

A Study of the Histone H1  
in Chromatin

This thesis was completed in partial  
fulfillment for graduation with honors  
in the Department of Biology at Carroll  
College, Helena, Montana

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April 1, 1986



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## ACKNOWLEDGEMENTS

First and foremost, I would like to thank my parents, Peter G. and Mary A. Jenks, for their constant love, understanding and support.

I wish to thank the entire staff at the University of California, Davis, School of Medicine, Department of Biological Chemistry, with special gratitude to Mr. John Breneman, and also to Dr. Peter Yau for his expert guidance and instruction in the techniques necessary to do proficient research on the histone H1 family. Furthermore, I am indebted to Dr. E. M. Bradbury for permitting me to work in his laboratory.

Finally, I wish to express my gratitude to my thesis director, Dr. James J. Manion, for his patience and advice, as well as to my readers, Dr. Jean E. Smith and Dr. Ruth G. Carrington, for their review of this thesis.

## ABSTRACT

After the isolation and fractionation of histones H1 and H1<sup>o</sup> from lamb thymus and bovine liver, these chromosomal proteins were purified for further research and experimentation as to how this family of histones functions in chromatin.

The relationship of the H1<sup>o</sup> subtype of histone H1 with DNA lengths was studied using a preparation of mouse liver nuclei. The H1<sup>o</sup> protein was found to be associated with longer lengths of DNA between 146 and 200 nucleotide base pairs.

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## INTRODUCTION

The basic structural unit of chromatin is the nucleosome. It consists of successive stretches of a DNA superhelix, approximately 146 to 200 nucleotide base pairs, wound twice around the outside of a core of histone proteins. These histones, called core histones, form octamers composed of two molecules of each of the four histones: H2A, H2B, H3 and H4. Along with a 146 nucleotide base pair segment of DNA they comprise the core particle of the nucleosome. This length of DNA, however, is only enough for one and three-quarter turns. In addition to the core particle, a single molecule of a fifth histone, H1, is bound simultaneously to both ends of the DNA stretch making up the full two-turn nucleosome. Histone H1 is actually outside of the nucleosome and is referred to as the linker histone because of its association with the DNA linking the core particles.

Histone H1 comprises a family of lysine-rich chromosomal protein. The function of H1 in chromatin is still unknown; it has been implicated to be involved with condensation of chromosomes during metaphase, causing chromatin to condense from small fibers to higher order

structured chromosomes. It is also shown to be modified by protein kinases and ADP-ribosylation enzymes in vivo. Furthermore, recent studies have also revealed histone H1<sup>o</sup>, a subfraction of histone H1, to be involved with active genes in mice while a similar protein, H5, found in chicken erythrocytes is associated with inactive genes.

This paper deals mainly with the isolation and fractionation of histone H1 with special attention being given to the subfraction H1<sup>o</sup>. Histone H1 can be extracted from various mammalian tissues and subsequently, the various subtypes of H1 can be purified. Preparations of polyclonal and monoclonal antibodies against each subtype of H1 can be used to perform immunological assays on the prepared sera. Also, specific gene association with these H1 containing chromatin using plasmid cloned genes and DNA:DNA hybridization may be studied. With further experimentation and research, the role of H1 in chromatin may be uncovered.

## LITERATURE REVIEW

Primary structure analysis of the lysine-rich histones H1 has shown them to be a relatively heterogenous family of histones (Von Holt et al., 1979), which shows both tissue and species specificity. H1 subfractions also have been designated as having functional significance (Cole, 1977). Although the precise role of H1 in chromatin is not fully understood, it is widely accepted that these histones are involved in the maintenance and control of higher order chromatin structure at the level of the nucleosome linker regions (Bradbury et al., 1973). Moreover, each H1 subfraction has been shown to interact differently with DNA or chromatin subfractions (nucleosomes) (Welch and Cole, 1980).

One of the prominent histone H1 subfractions is the H1<sup>o</sup> histone (Lennox and Cohen, 1980), which was first isolated by Panyim and Chalkley in 1969. It is similar to H1 and to H5, a class of lysine-rich histones present in avian and other erythrocytes, in size and amino acid composition. Like H1 and H5, H1<sup>o</sup> binds to the nucleosome (Smith and Johns, 1980). Histone H1<sup>o</sup> was assigned to the H1 class of very lysine-rich histones on

the basis of its amino acid composition and migration on polyacrylamide gels (Cary et al., 1981), but there is evidence suggesting that H1<sup>o</sup> and H5 proteins may be considered as belonging to a family distinct from H1 types (Smith et al., 1984). Moreover, the primary structure of histone H1<sup>o</sup> contains methionine whereas that of histone H1 does not.

In contrast to H1 and other classes of histones, the occurrence of H1<sup>o</sup> is generally associated with slowly growing tissues (Fanyim and Chalkley, 1969), which include ox liver, mouse liver and kidney, and hamster liver and kidney. It is present in small amounts or absent during the active proliferative growth of many developing tissues and high in the corresponding nondividing mature tissues (Varricchio, 1977). It also decreases during regenerative growth of adult tissues (Varricchio et al., 1977). Furthermore, studies of cells in culture have shown that H1<sup>o</sup> accumulates on inhibition of cell growth either by contact inhibition or serum deprivation (Pehrson and Cole, 1980). These observations have led to speculations that histone H1<sup>o</sup> might be involved in the repression of DNA synthesis (Larue et al., 1983). However, while there is circumstantial evidence that the accumulation of H1<sup>o</sup> histone protein parallels the decrease in cell division (Pehrson and Cole, 1980), there is no direct evidence that H1<sup>o</sup> may actually inhibit

the synthesis of DNA (Van Helden, 1984).

