, 1986). After 30 minutes of incubation, the poly(ADP-ribosyl)ation reaction was stopped with 1 mM benzamide (Catalog No. B-2009, Sigma, St. Louis, MO) solution (Rankin, et al., 1989). Immediately, the endonuclease (DNase I or MNase) was added to the poly(ADP-ribosyl)ated chromatin solution and allowed to digest the DNA according to the reaction times on the enzymatic degradation protocol. This time again, the enzymatic digestions were stopped with loading buffer containing EDTA.

An experiment of chromatin digestion with endonucleases (DNase I or MNase) in the absence of exogenous βNAD^+ was performed simultaneously, and run together with the previously poly(ADP-ribosyl)ated samples, in a 1.5% agarose gels. Three different controls were prepared and run in the same gel:

Control 1: fresh control (prepared immediately before running samples in electrophoretic gels); chromatin + TB + EDTA loading buffer

Control 2: no βNAD^+; chromatin + TB / 30 min incubation at room temperature; EDTA loading buffer

Control 3: with βNAD^+; chromatin + TB + βNAD^+ / 30 min. incubation at room temperature; benzamide / 30 min incubation; EDTA loading buffer

A DNA marker (Low Ranger DNA Marker, 100 bp-2000 bp, Fisher Bioreagents, Fisher Scientific FairLawn, NJ,) was used to compare migration of bands and estimate the sizes of oligonucleosomes produced after endonuclease digestion, both in absence or presence of exogenous βNAD^+. Electrophoresis was run at constant voltage of 200 volts, and the gel stained overnight with ethidium bromide solution.

RESULTS

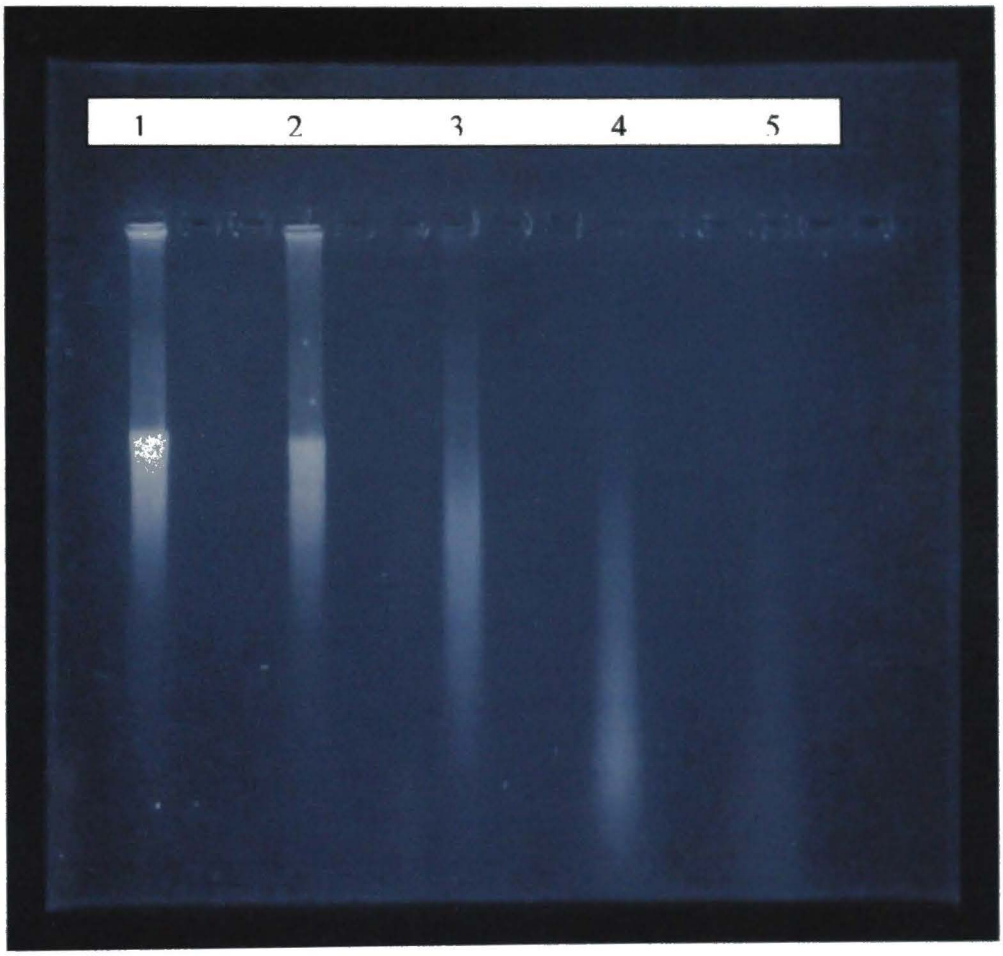
Determination of chromatin susceptibility to nuclease digestion by agarose gel electrophoresis

First, I established a suitable method to study the susceptibility of chromatin to endonuclease digestion. To do this calf thymus naked DNA was subjected to treatment with either DNase I or Micrococcal Nuclease and the patterns of polynucleosomal degradation produced were determined by agarose gel electrophoresis as described in the chapter of Materials and Methods.

In Figure 1, it can be seen that an increased degradation of chromosomal DNA occurred with increasing times of incubation with DNase I (Lanes 2-5) when compared to control DNA which was not subjected to the enzyme treatment (Lane 1). High molecular weight DNA can be observed as a band at the origin of the gel for the control sample as well as after only one minute of incubation with the enzyme (Lane 2). This band begun to disappear after 5 minutes of incubation (Lane 3) and smaller molecular weight smears resulted as digestion was continued for 30 minutes (Lanes 3-5). It is important to mention that although the control sample was not exposed to DNase, some degradation was still observed as a smear (Lane 1). Because some of the DNA is free of histone and non-histone proteins which provide some protection against nuclease attack, it is reasonable to expect that during the process of isolation of the DNA, and preparation of the sample, some degradation occurs. The pattern of digestion produced by MNase can be observed in Figure 2. Similarly to DNase I treatment (Fig. 1), the digestion of the DNA augmented as the incubation time with the enzyme was increased (Lanes 2-5). The

Figure 1: DNase I digestion of naked DNA. 1 % agarose gel electrophoresis of calf thymus naked DNA (20 μg/mL) digested with DNase I for 0 min (1), 1 min (2), 5 min (3), 15 min (4), and 30 min (5), at room temperature and 20 mM $MgCl_2$.

