Altered Chromatin Structure of Cerebral Nuclei in Experimental Diabetes Mellitus

(43357)

ARSHAG D. MOORADIAN[1]
Tucson VA Medical Center and Department of Medicine, University of Arizona College of Medicine, Tucson, Arizona 85719

Abstract. **To determine whether diabetes alters chromatin structure *in vivo*, micrococcal nuclease digestion kinetics were analyzed in cerebral cortical and hepatic nuclei of streptozotocin-induced diabetic rats. Cerebral nuclei of diabetic rats maintained for 6 weeks were less susceptible to micrococcal nuclease digestion compared with control rats. Insulin treatment reversed diabetes-related changes in nuclease digestion kinetics. There were no changes in the kinetics of digestion in hepatic nuclei. The reduced digestibility of cerebral DNA in diabetes could not be attributed to altered DNA fluorescence spectra, or altered distribution of most abundant chromatin proteins that were either solubilized or that remained insoluble immediately following nuclease digestion. It is concluded that chronic, uncontrolled hyperglycemia can alter chromatin structure of some tissues *in vivo*, and this change is probably related to subtle alterations in DNA-protein interactions.** [P.S.E.B.M. 1992, Vol 199]

In vitro studies have previously shown that elevated ambient glucose concentration can directly modify DNA structure (1–4). The biological significance of these changes in DNA has been demonstrated in *Escherichia coli* filamentous bacteriophage (2). In cultured human endothelial cells, high glucose concentrations induce DNA damage and cause cellular death (5, 6). We have previously found reduced alkali-induced DNA unwinding in various tissues of streptozotocin-induced diabetic rats and genetically obese (*db/db*) diabetic mice (7). A similar observation was made in white blood cells of diabetic patients (8). These studies indicated that chronic uncontrolled hyperglycemia can alter chromatin structure *in vivo*. The biochemical basis of these changes is unknown. To gain further insight into this problem, the present work was carried out to study the kinetics and the products of micrococcal nuclease digestion of rat brain and liver nuclei following 6 weeks of uncontrolled diabetes. Micrococcal nuclease has been used previously under certain conditions as a probe for transcriptionally active accessible DNA sites and is sensitive for DNA-protein interactions (9, 10). A change in micrococcal nuclease digestion kinetics, therefore, is evidence for a change in chromatin structure.

To whom correspondence and requests for reprints should be addressed at Division of Endocrinology, St. Louis University School of Medicine, 1402 S. Grand Boulevard, St. Louis, MO 63104.

Received May 6, 1991. [P.S.E.B.M. 1992, Vol 199]
Accepted September 23, 1991.

0037-9727/92/1993-0282$3.00/0
Copyright © 1992 by the Society for Experimental Biology and Medicine

Materials and Methods

Animals. Male Fischer 344 rats were obtained from Harlan (Indianapolis, IN) at 3 months of age. A 1.3% solution of streptozotocin (Upjohn, Kalamazoo, MI) in 0.05 *M* cold citrate buffer (pH 4.5) was injected at a dose of 45 mg/kg body wt ip. Control rats (n = 25) were concurrently injected with citrate buffer. Rats were maintained on regular rat chow and water *ad libitum*. Animals manifesting glucosuria, polydipsia, and polyphagia were considered diabetic (n = 25). The diabetic state was confirmed on the day of sacrifice by plasma glucose determination. Urinary excretion of ketones measured with Keto-Diastix (Ames, Miles, Elkhart, IN) was either trace or negative. Five days after streptozotocin injection, a group of diabetic rats was treated with NPH insulin (8 units/kg sc) twice a day until the end of the 6-week observation period. Plasma glucose in diabetic rats was 388 ± 33 mg/dl, whereas in insulin-treated diabetic rats and control rats plasma glucose levels were 152 ± 14 and 144 ± 7 mg/dl, respectively. Of the insulin-treated diabetic group, only those with plasma glucose of less than 200 mg/dl were included in the study (n = 8). Animals were sacrificed by exsanguination through the abdominal aorta under pentobar-

bital sodium anesthesia. The forebrain and liver were removed, frozen immediately on dry ice, and kept at −70°C until the day of the experiment. Preliminary experiments in our laboratory indicated that the results obtained in frozen-thawed tissues are similar to those seen in fresh tissue.