Histone H1<sup>o</sup> has also been associated with a gene repressed during liver development in the mouse. The alpha-fetoprotein gene has been found with H1<sup>o</sup> nucleosomes, but no H1<sup>o</sup> is found with the albumin gene. Also H1<sup>o</sup> synthesis is not directly coupled to DNA synthesis and is hormone dependent in some glands of adult rodents. The H1<sup>o</sup> protein is preferentially found with nuclease resistant chromatin and, therefore, may have a role in developmental gene control (Roche, et al., 1985).

The research done in this paper attempts to add to the information already accumulated through previous research done on these lysine-rich histones, H1 and H1<sup>o</sup>, so that more about the nature of their function in chromatin may be determined.

## METHODS AND MATERIALS

### Purification of H1/H1<sup>o</sup> From Lamb Thymus

A CM-25 Sephadex LKB column, 2.5cm in diameter x 25cm in length was washed with 7.5mM borate, 0.1M NaCl, 0.02% sodium azide, pH 8.8. A Pharmacia P3 three-channel peristaltic pump was used to set the flow rate at 30ml/h, and an Isco fraction collector was used to collect the fractions.

A 255mg sample of a previously isolated and fractionated lamb thymus H1 preparation was dissolved in borate buffer, containing 0.1M NaCl and loaded onto the CM-25 Sephadex column. The gradient used was 0.1M NaCl in PBS to 1.2M NaCl in PBS.

The column was run for 53h. Absorbance of fractions obtained from the column were read at 220nm on a Gilford Spectrophotometer 250 using a one-centimeter quartz cuvette to hold the samples. The fraction number versus the absorbance was plotted on a graph.

The fractions were analyzed by polyacrylamide gel electrophoresis: SDS (0.1%, w/v), polyacrylamide (17.5%, w/v) (Laemmli, 1970) in Schreier, Erni & Staehlin buffer (Schreier, Erni & Staehlin, 1977) and run at 150V. The gel

was stained in 0.03% coomassie blue R-250, 45% methanol, 10% acetic acid for 4hrs, The gel was destained in 25% methanol, 10% acetic acid for 1hr and 10% acetic acid, 1% ethanol until the gel background was clear.

Fractions were then combined with 4 volumes acetone and left to precipitate H1 and H1<sup>o</sup> in a cold room at 4°C.

The lamb thymus H1 and H1<sup>o</sup> acetone precipitates were then transferred to two 250ml bottles and spun down in a Sorvall RC-5B Refrigerated Superspeed Centrifuge at 7500xg for 20 minutes.

After centrifugation, each bottle was covered with a double layer of Kimwipes, fastened tightly with a rubber band, and placed in a vacuum to evaporate off the remaining acetone.

The purity of H1 and H1<sup>o</sup> was analyzed again using a polyacrylamide gel electrophoresis: SDS (0.1% w/v), polyacrylamide (17.5% w/v) (Laemmli, 1970) in Schreier, Erni & Staehlin buffer (Schreier, Erni & Staehlin, 1977) and run at 150V for 3h. The gel was stained in 0.03% coomassie blue R-250, 45% methanol, 10% acetic acid for 4h and destained in 25% methanol and 10% acetic acid for 1h and in 10% acetic acid, 1% ethanol until the gel background was clear.

### Mouse Liver Nuclei Preparation

Swiss Star male and female mice one year in age were used for this preparation. They were bought from the Charles River Company in Wilmington, Massachusetts, and raised in a bioclean, germ-free room at the University of California, Davis.

Sixteen mice were killed by neck dislocation. The mice were doused in 95% EtOH. Their livers were dissected out immediately, cut up into 1/4-inch pieces, and suspended in approximately 40ml of homogenizing buffer (0.34M sucrose, Buffer A, 2mM EDTA, 0.5mM EGTA, 1mM PMSF, 15mM B-mercaptoethanol). An ice bath was used to keep the suspension cold.

Buffer A is composed of the following: 60mM KCl, 25mM NaCl, 0.25mM spermine, 0.5mM spermidine, 25M Tris HCl pH 7.4.

In a Sorvall Omni-mixer, livers were homogenized four times, 30 seconds each time, with a 30-second "rest" between homogenations. This was done in a cold room at 4°C with homogenate kept on ice.

Fifteen milliliters underlayering buffer (1.37M sucrose, Buffer A, 1mM EDTA, 0.25mM EGTA, 15mM B-mercaptoethanol, 1mM PMSF) was pipetted into eight 40ml Sorvall centrifuge tubes. The homogenate was distributed equally among the eight tubes. This mixture was spun in a Sorvall RC-5B Refrigerate Superspeed Centrifuge at 7000 rpm

(approximately 9500xg) for 10 minutes. The supernatant was carefully decanted, and the nuclear pellet was suspended in 10ml of 24M sucrose solution (Buffer A, 2.4M sucrose, 0.1mM EDTA, 0.1mM EGTA, 15mM B-mercaptoethanol, 1mM PMSF).