Figure 1



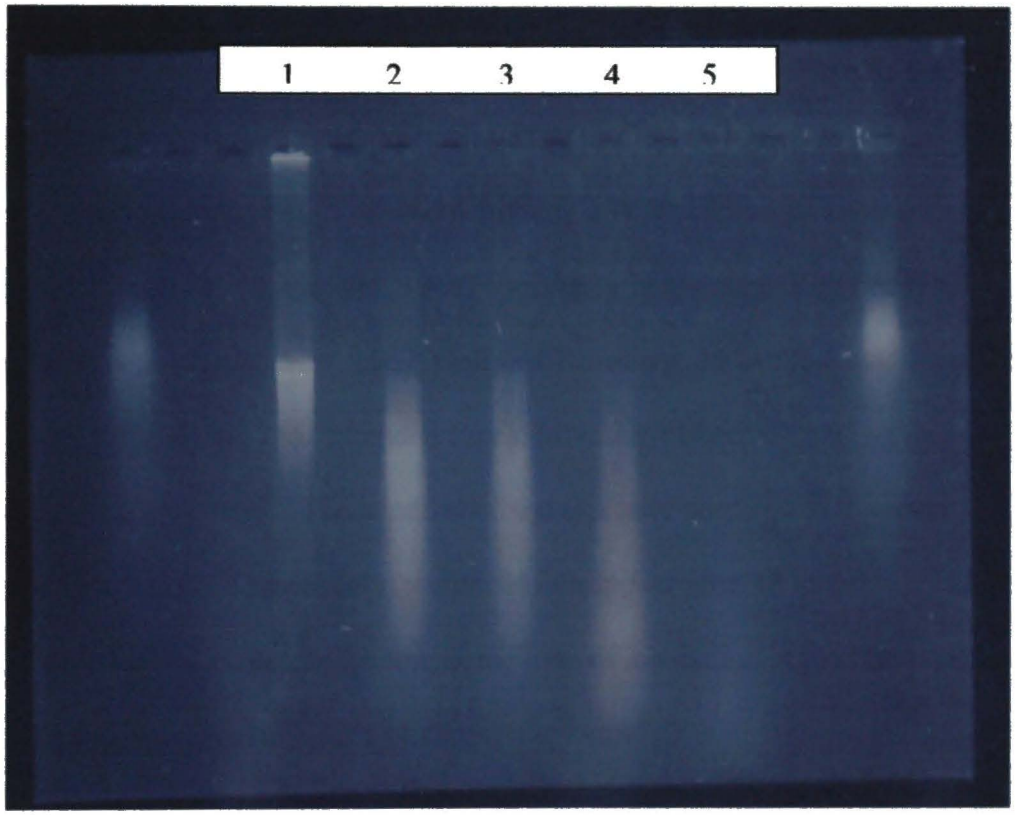
bands at the extremes of the gel on Figure 2 correspond to chromatin directly loaded on the gel (no EDTA, sucrose). These samples represented markers to compare the mobility of native chromatin in solution with chromatin subjected to enzymatic digestion. In these, experiments, MNase seems to easily digest the high molecular weight naked DNA as no trace of it was observed at the origin of the gel for any of the samples incubated. The band in the origin of the gel can also be observed for the control sample (Lane 1) as was the case with DNase I (Fig.1 Lane 1). However, this band completely disappeared after only 1 min incubation (Figure 2, Lane 2; compare with Fig.1 Lane 2).

Electrophoresis of chromatin degradation patterns following incubation with DNase I and MNase

After establishing agarose gel electrophoresis as a suitable method to study degradation patterns of DNA and chromosomal digestion kinetics of both DNase I and MNase, experiments were then performed with chromatin samples obtained from rat liver. In this case, chromatin was exposed to incubation times of 0, 1, 5, 15, and 30 minutes as detailed in a diagram at the section of Materials and Methods. Therefore, in order to further characterize this system, parameters of temperature and ionic strength were varied.

Figure 2: MNase digestion of naked DNA. 1 % agarose gel electrophoresis of calf thymus naked DNA (20 μg/mL) digested with MNase for 0 min (1), 1 min (2), 5 min (3), 15 min (4), and 30min (5), at room temperature and 20 mM $MgCl_2$.

Figure 2



Effect of low ionic strength (20 mM $MgCl_2$) at Room Temperature

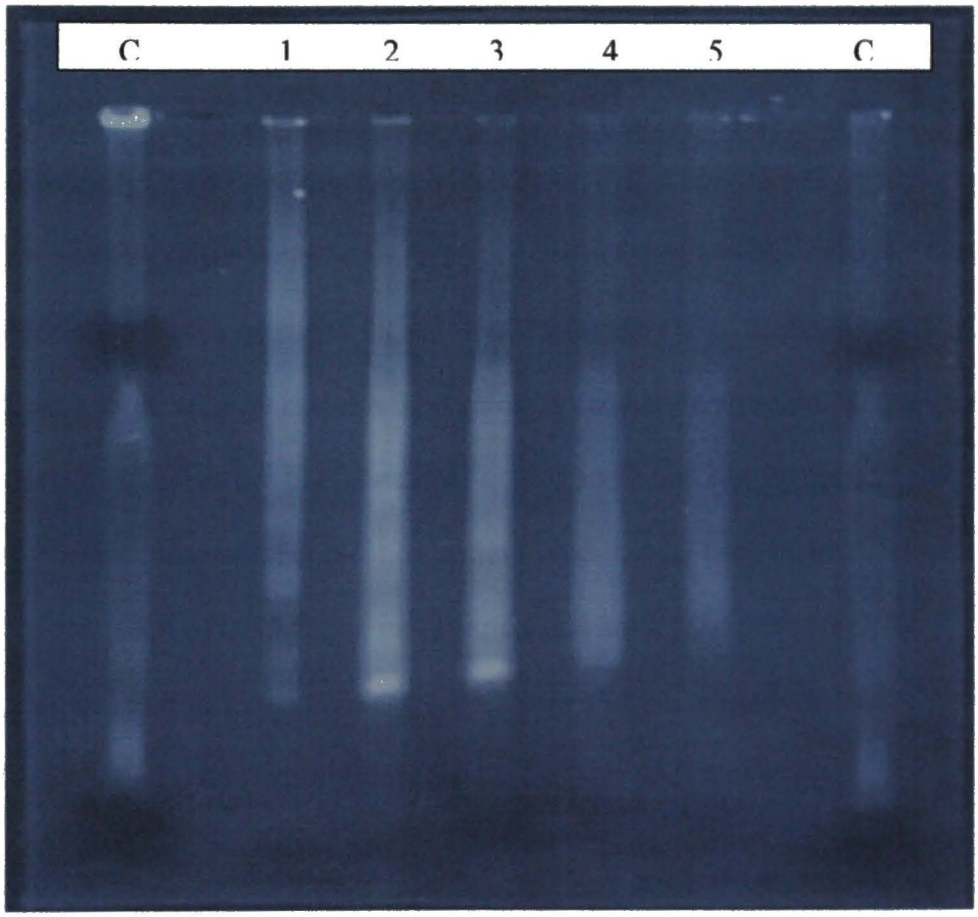
As it happened with naked DNA, the degradation of chromatin increased with time of endonuclease incubation (data not shown). The pattern produced by DNase I treatment appeared as a wide and diffuse single band that consistently became smaller and less intense as digestion proceeded. On the other hand, with MNase in Figure 3, at least three discrete bands could be distinguished against the smear background, on lanes corresponding to 1 and 5 minutes of incubation. Micrococcal nuclease is known to produce oligonucleosomal patterns because of its preference to cleave DNA in the linker region between nucleosomes (Noll and Kornberg, 1977). In fact, its subsequent exonucleolytic activity (Alexander, 1961) apparently keeps degrading free DNA up to the nucleosomal core (Cuatrecasas, 1967; Noll and Kornberg, 1977). It should be noted that if the digestion is allowed for extended times, MNase will keep degrading the nucleosomal DNA as well. The bands obtained typically correspond to mono-, di-, tri-, and tetranucleosomes, respectively, which also disappear after 15 minutes of digestion (Lanes 4 and 5). As expected, the longer the time of incubation in the presence of endonuclease, the more the extent of chromatin degradation.