Preparation of Nuclei. Tissue was diluted 1/10 (w/v) with ice-cold buffer A (0.34 *M* sucrose, 15 m*M* Tris HCl [pH 7.5], 60 m*M* KCl, 15 m*M* NaCl, 150 μ*M* spermidine, 500 μ*M* spermine, 500 μ*M* dithiotrietol, 2 m*M* EDTA, 500 μ*M* EGTA, and 1 m*M* phenylmethylsulfonyl fluoride) and gently hand-homogenized with seven strokes in a glass homogenizer specially milled to give 0.25-mm clearance. The homogenate was filtered through cheesecloth, centrifuged at 600*g* for 10 min, washed twice successively in buffer B (which is identical to buffer A except for 1.0 *M* sucrose and 0.5% triton) and buffer C (which is identical to buffer B except for 0.25 *M* sucrose), and resuspended in buffer D nuclease digestion buffer (0.25 *M* sucrose, 15 m*M* Tris HCl [pH 7.5], 60 m*M* KCl, 15 m*M* NaCl, 500 μ*M* dithiotreitol, 1 m*M* $CaCl_2$, and 3 m*M* $MgCl_2$). The isolation of nuclei was carried out at 4°C.

Nuclease Digestion for Kinetic Experiments. The kinetic experiments were carried out as described previously (11). Nuclear suspension was adjusted to a concentration of 150 μg/ml. This concentration was determined by suspending a known aliquot in 2 *M* NaCl and 5 *M* urea, and determination of absorbance at 260 nm. $CaCl_2$ was added to a final concentration of 1 m*M*, followed by 100 units/ml of micrococcal nuclease (Worthington, Freehold, NJ). The incubation was carried out at 37°C in triplicate aliquots. The reaction was terminated at intervals between 30 sec and 120 min by adding perchloric acid to a final concentration of 7% and chilling on ice. Precipitated material was removed by centrifugation at 10,000 rpm for 30 min and the absorbance of the acid-soluble material in the supernatant was determined at 260 nm. Pellets were resuspended in 1 ml of 7% perchloric acid, heated for 10 min at 70°C, and cooled, and the absorbance was measured to determine the nucleotide concentation of hydrolyzed acid-insoluble material (11). The total acid-soluble DNA concentrations were also performed in representative samples using diphenylamine colorimetric assay (12).

To verify the positive findings in these measurements, additional experiments on the time course of nuclease digestion were carried out. Nuclei were suspended in digestion buffer at a concentration of 1 mg/ml and were digested with 50 units/ml of micrococcal nuclease at 23°C for 2, 5, 15, 30, and 60 min. These experimental conditions were best suited for identifying diabetes-related changes. DNA was purified after pronase digestion by phenol extraction, followed by chloroform-isoamyl alcohol reextraction and ethanol precipitation. Approximately 15 μg of DNA was applied to each slot of 17.5-cm 1% agarose horizontal slab gel. Electrophoresis was carried out at 30 V for 18 hr using Tris-acetate buffer (13). The gels were stained with ethidium bromide (0.5 μg/ml) and photographed through a red filter.

Measurement of Fluorescence Absorbance of DNA. DNA purified from either intact cerebral nuclei or following micrococcal nuclease digestion for 5, 30, and 60 min were subjected to fluorescence spectral analysis. The fluorescence was measured with emission wavelengths between 400 and 470 nm and excitation was fixed at 370 nm, or emission wavelength was fixed at 440 nm and excitation wavelength varied from 330 to 410 nm. This approach has been used previously to detect modification of DNA by reducing sugars *in vitro* (2).

Determination of Chromatin Proteins. Nuclei were suspended in digestion buffer at a concentration of 1 mg/ml and digested with micrococcal nuclease (100 units/ml) at 37°C for 10 min. The reaction was terminated with rapid chilling on ice and centrifugation at 1,000*g* for 5 min. The pellet was resuspended in 0.2 m*M* EDTA, pH 7.5, and centrifuged at 12,000*g* for 30 min. This pellet and the first supernatant (S) were adjusted to 0.2 *M* H_2SO_4, incubated for 1 hr at 4°C, and centrifuged at 10,000*g* for 10 min. The solubilized proteins in the supernatant were precipitated with 2 vol of 95% ethanol at −40°C overnight. The proteins were recovered by centrifugation at 10,000*g* for 30 min at −5°C, washed twice with cold 70% ethanol, and lyophilized. The protein quantitation was carried out in all of these fractions using Lowry's assay (14). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis slab gels (12%) were used for electrophoretic separation of different protein fragments. Electrophoresis was carried out at 30 mA/gel constant current for 3 to 4 hr using glycine buffer (15). The gels were stained with silver nitrate as described previously (16). The purpose of this approach was to determine whether chronic, uncontrolled hyperglycemia would alter the electrophoretic mobility of various protein components of chromatin.