Next, 10ml of the 2.4M sucrose solution was pipetted into four polyallomer Beckman centrifuge tubes. Equal amounts of the resuspended nuclear pellet were carefully pipetted into each of the four tubes and spun in a Beckman L5-65 Ultracentrifuge at 25,000 rpm (approximately 120,000xg) for one hour.

The top layer of reconstituted liver cells was carefully scraped off. The supernatant was carefully decanted, and the nuclear pellet was resuspended in 10ml of nuclei wash (Buffer A, 0.34M sucrose, 15mM B-mercaptoethanol, 1mM PMSF). This mixture was transferred to two 40ml Sorvall centrifuge tubes and spun at 7000 rpm (approximately 9500xg) for 10 minutes. After the supernatant was carefully decanted, the nuclear pellet was stored at -80°C for further use.

#### Varshavsky Particle (DNF) Gel of Mouse Liver Nuclei Preperation

A sample of mouse liver nuclei was fractionated by electrophoresis on a 7% polyacrylamide Varshavsky particle gel in 100mM triethanolamine, 20mM sodium EDTA buffer. The Varshavsky particle gel was prerun for 20h at 100V at

4°C with a continuous circulation of the buffer between the two reservoirs.

The mouse nuclei were washed once in nuclei digestion buffer (Hewish and Burgoyne Buffer A: 1.37M sucrose, 1mM EDTA, 0.25mM EGTA, 15mM B-Mercaptoethanol, 1mM PMSF). The sample was loaded on to the particle gel and run for 16h at 300V at 4°C with a continuous circulation of buffer between the two reservoirs.

Three 1.5cm x 15cm lanes were cut off lengthwise from the DNP gel.

#### 2-Dimension Gel of DNP Gel Lane

The first lane from the DNP gel was analyzed by polyacrylamide gel electrophoresis: SDS(0.1%, w/v polyacrylamide (17.5% w/v) (Laemmli, 1970) in Schreier, Erni & Staehlin buffer (Schreier, Erni & Staehlin, 1977) and run at 150V. The gel lane was run against a standard of chicken erythrocytes histones (2mg/ml). The gel was stained in 0.03% coomassie blue R-250, 45% methanol 10% acetic acid for 1h and destained in 25% methanol, 10% acetic acid for 1h in 10% acetic acid, 1% ethanol until the gel background was clear.

#### Blotting of DNP Gel Lane

A second lane from the DNP gel was soaked for 15-20 minutes in Shreier, Erni and Staehlin buffer using a 12V

power supply. The gel lane was then transblotted in transblot buffer (20mM Tris base, 150mM glycine, 20% methanol) for 30 minutes. The nitrocellulose paper from the transblot was stained for 2 to 3 minutes with 0.1% amido black, 45% methanol, 10% acetic acid and destained with three changes in Western blot Destain (50% methanol, 5% acetic acid) for 15 minutes.

Next, the membrane was blocked with PBS, 0.5% gelatin for 40 minutes.

To probe the protein blot, chicken anti-H5 antibodies were used. The probe was first diluted by a factor of 200 in PBS 0.25% gelatin, 0.02% sodium azide. The membrane was then incubated for 15.5h with this solution in a heat-sealed plastic bag. The nitrocellulose membrane was washed three times in PBS 0.02% azide, 0.1% v/v Tween for 1h with shaking.

Along with the membrane, PBS 0.25% gelatin 0.02% azide and radioactive labelled <sup>125</sup>I protein A were placed in a heat-sealed plastic bag and mixed for 5h at room temperature. The nitrocellulose membrane was then removed from the bag and washed twice in PBS 0.1% v/v Tween for 25 minutes with shaking.

Autoradiography of the nitrocellulose membrane was carried out using XS-5 X-ray film at -60°C for approximately 86h.

### DNA Precipitates of Gel Lane

A third lane from the DNP gel was cut up into fifteen one-centimeter pieces. Each piece was placed into a separate Eppendorf tube, and 1% SDS was added to each tube. The tubes were placed on a shaker table.

After approximately twenty-four hours, all 15 tubes were spun in a Brinkman Eppendorf Centrifuge 5414 for 5 minutes on dial setting 6. The liquid layer was removed and transferred to a second Eppendorf tube.

Equal volumes of phenol, 0.1 Tris pH 8.0, 100mM NaCl were added to the supernatant with shaking for 1h. The sample was then spun down in a table top centrifuge for 5 minutes at approximately 1000xg. The supernatant was carefully removed, added to 2.5 volumes of 95% ethanol, and stored at -20°C overnight.

The samples were then analyzed on a 5% polyacrylamide particle gel (DNA gel) in Tris borate buffer (0.089M Tris base, 0.089M boric acid, 2.5mM Na<sub>2</sub>EDTA). The gel was prerun at 50mA constant current for 1h at 4°C.

The DNA precipitates were spun down in a Brinkman Eppendorf Centrifuge 5414 for 10 minutes. The supernatant was decanted and the pellet was resuspended in 10% glycerol. These samples were then loaded onto the prerun DNA gel. The gel was run at 50 mA, 330V at 4°C for approximately 1.5h. Next, the gel was stained in a 1 ug/ml solution of ethidium bromide (in water) for 30 minutes.

### Bovine Liver Preparation

One whole fresh bovine liver, weighing approximately 15 pounds, was removed from the animal at Armour Meat Packing Company, Dixon, California. The liver was immediately put on ice, transported to the medical school at the University of California, Davis, and stored in a cold room at 4°C.