Effect of low ionic strength (20 mM $MgCl_2$) at 37 °C

To test the influence of a higher temperature in the activity of chromatin degradation with both endonucleases, digestion kinetics was also performed at 37 °C instead of room temperature. As noted above, the ionic strength of the samples was kept the same, at

Figure 3: MNase digestion of chromatin. 1.5% agarose gel electrophoresis of rat liver chromatin (100 μg protein/mL) digested with MNase for 0 min (1), 1 min (2), 5 min (3), 15 min (4), and 30 min (5), at room temperature and 20 mM $MgCl_2$.

Figure 3



20 mM $MgCl_2$. Figures 4 and 5 show the digestion patterns produced by DNase I and MNase, respectively. For DNase I (Fig. 4), an unexpected digestion pattern resulted. The digestion seems almost identical as the one produced by Micrococcal Nuclease in Figure 5. In fact, for the first time, discrete bands corresponding to mononucleosomal DNA can be distinguished at levels that would correspond to oligonucleosomal fragments as observed also for MNase (Compare Fig. 4 and Fig. 5). Again, chromatin degradation consistently increased with the time of incubation for both DNase I and MNase. Once again, the chromatin appears to be degraded a little faster under MNase activity than for DNase I. If the intensity of the bands at the origin of the gels and the smears are compared, especially after an extended digestion of 15 minutes (Fig. 4, Lane 4 and Fig.5, Lane 4), and much more after 30 minutes incubation, it can be seen that for MNase there is almost no chromatin left in the gel (compare Fig. 4, Lane 5 and Fig. 5, Lane 5). The great similarity of the chromatin degradation pattern produced by both endonucleases can be explained as the result of DNA being more exposed at higher temperatures. Supposedly, this makes digestion at linker regions, where DNA is less protected, more effective. Consistent with this hypothesis is the well established notion that an endogenous endonuclease actually generates very similar patterns to those produced by MNase (Hewish and Burgoyne, 1973 a). Alternatively, the increased temperature of incubation could have played an important role in the activity of this endonuclease as well.

Figure 4: DNase digestion of chromatin at low ionic strength and 37 °C. 1.5% agarose gel electrophoresis of rat liver chromatin (100 μg protein/mL) digested with DNase I for 0 min (1), 1 min (2), 5 min (3), 15 min (4), and 30 min (5), at 37 °C and 20 mM $MgCl_2$.

Figure 4

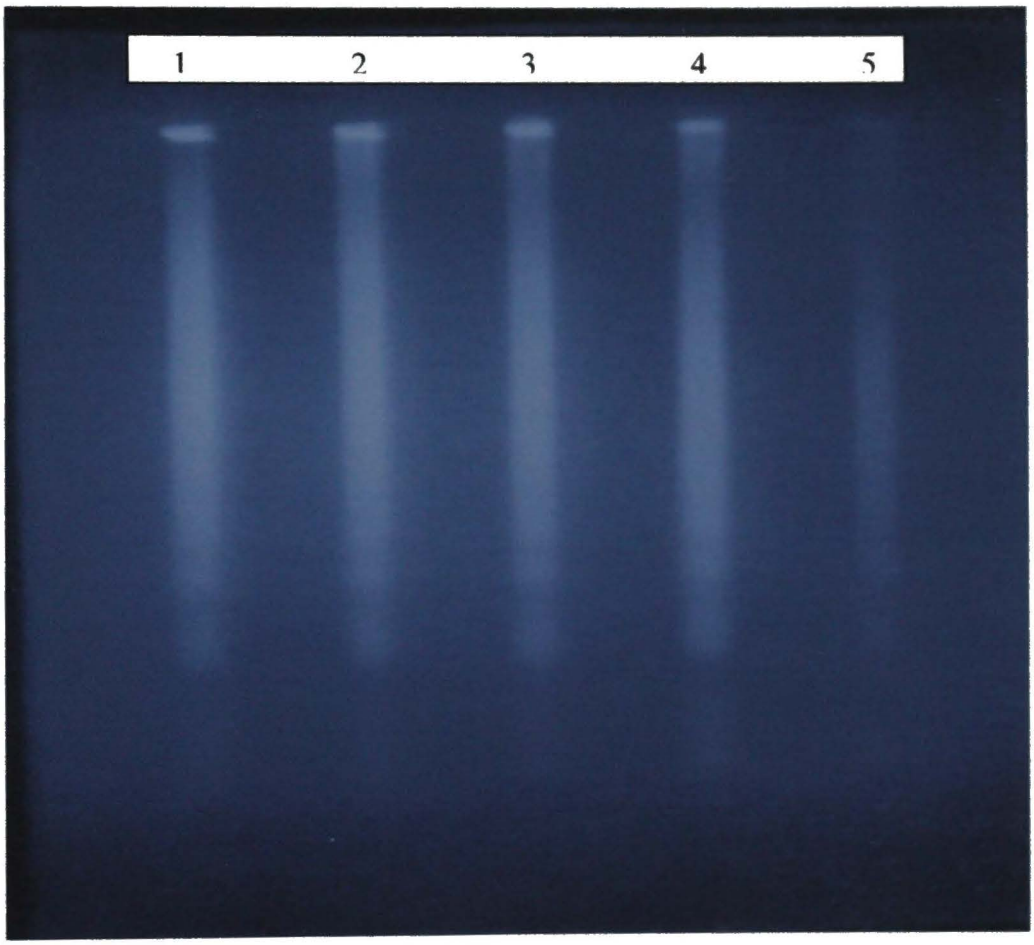
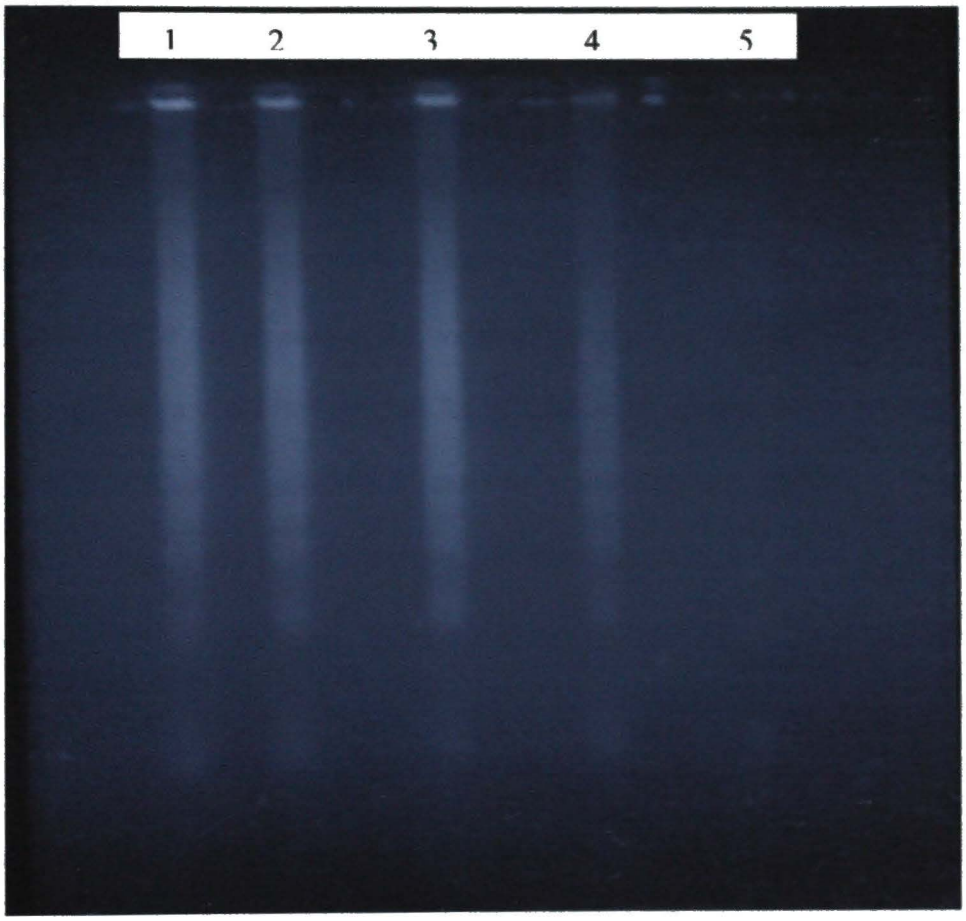