The statistical significance of the differences was determined by analysis of variance, followed by Student's *t* test for unpaired variables.

Results

Digestion kinetics of rat forebrain nuclei with micrococcal nuclease are shown in Figure 1. The amount of acid-soluble material at zero time (background) was subtracted from all the experimental points. Endogenous nuclease activity (i.e., acid-soluble material in aliquots without added enzyme) could not be detected at up to 120 min of incubation. Chromatin digestion of diabetic rat brain was slower than in control rats. The percentages of DNA digested at 5, 10, and 30 min

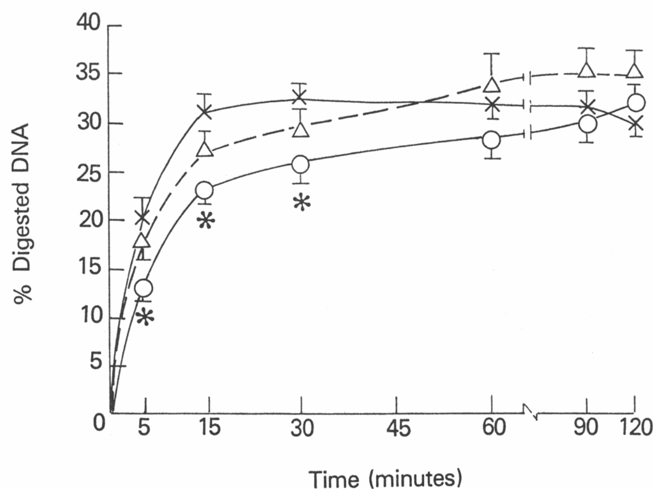

Figure 1. Digestion kinetics of rat brain cerebral nuclei with micrococcal nuclease. x----x, Control rats (n = 9); O----O, diabetic rats (n = 9); Δ----Δ, diabetic rats treated with insulin (n = 7). Values are mean ± SE. *$P < 0.01$ compared with control rats using analysis of variance followed by Student's t test.

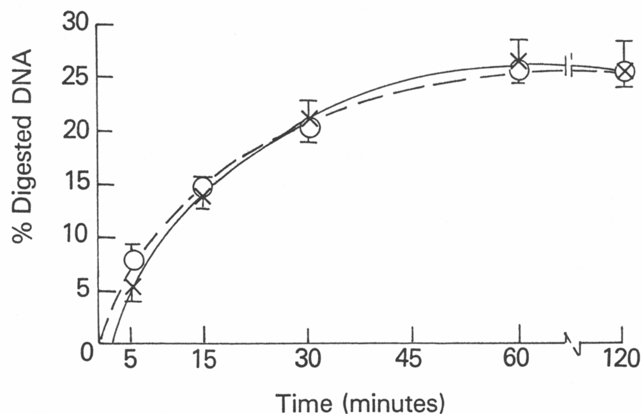

Figure 2. Digestion kinetics of rat hepatic nuclei with micrococcal nuclease. x----x, Control rats (n = 7); O----O, diabetic rats (n = 7). Value are mean ± SE.