The liver was cut into 250g pieces which were further cut into approximately one-inch pieces.

In a one gallon capacity commercial duty Waring blender, a 250g portion of the liver was added, followed by the addition of 600ml of 5% Perchloric Acid, and blended in a cold room (4°C) at low speed for two minutes, at medium speed for one minute, and at high speed for one minute. Another 250g portion of the bovine liver was added to the blender and blended at low speed for two minutes, at medium speed for one minute, and at high speed for one minute. A third 250g portion of the bovine liver was added to the blender and blended at low speed for two minutes, at medium speed for one minute, and at high speed for one minute. The contents of the blender were poured into one-liter Sorval RC 3 centrifuge bottles.

The remaining liver was processed by repeating the procedure described above.

The homogenate in 5% PCA was centrifuged for 10 minutes in Sorval RC 3 centrifuge at 2000xg (3000 rpms).

The supernatant was carefully decanted and kept on ice in a cold room at 4°C. The volume of the supernatant was measured by using a graduated cylinder and combined with 3.5 volumes acetone and put into the 4°C cold room to precipitate the H1 and H1<sup>o</sup> chromosomal proteins.

The acetone precipitate of H1 and H1<sup>o</sup> proteins were collected by centrifugation, washed twice with reagent grade acetone, followed by centrifugation, and dried under a vacuum for further use.

#### Purification of H1 and H1<sup>o</sup> from Bovine Liver

A BioRex 70 anion exchange column was washed in 40% w/v GuCl, .1M KPO<sub>4</sub> pH 6.8 and was equilibrated to pH 6.66 in UV transparent 8% GuCl (refractive index = 1.3508) pH 6.8 in 0.1M NaPO<sub>4</sub>. A Pharmacia P3 three channel peristaltic pump was used to set the flow rate at 20 ml/h and an Isco fraction collector was used to collect 140 drop samples.

Approximately 2.52g of the H1/H1<sup>o</sup> bovine liver perchloric acid liver extract acetone precipitate was dissolved in 0.1M KPO<sub>4</sub>, 8% GuCl pH 6.8 and loaded onto the column. The gradient used was 8.2% GuCl (refractive index = 1.3496) .1M NaPO<sub>4</sub> pH 6.8 to 15.8% GuCl (refractive index = 1.3622) .1M NaPO<sub>4</sub> pH 6.8.

The column was run for approximately 40h. The absorbance of each fraction from the BioRex 70 column were read at 230nm on a Gilford Spectrophotometer 250, using a one-centimeter quartz cuvette to hold the samples. The fraction number versus the absorbance was plotted on a graph.

The fractions were then analyzed by polyacrylamide gel electrophoresis: SDS (0.1%, w/v), polyacrylamide (17.5%, w/v) (Laemmli, 1970) in Schreier, Erni & Staehlin buffer (M. Schreier, Erni & Staehlin, 1977) and run at 150V. The gel was silver stained using chicken erythrocyte histones as the standard. The procedure for silver staining was as follows: (1) the gel was soaked for 1 hour in 50% methanol on shaker; (2) Solution A (.8g silver nitrate in 4ml distilled water) was added dropwise to Solution B (21mls .36% (w/v) sodium hydroxide mixed with 1.4 ml of 14.8M ammonium hydroxide) to make Solution C; (3) the gel was stained in Solution C for 15 minutes with constant shaking in Solution C; (4) it was then washed in water with gentle agitation for 5 minutes and developed in Solution D (2.5 ml 1% citric acid, 0.25 ml 38% formaldehyde, water to 500 ml); (5) the development was stopped by placing the gel in 50% methanol (with or without 10% acetic acid).

Fractions were then pooled and analyzed against 1% acetic acid in pre-prepared dialysis bags with a molecular

weight capacity of 11,000g/mole. The sample of H1 was lost, and the volume of H1<sup>o</sup> was greatly reduced in the dialysis tubing. The H1<sup>o</sup> was then lyophilized.

## RESULTS

### Purification of H1 and H1<sup>o</sup> from Lamb Thymus

Fig. 1 shows the elution profiles of lamb thymus H1 and H1<sup>o</sup> proteins. The graph indicates that the H1 peak was around fraction number 160 (A<sub>220</sub> = 2.869). The H1<sup>o</sup> peak was around fraction number 180 (A<sub>220</sub> = 1.020).

Fig. 2 analysis by polyacrylamide gel electrophoresis confirms the previous observations and illustrates the identity and purity of the H1 and the H1<sup>o</sup> proteins. The three top bands on the left of the picture represent Histone H1; the two bottom bands to the left of these three represent Histone H1<sup>o</sup>. It was from the combined information obtained from the graph and the gel pattern that the fractions were pooled correctly.