Figure 5: MNase digestion of chromatin at low ionic strength and 37 °C. 1.5% agarose gel electrophoresis of rat liver chromatin (100 μg protein/mL) digested with MNase for 0 min (1), 1 min (2), 5 min (3), 15 min (4), and 30 min (5), at 37 °C and 20 mM $MgCl_2$.

Figure 5



Effect of high ionic strength (75 mM $MgCl_2$) at 37 °C

Based on a notable difference in chromatin structure behavior under high ionic strengths (Thoma, 1979), the endonuclease digestion was also performed at 75 mM $MgCl_2$, keeping the temperature at 37 °C, to determine if this parameter could affect the accesibility of polynucleosomal DNA to endonuclease digestion. As shown in Figure 6, no significant difference is observed in the degradation by DNase I. The same proportional relationship between the extent of degradation and the time of incubation with DNase I is observed when digested samples are compared with the control. Indeed, a very intense band of high molecular DNA can be seen at the origin of the gel (Fig. 6, Lane C). On the contrary, a difference could be observed for MNase when comparing the patterns produced at 20 mM and 75 mM $MgCl_2$, especially after extended periods of incubation (Lanes 4 and 5, Fig. 5 and 7, respectively). At 75 mM $MgCl_2$, the bands at the origin of the gel appear much more intense, especially at incubation times of 15 and 30 minutes (Fig. 7, Lanes 4 and 5). Moreover, the difference is very noticeable after 30 minutes of digestion, whereas at 20 mM $MgCl_2$ almost no trace of chromatin was observed (Fig. 5, Lane 5). This result is consistent with the notion that a more condensed state of chromatin exists at higher ionic strengths, which could make more difficult the access of the endonuclease to the DNA because of the more compacted form. Although the degradation rate of chromatin appears to increase with incubation time, this effect seems smaller at high ionic strength than at low ionic strength (compare Fig 5 and 7). Therefore, the change of ionic strength in the sample solution seems to influence slightly more the action of MNase than for DNase I.

Figure 6: DNase digestion of chromatin at high ionic strength and 37 °C. 1.5% agarose gel electrophoresis of rat liver chromatin (100 μg protein/mL) digested with DNase I for 0 min (1), 1 min (2), 5 min (3), 15 min (4), and 30 min (5), at 37 °C and 75 mM $MgCl_2$.