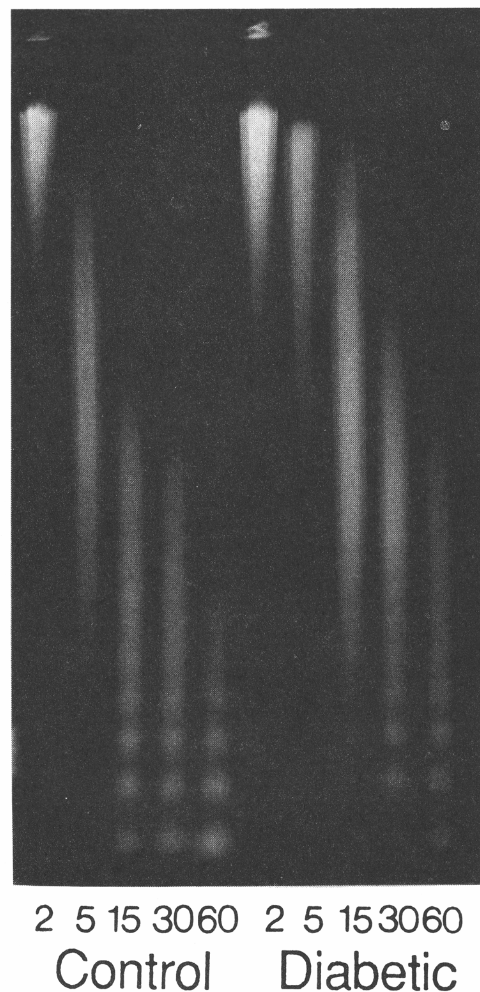

Figure 3. DNA analysis of 1% agarose horizontal slab gel of micrococcal nuclease (50 units/ml) digestion of control and diabetic rat cerebral nuclei (1 mg/ml) for 2, 5, 15, 30, and 60 min of incubation at 23°C.

were significantly lower in diabetic animals. In control rats, the reaction is complete by 30 min and reaction midpoint (50% of digestion) occurs in 3–4 min. In diabetic rats, the digestion reaction is complete by 60 min and reaction midpoint occurs at 13 min. The kinetics of digestion seen in insulin-treated diabetic rats were not significantly different from those in controls. The percentage of DNA digested at 60 min and beyond was not significantly reduced in diabetic rats.

Digestion kinetics of hepatic nuclei with micrococcal nuclease were not altered in diabetic rats (Fig. 2). Endogenous nuclease activity in the liver resulted in up to 20–30% digestion of DNA. This background activity was subtracted from the total DNA digested at each time point.

The relative resistance of cerebral nuclei of diabetic rats to micrococcal digestion is also shown in Figure 3. Whereas the cerebral nuclei of control rats show cleavage at 5 min of incubation, the first sign of cleavage in nuclei of diabetic rats is evident at 15 min of incubation under these experimental conditions.

The DNA isolated from the cerebrum of diabetic rats, like DNA isolated from control rats, did not show fluorescence (data not shown). The electrophoretic pattern of proteins isolated from the first supernatant (S) following micrococcal nuclease digestion is shown in Figure 4. There were no differences between control and diabetic rats. The proteins isolated from the pellet fraction are shown in Figure 5. As was found in the S fraction, the 65-kDa band is also a prominent protein in the pellet. The 92-kDa band seen in S proteins is now replaced by a smaller protein band at 14–18 kDa. There were also no significant differences between control and diabetic rats. The comparisons of protein profiles of various fractions were repeated in five experiments.

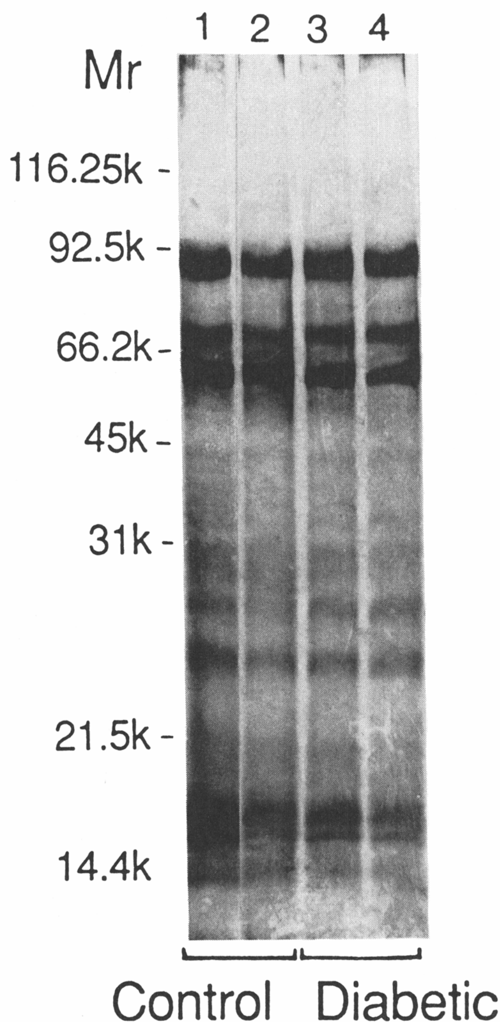

Figure 4. Analysis of protein content of the first supernatant fraction by sodium dodecyl sulfate-polyacrylamide (12%) gel electrophoresis. Lanes 1 and 2 are for control rats and lanes 3 and 4 are for diabetic rats.