# CM-25 SEPHADEX H1/H1<sup>o</sup>

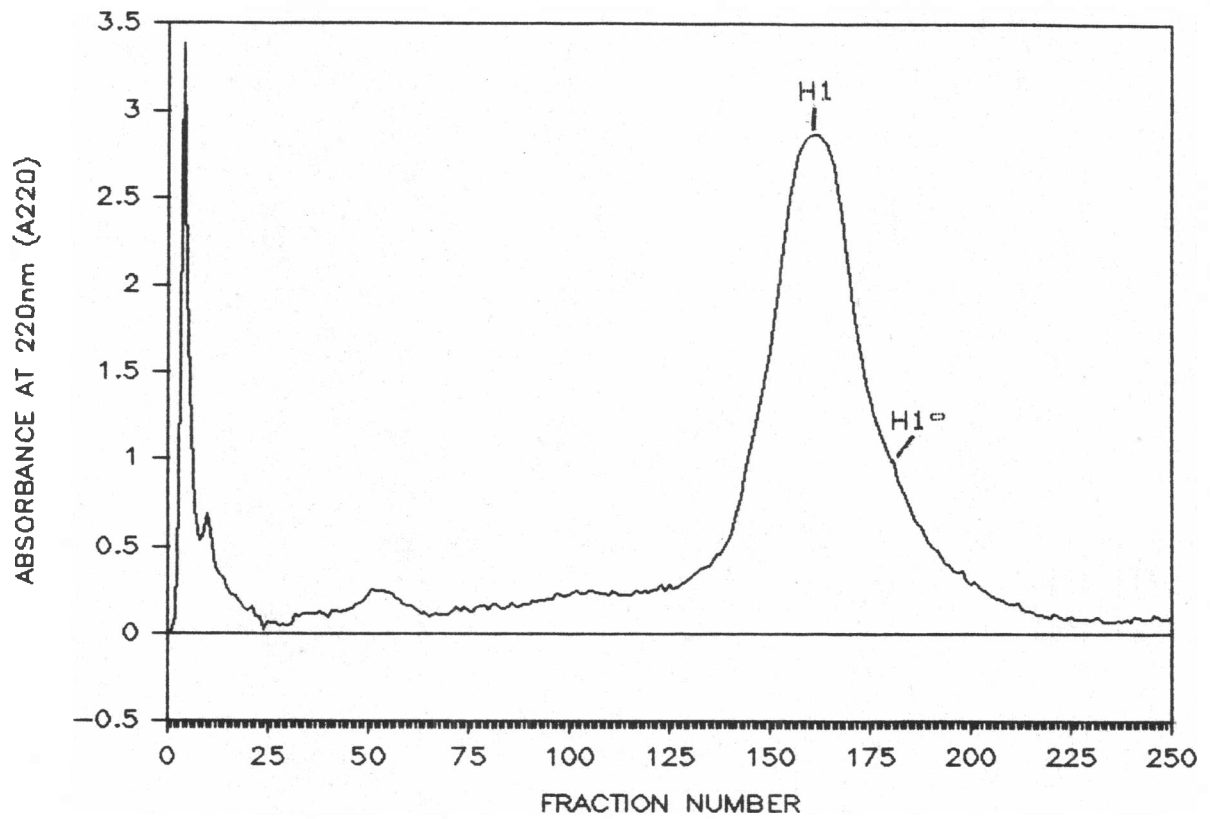


Fig. 1. Elution profiles of histones H1 and H1<sup>o</sup> from CM-25 Sephadex column of lamb thymus preparation.

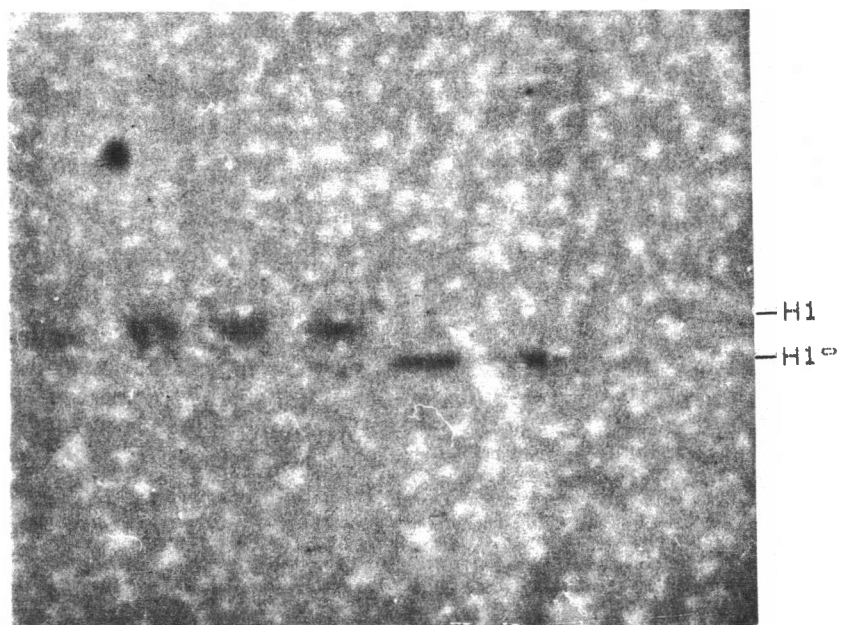


Fig. 2. SDS polyacrylamide gel showing histones H1 and H1<sup>0</sup> from lamb thymus preparation.