Figure 6

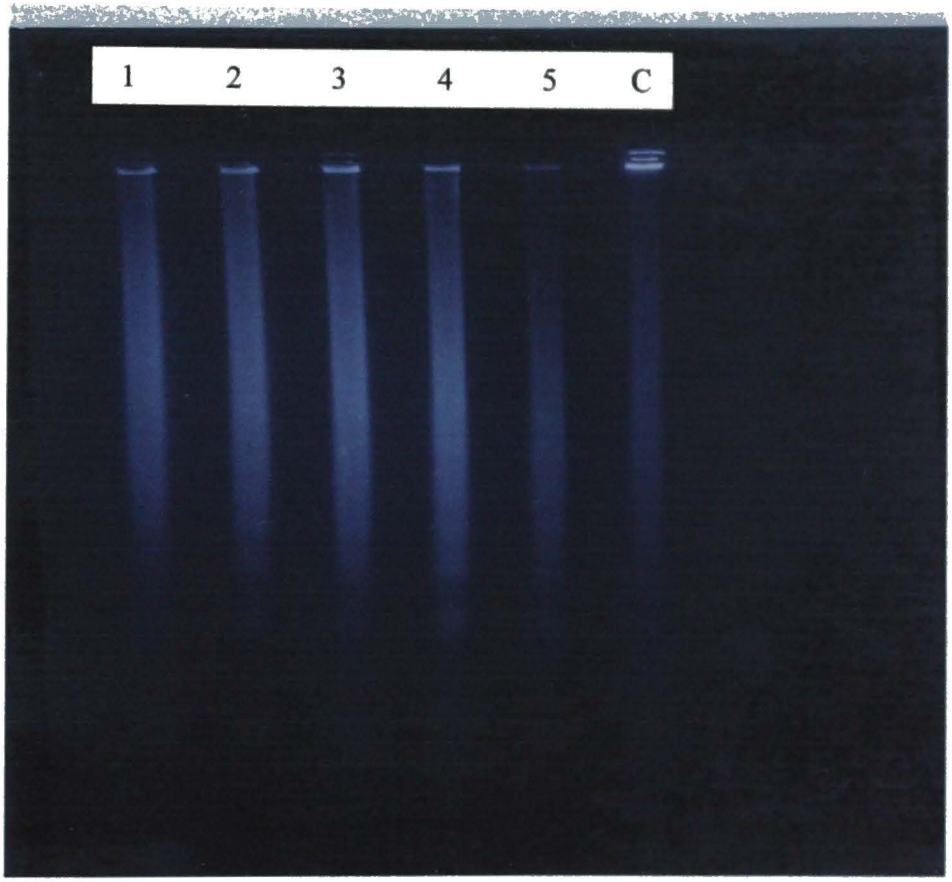
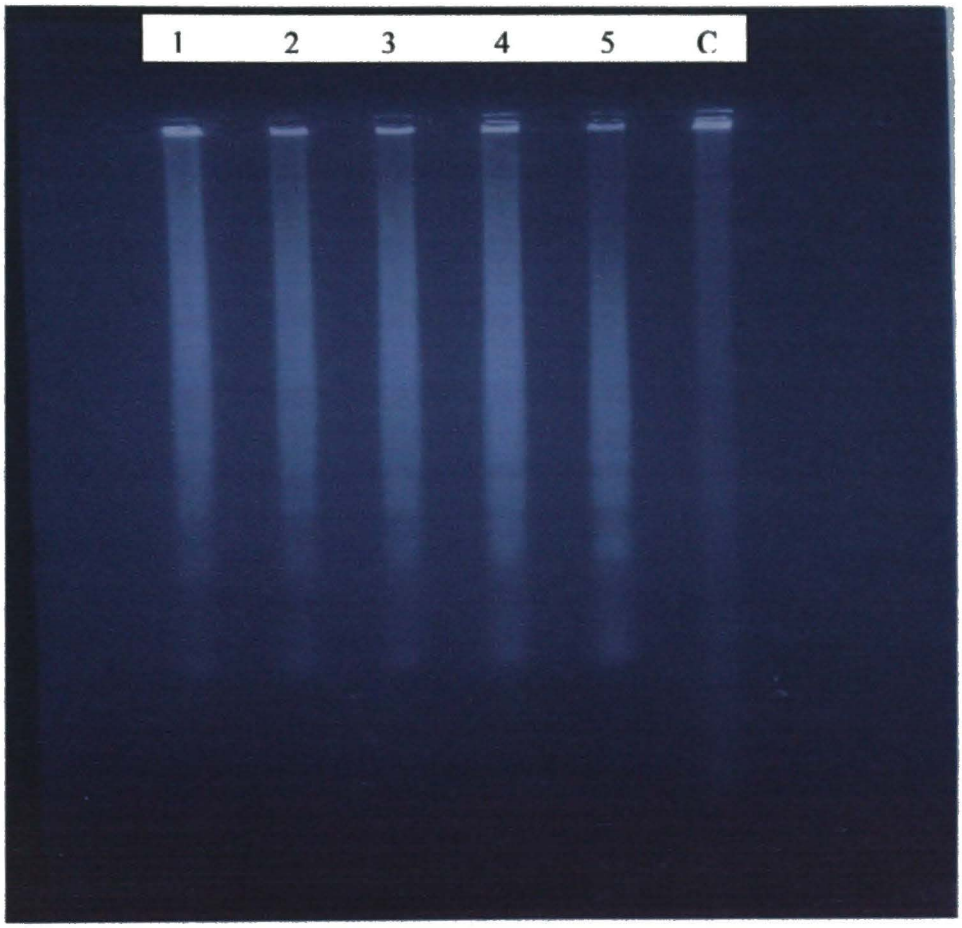


Figure 7: MNase digestion of chromatin at high ionic strength and 37 °C. 1.5% agarose gel electrophoresis of rat liver chromatin (100 μg protein/mL) digested with MNase for 0 min (1), 1 min (2), 5 min (3), 15 min (4), and 30 min (5), at 37 °C and 75 mM $MgCl_2$.

Figure 7



Influence of protein-poly(ADP-ribosyl)ation on chromatin structure and subsequent accessibility to endonuclease digestion

Once a methodology for chromatin-digestion kinetics was established, and the patterns of degradation by both DNase I and MNase were obtained, the effect of protein-poly(ADP-ribosyl)ation on chromatin structure and its consequence on its susceptibility to digestion by the endonucleases was studied. Chromatin was incubated with 200 μM βNAD^+ for 30 minutes to allow for ADP-ribose polymers to form, and then samples were subjected to the same enzymatic digestion by DNase I and MNase, as described above. Digestions by both endonucleases were simultaneously performed on chromatin in the absence or presence of βNAD^+, and the samples were processed in the same gel to observe any difference on the chromatin degradation patterns generated. It should be noted that control incubations were also run at both extremes of the gel.

Figure 8 and 9 show the effect of the poly(ADP-ribosyl)ation on the digestion kinetics of chromatin by DNase I. Digestion of chromatin in the absence of exogenous βNAD^+ appears in Lanes 1 to 5 (Fig. 8). For digested chromatin previously incubated with βNAD^+, the samples were run in lanes 1 to 5 (Fig. 9). Both reactions showed a consistency in the increased degradation with longer incubation with the DNase. Nevertheless, a marked difference at high molecular weight levels of the gel and at extended periods of digestion, i.e., 15 and 30 minutes can be seen. When comparing lanes 1 to 5 of figures 8 and 9, respectively, at the same times of DNase I incubation, those samples corresponding to poly(ADP-ribosyl)ated chromatin (Fig. 9) have less intense bands at the origin of the gel, while almost completely disappeared after 15 min

Figure 8: Chromatin digestion by DNase I in the absence of βNAD$^+$. 1.5% agarose gel electrophoresis of rat liver chromatin (100 μg protein/mL) digested with DNase I, in the absence of βNAD$^+$, for 0 min (1), 1 min (2), 5 min (3), 15 min (4), and 30 min (5), at room temperature and 20 mM $MgCl_2$.