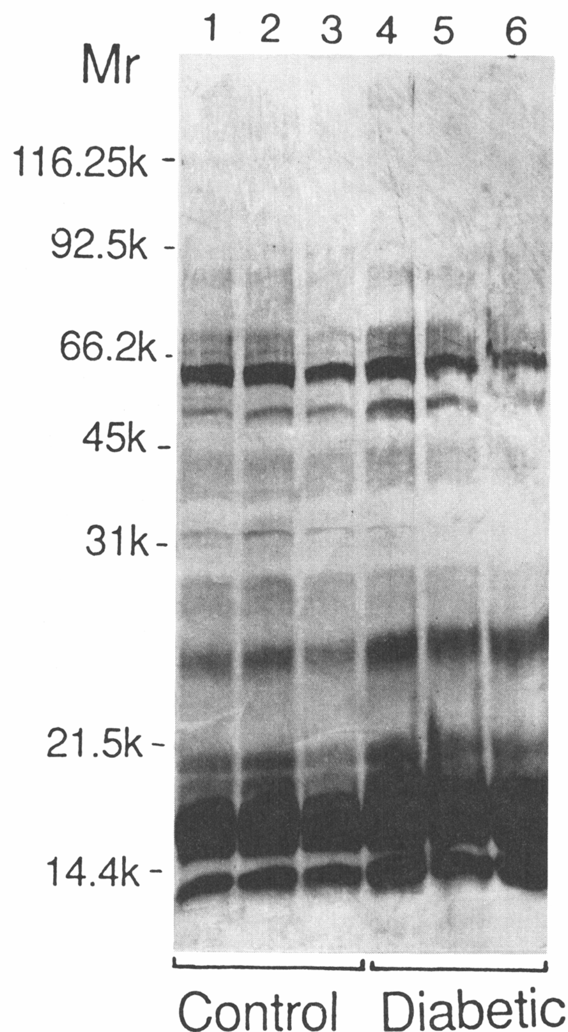

Figure 5. Analysis of protein content of the pellet by sodium dodecyl sulfate-polyacrylamide (12%) gel electrophoresis. Lanes 1 to 3 are proteins isolated from control rats and Lanes 4 to 6 are isolated from diabetic rats.

Discussion

The decreased susceptibility of DNA to micrococcal nuclease digestion in cerebral nuclei of diabetic rats is consistent with our previous observation of reduced alkali-induced unwinding of DNA in cerebral nuclei of diabetic rats and mice (7). Near normalization of serum glucose with insulin therapy corrected the diabetes-related changes in DNA digestibility. The susceptibility of DNA to nuclease digestion in hepatic nuclei was not altered in diabetes. This suggests that cerebral nuclei are more susceptible than liver nuclei to glucose-induced changes. However, the inability to document diabetes-related changes in liver nuclei can also be due to high endogenous nuclease activity in the liver, making the measurements of micrococcal nuclease digestion kinetics in this tissue less sensitive. In contrast, endogenous nuclease activity in diabetic or control rat cerebral nuclei was essentially undetectable.

The contribution of nuclear RNA to the acid-soluble material appeared to be small. Direct measurement of acid-soluble material with diphenylhydramine reaction in representative samples indicated that over 85% of the acid-soluble material was DNA. This is consistent with the fact that micrococcal nuclease has substantially lower affinity to RNA (17).

It is noteworthy that there is a remarkable similarity between the changes observed in diabetic rat cerebral and hepatic nuclei and those previously found in aged animals (11). The decreased sensitivity to micrococcal nuclease digestion is found in the cerebral nuclei of aged rats, but not in the hepatic or cardiac nuclei of aged mice (18). It remains to be seen whether diabetes-related changes in gene expression can be related to alterations in chromatin structure.