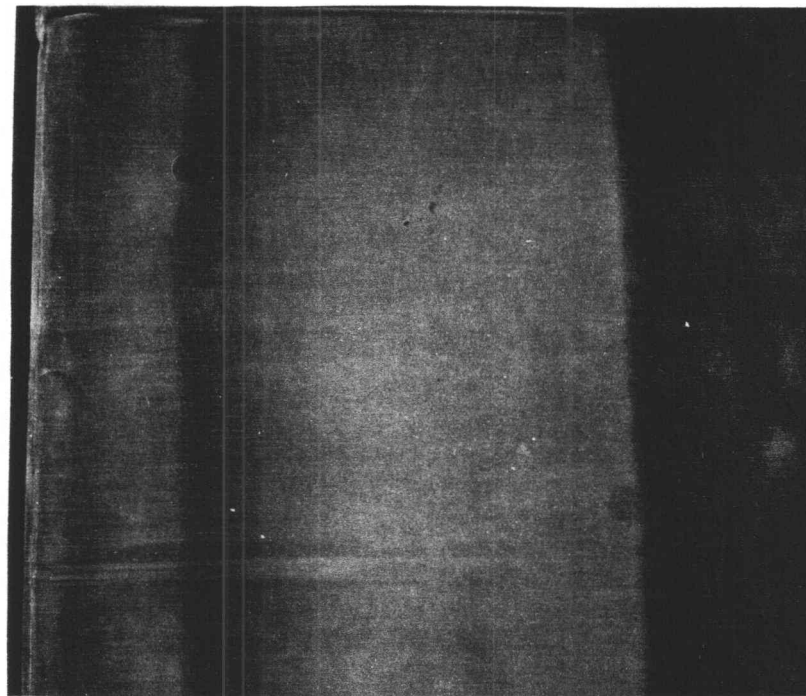
## DNA Associated with Histone H1<sup>o</sup>

Fig. 3 shows the first dimension of a 2-dimension DNP gel of the digest of adult mouse liver nuclei. This Varshavsky particle gel illustrates the separation of DNA into monomers, dimers, trimers, higher order structures and free DNA.

The second dimension of the 2-dimension DNP gel is pictured in Fig. 4 and further characterizes what is happening to the H1 and H1<sup>o</sup> chromosomal proteins. This polyacrylamide gel of the DNP gel lane demonstrates that there is a separation between the monomers and the dimers in the migration of the H1 and the H1<sup>o</sup> histones.

The result from the transblotted and immunoblotted DNP gel lane is shown in Fig. 5. The anti-chicken H5 antibodies labelled the area of monomers and dimers on the strip of the DNP gel, indicating the presence of histone H1<sup>o</sup>.

In Fig. 6 the DNA extracted from the third DNP gel lane from the mouse nuclei DNP gel is shown with the known DNA lengths of the HAE III PM-2 standard. When Fig. 4, Fig. 5 and Fig. 6 are compared, it can be seen that the chromosomal protein, histone H1<sup>o</sup>, is associated with longer lengths of DNA, approximately 146 to 200 nucleotide base pairs, which are longer than those DNA lengths typically associated with the core particle.



Trimers

Dimers

Monomers

Fig. 3. First dimension Varshavsky particle gel of mouse liver nuclei preparation. The direction of migration is from left to right.

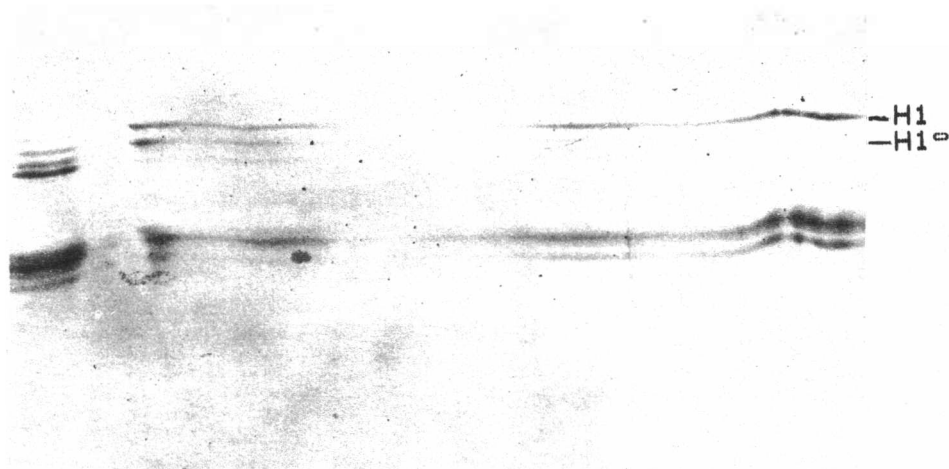


Fig. 4. Second dimension SDS polyacrylamide gel of mouse liver nuclei preparation. The chicken erythrocyte standard is visible in far left lane.

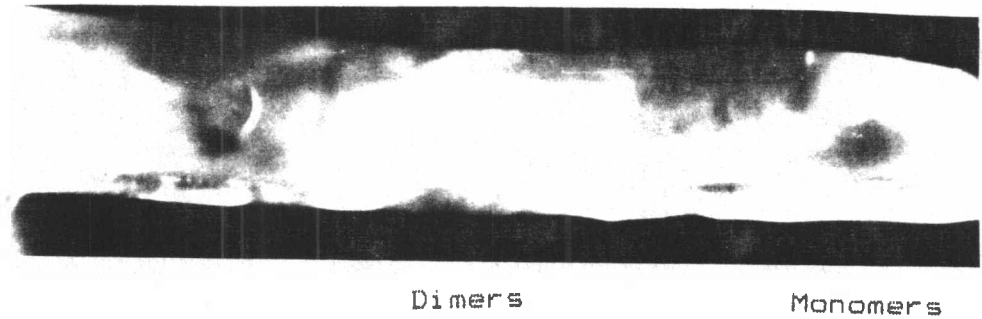


Fig. 5. Transblotted and immunoblotted DNP gel lane showing H1<sup>o</sup> protein is labelled at migration levels of monomers and dimers.