Figure 8

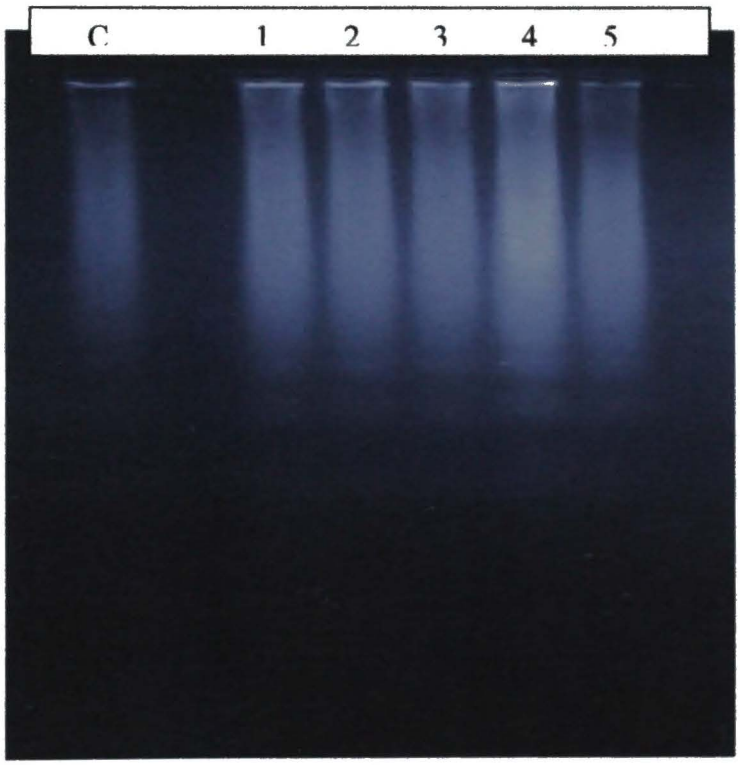


Figure 9: Chromatin digestion by DNase I in the presence of 200 μM βNAD$^+$. 1.5% agarose gel electrophoresis of rat liver chromatin (100 μg protein/mL), digested with DNase I, for 0 min (1), 1 min (2), 5 min (3), 15 min (4), and 30 min (5), at room temperature and 20 mM $MgCl_2$. Samples were incubated in the presence of 200 βNAD$^+$, prior to enzymatic digestion, to promote protein-poly(ADP-ribosyl)ation.

Figure 9

of incubation (Fig. 9, Lanes 4 and 5); and high molecular weight smears are notably much less intense even after only 1 minute of DNase I digestion, if compared at the same time for non-poly(ADP-ribosyl)ated chromatin (Fig. 8). The most pronounced difference is seen when comparing lane 5, in both Fig. 8 and Fig. 9 (30 min DNase digestion), where an intense smear and oligonucleosomal bands can be distinguished for lane 5 on Fig. 8, while the smear is very faint for the same time of incubation in Fig. 9, and no oligonucleosomal band appears on the gel. Also the band at the origin after 30 min. incubation (Fig. 8, Lane 5) is not present in Fig.9 (Lane 5). These results clearly confirm a faster and increased degradation of the chromatin after being poly(ADP-ribosyl)ated prior to DNase I digestion. These same general results appear for MNase digestion in Figure 10. As for DNase I, lanes 1 to 5 of Fig. 10 and Fig. 11 are compared. This time, a greater effect seems to influence susceptibility of chromatin to MNase activity. The bands and smears corresponding to high molecular weight are much less intense for chromatin samples generated in presence of βNAD^+ (Fig 11) than for chromatin samples incubated in the absence of βNAD^+ (Fig 10). A higher level of digestion could be observed at earlier times of incubation which would correspond to the longest enzymatic digestions for non-poly(ADP-ribosyl)ated chromatin (compare lanes 4-5 in Fig. 10 against 4-5 in Fig. 11). In lane 1 (Fig. 11), which corresponds to control poly(ADP-ribosyl)ated chromatin (no MNase), a high degradation can be observed that compares with MNase digested chromatin in absence of βNAD^+(Lanes 4 and 5, Fig. 10). The same pattern should not be expected for sample in lane 1 of Fig. 11 (0 min), since no MNase was added to the tube. The marked degradation of chromatin in this case can be

Figure 10: Chromatin digestion by MNase in the absence of βNAD$^+$. 1.5% agarose gel electrophoresis of rat liver chromatin (100 μg protein/mL) digested with MNase, in the absence of βNAD$^+$, for 0 min (1), 1 min (2), 5 min (3), 15 min (4), and 30 min (5), at room temperature and 20 mM $MgCl_2$.

Figure 10

Figure 11: Chromatin digestion by MNase in the presence of 200 μM βNAD$^+$. 1.5% agarose gel electrophoresis of rat liver chromatin (100 μg protein/mL), digested with MNase, for 0 min (1), 1 min (2), 5 min (3), 15 min (4), and 30 min (5), at room temperature and 20 mM $MgCl_2$. Samples were incubated in the presence of 200 βNAD$^+$, prior to enzymatic digestion, to promote protein-poly(ADP-ribosyl)ation.

Figure 11

explained by the activity of an endogenous endonuclease present in RLC as mentioned earlier (Hewish and Burgoyne, 1973). If compared the chromatin digestion patterns by MNase, in the absence or presence of βNAD^+ (Fig 10 and Fig. 11) with those of DNase I (Fig. 8 and Fig. 9), once again MNase seems to digest in a more productive way than DNase I: digestion is faster and more efficient. The results obtained in Figure 11 strengthen what was confirmed with DNase I in Figure 9, that poly(ADP-ribosyl)ation exposes linker DNA to endonuclease digestion, because chromatin relaxes upon protein modification.

Experimental evidence for the presence of an endogenous endonuclease activity that degrades freshly isolated chromatin samples

To confirm the presence and activity of endogenous endonuclease on chromatin, simultaneous digestion by MNase or DNase I, in the absence or presence of βNAD^+ were performed. However, three different controls were prepared and run in the same gel (see Material and Methods for each preparation), to compare changes in digestion patterns of the chromatin based on the time that chromatin was left in solution before the final endonuclease digestion by either MNase or DNase I.