The precise biochemical basis of reduced DNA accessibility to micrococcal nuclease is not known. Changes in DNA, per se, or in chromatin proteins or the changes in the interaction of the two may alter

DNA digestibility. Alternatively, the observed changes can also be due to differences in the characteristics of the nuclear envelope that nuclease must traverse to reach the chromatin. It was tempting to postulate that DNA or chromatin proteins can undergo glycosylation which would then alter chromatin digestibility. To test this postulate, the DNA fluorescence spectra were examined and the electrophoretic patterns of chromatin proteins were studied. The fluorescence spectra of DNA isolated from diabetic rat brain were not different from that of controls. This is in line with *in vitro* studies showing that glucose adduction to DNA can be demonstrated for single-stranded DNA and various nucleotides, while the double-stranded DNA is protected against glycation (2).

The first supernatant fraction following nuclease digestion represents the chromatin proteins that are readily solubilized with the micrococcal nuclease. The pellet fraction represents chromatin resistant to micrococcal nuclease. There were no significant diabetes-related changes in the chromatin proteins identified in this study that would explain the altered DNA digestibility with micrococcal nuclease.

It appears that the most abundant proteins of nuclear chromatin are not significantly altered in diabetes. The reduced DNA susceptibility to micrococcal nuclease, therefore, is probably related to more subtle changes in less abundant proteins or in the DNA-protein interactions.

Chronic, uncontrolled hyperglycemia can alter chromatin structure *in vivo*. Certain tissues appear to be more susceptible to these changes than others. Further studies are needed to clarify the precise biochemical basis of these changes.

The work was supported by the Medical Research of Veterans Affairs and The Flinn Foundation. The author thanks Tatyana Yetskievych, Catherine Oder, and Sherry Atkins for excellent technical assistance.

1. Morita J, Ueda K, Nanjo S, Komano T. Sequence specific damage of DNA induced by reducing sugars. Nucleic Acids Res **13**:449–458, 1985.
2. Bucala R, Model P, Cerami A. Modification of DNA by reducing sugars: A possible mechanism for nucleic acid aging and age-related dysfunction in gene expression. Proc Natl Acad Sci USA **81**:105–109, 1984.
3. Lee AT, Cerami A. Elevated glucose-6-phosphate levels are associated with plasmid mutations in vivo. Proc Natl Acad Sci USA **84**:8311–8314, 1987.
4. Lee AT, Cerami A. The formation of reactive intermediate (S) of glucose 6-phosphate and lysine capable of rapidly reacting with DNA. Mutat Res **179**:151–158, 1987.
5. Lorenzi M, Cagliero E, Toledo S. Glucose toxicity for human endothelial cells in culture: Delayed replication disturbed cell cycle and accelerated death. Diabetes **34**:621–627, 1985.
6. Lorenzi M, Montisano DF, Toledo S, Barrieux A. High glucose induces DNA damage in cultured human endothelial cells. J Clin Invest **77**:322–325, 1986.
7. Hartnell JM, Storrie MC, Mooradian AD. Diabetes-related changes in chromatin structure of brain, liver, and intestinal epithelium. Diabetes **39**:348–353, 1990.
8. Hartnell JM, Morley JE, Mooradian AD. Reduction of alkali-induced white blood cell DNA unwinding rate: A potential biomarker of aging. J Gerontol Biol Sci **44**:B125–B130, 1989.
9. Bloom KS, Anderson JN. Fractionation of hen oviduct chromatin into transcriptionally active and inactive regions after selective micrococcal nuclease digestion. Cell **15**:141–150, 1978.
10. Levy WB, Dixon GH. Partial purification of transcriptionally active nucleosomes from trout testis cells. Nucleic Acids Res **5**:4155–4163, 1978.
11. Berkowitz EM, Sanborn AC, Vaughan DW. Chromatin structure in neuronal and neuroglial cell nuclei as a function of age. J Neurochem **41**:516–523, 1983.
12. Burton K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J **62**:315–323, 1956.
13. Loenig UE. The fractionation of high-molecular weight ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem J **102**:251–257, 1967.
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem **193**:265–275, 1951.
15. O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem **250**:4007–4021, 1975.
16. Oakley BR, Kirsch DR, Moris NR. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal Biochem **105**:361–363, 1980.
17. Cuatrecases P, Fuchs S, Anfinsin CB. Catalytic properties and specificity of the extracellular nuclease of Staphylococcus aureus. J Biol Chem **242**:1541–1547, 1967.
18. Gaubatz J, Ellis M, Chalkley R. Nuclease digestion studies of mouse chromatin as a function of age. J Gerontol **34**:672–679, 1979.