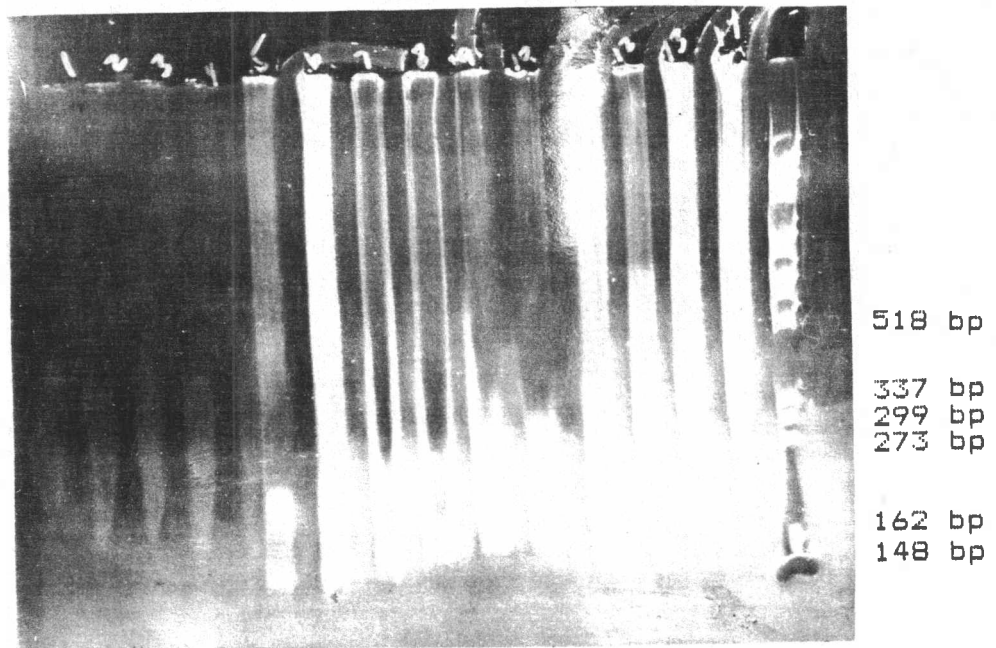


Fig. 6. DNA gel of mouse liver nuclei extracts from DNP gel lane. HAE III PM-2 standard is visible in far right lane. DNA lengths are expressed in base pairs (bp).

### Bovine Liver Preparation

Fig. 7 shows the elution profiles of the bovine liver H1 and H1<sup>o</sup> histone proteins. The graph indicates that there are a lot of other proteins which are eluted with the first fractions in addition to the H1 and the H1<sup>o</sup> proteins which eluted with the later fractions. Also the H1 peak was seen around fraction number 65 (A220 = 1.093); a weak H1<sup>o</sup> peak can be seen around fraction number 80 (A230 = .369). In Fig. 8 analysis by polyacrylamide gel electrophoresis for identification of the proteins being eluted showed that histone H1 actually eluted from the BioRex 70 column around fraction number 86 (A230 = .199) and that histone H1<sup>o</sup> eluted from the column around fraction number 135, which gave a negative absorbance. Also, other subtypes of the histone family, H1a and H1b were visible after electrophoresis; in Fig. 8 they are the band seen directly under the H1 band in the first SDS polyacrylamide gel. The H1<sup>o</sup> band can be seen lying below the H1 band in the second SDS polyacrylamide gel of Fig. 8. The fractions were pooled according to the electrophoretic results. Furthermore, the results from the polyacrylamide gel electrophoresis illustrate the relative purity of these two chromosomal proteins.