The results shown in Figure 12 are consistent with those presented above (Figure 11), where MNase digestion was faster and increased when proteins were poly(ADP-ribosyl)ated. Control 1 (C1), as should be expected, shows a pattern identical to Lane 1, which corresponds to control chromatin incubated without MNase and in the absence of

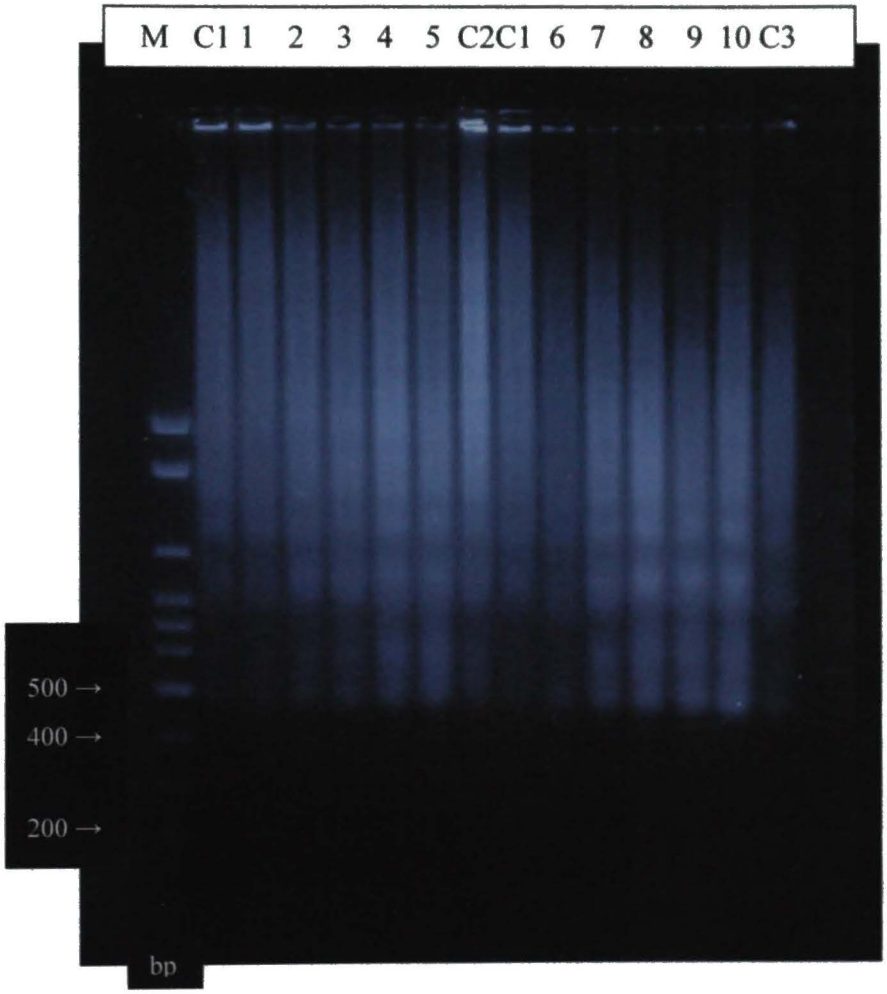
βNAD^+. Control 2 (C2), after a 30 min incubation at room temperature, shows a much more intense degradation smear, which can be explained by the activity of an endonuclease endogenous to chromatin in solution. Control 3 (C3), that was previously incubated with βNAD^+, and then allowed for another 30 min at room temperature, produced the most degraded pattern of all three controls, as expected after protein-poly(ADP-ribosyl)ated chromatin is more susceptible to activity by endogenous endonuclease. When comparing C3 with any of the samples incubated with βNAD^+ and then subjected to MNase digestion (Fig. 12, Lanes 6-10), a faint band, still a little more intense than the ones in lanes 6-10, is observed at the origin of the gel for the control (C3). Also a less intense smear and not very distinguishable oligonucleosomal bands were produced under C3 if compared with lanes 6-10 (Fig. 12). This could be indicative that poly(ADP-ribosyl)ation renders chromatin more susceptible to the exogenous MNase than to the endogenous endonuclease. In Figure 12 is also very notable the increased intensity in the smears and bands at low molecular weigh levels for chromatin in the presence of βNAD^+ (Fig. 12, Lanes 6-10), than for samples in the absence of βNAD^+ (Fig. 12, Lanes 1-5). The oligonucleosomal bands can be seen to acquire a ladder tendency, from sample 1 to 10, indicating that the bands probably correspond to mono-, di-, and trinucleosomes, respectively. Very important to notice too, is the shifting of the oligonucleosomal band toward higher molecular weight. Bands corresponding to mononucleosomes are appearing at levels around 450 bp, instead of the expected 200 bp.

The mobility of these bands is delayed at some extent, due to the complexes formed by histone-DNA complexes and the influence of addition of mass by modifying ADP-ribose polymers.

Figure 12: Evidence for presence of endogenous endonuclease activity.

1.5% Agarose gel electrophoresis of rat liver chromatin (100 μg protein/mL) digested with MNase in the absence of 200 μM βNAD^+, for 0 min (1), 1 min(2), 5 min (3), 15 min (4), and 30 min (5), at room temperature and 20 mM $MgCl_2$. Lanes 6-10 are chromatin previously incubated with 200 μM βNAD^+ for 30 min. C1 is a fresh control prepared right before running the samples in the gel: chromatin with TB buffer and loading buffer with EDTA. C2 is chromatin with TB, left at room temperature for 30 min before adding loading buffer with EDTA. C3 contains chromatin with TB buffer, incubated with 200 μM βNAD^+ for 30 min, stopped with 1 mM benzamide, and left for another 30 min at room temperature, before adding loading buffer with EDTA.

Figure 12



DISCUSSION

During the life cycle of a cell, chromatin structure undergoes transitions between several stages of condensation and relaxation, in order for the different nuclear processes to occur. These transitions take place, when non-histones proteins and, especially histones, are biochemically modified in order to produce a change in the structure of chromatin that will allow the factors, enzymes, and other proteins necessary for the nuclear processes to access DNA. The modification of a histone protein occurs when a specific chemical moiety is added to the tails of histones, interfering this way with its interaction with the DNA and allowing for the change in structure to happen, either making chromatin more compact or more relaxed. Among the most studied chemical modifications of histone proteins are methylation, acetylation, phosphorylation, and poly(ADP-ribosyl)ation. Of all the modifications identified so far, it is poly(ADP-ribosyl)ation the one that seems to influence the most the structure of chromatin. The reason for this is the formation of a highly branched polyanionic ADP-ribose polymer that could reach an average of 200 ADP-ribose residues (Alvarez-Gonzalez and Jacobson, 1987). The size and the high density of negative charges of this nucleic acid, as expected, will weaken and transiently avoid the interactions between the positively charged amino acids of the histones (i.e., Arg and Lys) and the negative phosphate backbone of the DNA, leading chromatin to adopt a more open structure. Of course, this modification must be reversible in order for recondensation to occur. This is possible by the degradation of the poly(ADP-ribose) modifying the protein. This way, the polymer that has been added to the histone is being degraded by the counterpart of PARP, a