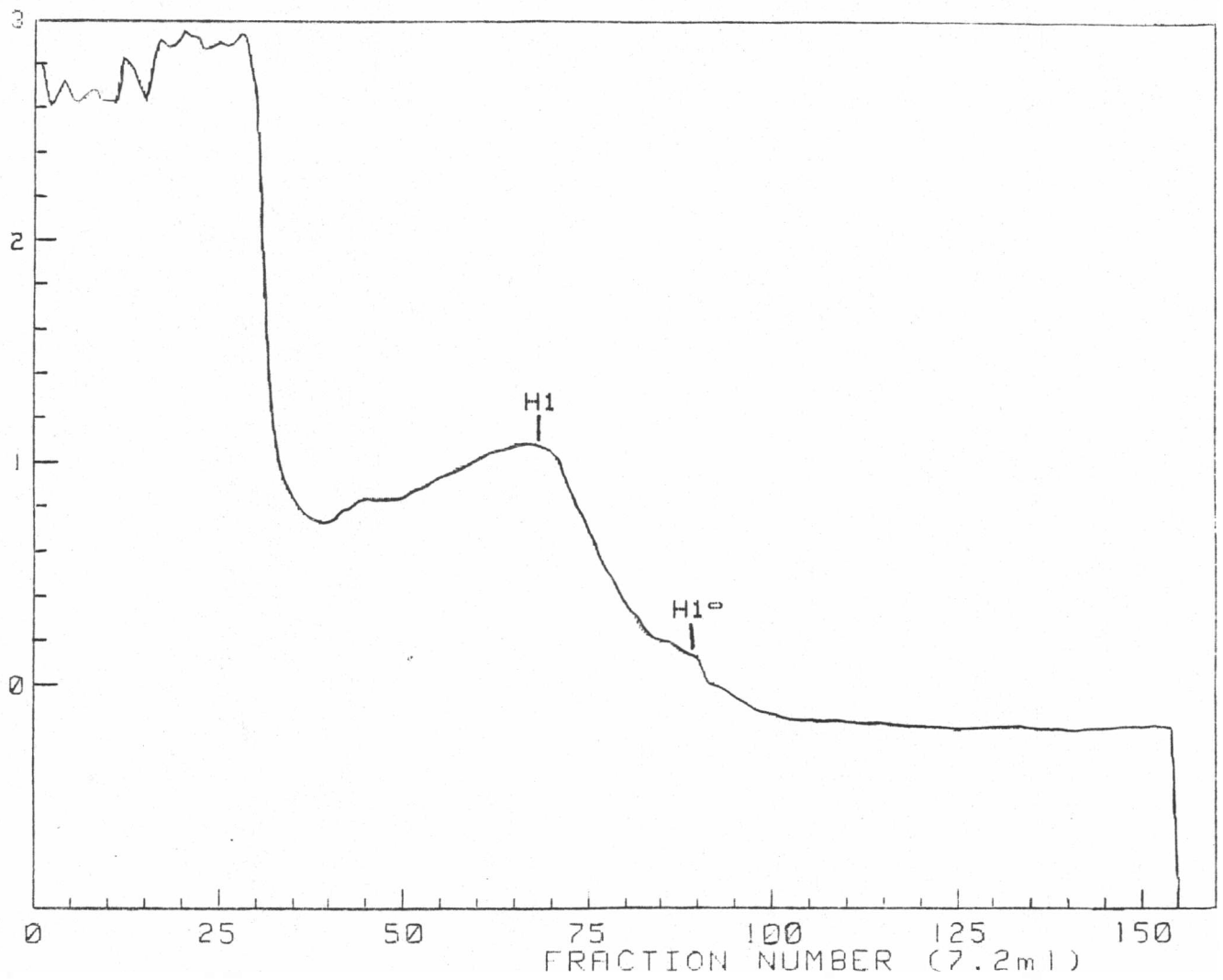
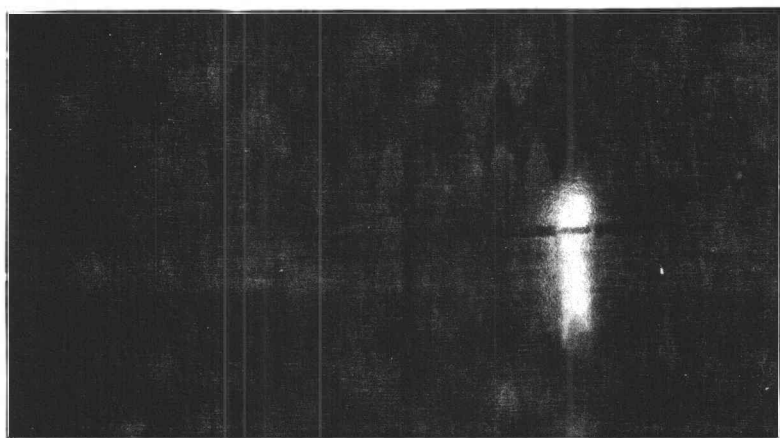


Fig. 7. Elution profiles of histones H1 and H1<sup>0</sup> from BioRex 70 column of bovine liver preparation.



-H1  
-H1a/H1b



-H1  
-H1<sup>o</sup>

Fig. 8. Two SDS polyacrylamide gels showing histones H1 and H1<sup>o</sup> from bovine liver preparation.

## DISCUSSION AND CONCLUSION

Histones H1 and H1<sup>o</sup> were isolated and fractionated from lamb thymus and bovine liver. The purification of these proteins was achieved by using two different ion exchange columns. Analysis by polyacrylamide gel electrophoresis showed that these histones were relatively pure. However, there was very little H1<sup>o</sup> protein obtained and no H1 protein obtained from the bovine liver preparation. For some unknown reason the H1 leaked out of the dialysis bag and much of H1<sup>o</sup> was lost; therefore, no H1 and very little H1<sup>o</sup> was recovered.

These purifications were to be used in preparing monoclonal and polyclonal antibodies and in other types of experimentation. The antibodies will serve as probes against the various subtypes of H1. Also the monoclonal antibodies would provide for the specific identification and labelling of the various subtypes of H1.

In addition the mouse liver nuclei preparation and the subsequent experiments showed that the H1<sup>o</sup> chromosomal protein is associated with longer length DNA stretches, typically 146 to 200 nucleotide base pairs. Locating the position of histone H1<sup>o</sup> on the DNA strand

is important to determining its function in chromatin. Moreover, further experimentation to reveal the specific gene association of histones H1 and H1<sup>o</sup> will be conducted.

Furthermore, other researchers have shown histone H1<sup>o</sup> to be associated with decreased cell growth in culture, mitotic activity, DNA synthesis, and cloning efficiencies. This decrease may be correlated with presence of H1<sup>o</sup> and could possibly be linked to Progeria, a disease causing rapid aging of children. Only time and continued research will determine the importance of the H1 family in chromatin.

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