glycohydrolase that digests the ADP-ribose polymer (PARG), and returning the histone to an unmodified state. Extensive experimental evidence suggests the involvement of poly(ADP-ribosyl)ation in processes like DNA repair, transcription, and differentiation (Kraus, 2003; Atorino, 2000). Many studies from different laboratories show that poly(ADP-ribosyl)ation causes decondensation of native chromatin (De Murcia, et al 1986; Poirier, et al., 1982; D'Erme, et al., 2001). Other factors seem to influence this effect, like the source of chromatin, and ionicity of the environment. Nevertheless, when chromatin has been poly(ADP-ribosyl)ated the typical condensation under high ionic strength does not occur, and chromatin stays open, resembling the beads-on a string fiber (Poirier, et al 1982). Based on the evidence from these studies, I decided to subject chromatin to digestion by the endonucleases Deoxyribonuclease I (DNase I) and Micrococcal Nuclease (MNase) in the presence or absence of βNAD^+, the substrate for poly(ADP-ribosyl)ation, and study how this modification could affect the accessibility and kinetics of degradation of chromatin by these two enzymes, as a function of their concentration and the time of incubation. In order to have a negative control for comparison of the degradation patterns of chromatin, naked DNA was subjected to digestion with both DNase I and MNase, for 0, 1, 5, 15 and 30 minutes, and at room temperature. As expected for DNA free of all histone and non-histone proteins, the pattern for both nucleases was only a smear without any discrete band. This smear decreased in intensity as the time of incubation with the nucleases was longer, which indicates that digestion of DNA increased proportionally with the time of incubations. Then, chromatin was incubated also with both DNase I or MNase, for the same times and

at the same temperature, and a different pattern for each of the endonucleases resulted. In the case of DNase I, only one wide band was seen, that decreased in intensity and size with time (data not shown). It is known that DNase I is a non-specific endonuclease that cuts in those areas where the DNA is less protected and more exposed. On the other hand, MNase produced a pattern where some discrete bands could be seen, that would correspond to the mono-, di-, and trinucleosome; that is, approximately 200 bp, 400 bp, 600 bp, etc. MNase has a recognized specificity for cutting on areas of linker DNA, giving rise to the typical bands of multiples of 200 bp that comprises the nucleosomal particle (Noll, 1977). This time also, degradation of chromatin increased with time of incubation (Fig. 3), but the oligonucleosomal bands disappeared after 15 minutes of MNase activity. These results are explained by the exonucleolytic activity of MNase (Alexander, 1961), which continues digesting the DNA on the nucleosome core after extended periods of times (Noll, 1977). As done in the studies where the hypothesis of this project is based, the ionic strength of the solution was increased from 20 mM $MgCl_2$, to 75 mM $MgCl_2$, and then the chromatin was subjected to the same digestion process by either DNase I or MNase. Also, the temperature was raised from room temperature to 37°C to observe any effect of temperature increase on the digestion by the endonucleases. Nevertheless, an unexpected pattern resulted for digestion with DNase I (Figs. 4 and 6), where bands could be observed, that seemed very similar to the ones produced by MNase. Considering that the source of the chromatin used in the experiments is rat liver chromatin, and the liver is an organ where a great deal of the cells is constantly undergoing apoptosis, some fragmentation pattern typical of this process can result. The

apoptotic fragmentation pattern happens to be also very similar to the one produced by MNase because of the internucleosomal cleavage that occurs in both cases. There is extensive evidence of the presence in rat liver chromatin of high levels of certain Ca^{+2}/Mg^{+2} dependent endonucleases (CME's) that produce these internucleosomal cleavage patterns, and are also responsible for apoptotic degradation (Hewish, 1973; Giannakis, 1991; Walker, 1994; Pandey, 1997). There is evidence too of the need of only traces of Ca^{+2} in the solution to be able to see the activity of some of these endonucleases (Hewish, 1973). It is understandable therefore that even before submitting the chromatin to DNase I or MNase digestion, some degradation may occur as a result of the activity of endogenous endonucleases present in the chromatin solution. It is also important to mention that this unexpected pattern for DNase I was seen when incubations were done at 37 °C instead of room temperature, and independent of the ionic strength, which could have activated the CME's to an extent that their pattern of cleavage darkened that of DNase I. For the experiments of poly(ADP-ribosyl)ation, the ionic strength was kept at 20 mM $MgCl_2$ and the temperature of incubation was room temperature again. If we compare the bands for DNase I in Figure 9, with those where incubations were performed at 37 °C (Figs. 4 and 6), even though chromatin was previously poly(ADP-ribosyl)ated (Fig. 9), the bands at low molecular weight levels seen in Figs. 4 and 6 (in absence of βNAD^{+}), that would correspond to the fragments of internucleosomal cleavage, are very faint, almost absent. This supports the action of endogenous endonucleases on chromatin and its consequent apoptotic-like fragmentation pattern, at 37 °C, but absent at room temperatures.

As mentioned before, the main focus of this project, was to see how poly(ADP-ribosyl)ation could affect the susceptibility of chromatin to DNase I and MNase digestion, in such a way that differences could be observed in the band patterns. For this part, chromatin was incubated with 200 μM βNAD^+ for 30 minutes to allow formation and modification by the ADP-ribose polymer. To stop this reaction, a solution of 1 mM benzamide, a potent PARP inhibitor (Rankin, 1989), was added to each sample. Then the same protocol for the enzymatic digestion by DNase I and MNase was followed (See Materials and Methods). For both endonucleases, samples that were previously poly(ADP-ribosyl)ated showed increased degradation when compared to the samples that were not, at the same times of incubation. The effect could be clearly seen especially at high molecular weights, where the bands at the origin of the gels and the smears decreased steeply in intensity, while the bands of lower molecular weights increased in intensity. In fact, digestion patterns after 15 and 30 minutes for samples in absence of βNAD^+ show a great similarity with those at very early times when chromatin was first incubated with βNAD^+, even in the control, when no DNase I or MNase was added. These results support the hypothesis that modification of histone proteins by poly(ADP-ribosyl)ation indeed make chromatin more susceptible to attack and digestion by endonucleases, including endogenous ones, facilitating their access to more open areas on the DNA, destabilizing chromatin structure, and therefore degrading it much faster. The findings during this project are consistent with previous studies where poly(ADP-ribosyl)ation is involved in the condensation-relaxation transition of chromatin and strengthens the idea of its involvement in other DNA metabolism processes where some

modulation of chromatin structure is required, like DNA replication, DNA repair, and gene expression, etc. In conclusion my experimental results agree with the notion that poly(ADP-ribosyl)ation of chromatin proteins could play a fundamental regulatory step in protein-DNA interactions to allow and facilitate the attachment/detachment cycle of all the machinery necessary to access DNA during the different nuclear events.

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