CRC

CHROMOSOMAL NONHISTONE PROTEINS

Volume II
Immunology

Lubomir S. Hnilica

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Chromosomal Nonhistone Proteins

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Immunology

Editor

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INTRODUCTION

The second volume of the *Chromosomal Nonhistone Proteins* treatise is dedicated in its entirety to the immunobiology of these proteins. Since immunological methods are adding rapidly to our knowledge of the specificity and intranuclear localization of chromosomal nonhistone proteins, a collection of chapters dealing with various aspects of this important area was judged appropriate. The reader can select a variety of topics dealing with techniques, cell and tissue specificity, autoimmune disease, virus associated nuclear antigens, and interesting localizations of select chromosomal proteins to transcriptionally active sites on polytene chromosomes. It is certain that immunological methods will contribute immensely to future inquiries into the complex puzzle of chromatin structure and function.

THE EDITOR

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Chapter 1

IMMUNOCHEMICAL METHODS IN NONHISTONE PROTEIN RESEARCH

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I. INTRODUCTION: HETEROGENEITY OF NONHISTONE PROTEINS

Chromosomal proteins have been assigned to two major categories, histones and nonhistones. The five common histones are relatively small basic proteins which are almost universally found in chromatin, and exhibit limited heterogeneity between all eukaryotic cells.[1-3] Yet only recently have their roles in chromatin structure become

very well understood.[3-4] The nonhistone chromosomal proteins are generally larger and neutral or acidic in overall charge. They are also far more heterogeneous in quantities and numbers than are the histones. Further, only a few specific functions have been assigned to any of them; rather they are understood largely in terms of their associations with nuclear structures and fractions.

Though nearly all eukaryotic cells contain histones in about a 1:1 ratio by mass with DNA,[2] total quantities of nonhistone protein range widely from as little as 0.25:1 in chicken erythrocyte chromatin,[5] to greater than 2.4:1, in HeLa chromatin.[2] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) may reveal only 20 to 115 bands of nonhistone proteins,[1] but expansion to two dimensions, isoelectric focusing followed by SDS-PAGE, has allowed visualization of 450 nonhistone protein species in HeLa chromatin.[6]

The great heterogeneity of the nonhistone proteins and the inability to readily determine their biological activities has made it difficult to identify and follow individual proteins with respect to distributions in different tissues and states of differentiation. Also difficult to ascertain is whether proteins that migrate similarly in electrophoretic separations and are found in different nuclear fractions are actually the same or different proteins. In some cases the presence of specific nuclear proteins only in certain cell types and differentiation states has been used to suggest their probable involvement in cellular differentiation.[7] Proteins common to all or many classes of cells may have roles in regulation of commonly expressed genes or shared nuclear structures and functions.

To study an individual protein in a complex mixture of proteins one must have a means of identifying that protein. The best properties by which to identify individual proteins are intrinsic enzyme or binding activities unique to each. However, many nuclear proteins may be of interest although their unique intrinsic properties are unknown. Two approaches widely used to study such proteins have been to use polyacrylamide gel electrophoresis[6,8,9] and immunological methods.[7] The former method has the disadvantage that numerous proteins in crude preparations can have similar mobilities, particularly in a single dimension. Two-dimensional methods involving isoelectric focusing have greatly expanded the resolving power of electrophoretic methods. One disadvantage in its application is that the mobility of a single protein can be shifted by modifications, thereby complicating the interpretation of experimental results where new proteins may also appear or some may be lost. Although two-dimensional gels may be used to identify interesting and specific proteins, comparison of spots between many preparations may be rather tedious and time consuming in routine small scale experimentation.

Immunological methods provide means by which specific proteins can be identified, characterized, and localized in the presence of a multitude of other proteins. In spite of the fact that identification of the intrinsic activities associated with interesting proteins may not be immediately possible, marker activities specific to each can be "created" by eliciting antibodies to them. The most frequently used immunological methods include

1. Identification of antigenic proteins separated by polyacrylamide gel electrophoresis
2. Complement fixation (Cf)
3. Radioimmunoassay (RIA)
4. Immunocytochemical localization
5. Immunodiffusion

The first category is particularly useful for identifying antigens using antisera prepared

to heterogeneous fractions of proteins. It can be used to obtain information about the distributions between subcellular fractions and tissues of numerous antigens simultaneously. If a standard preparation of a given antigen is available it can also be used for quantitation.

Complement fixation is dependent on the arrangement in space of bound antibodies and is therefore useful for studying antigen conformation. This plus the fact that it is also dependent on antibody affinity make it useful for studying antigenic differences between similar proteins.

Radioimmunoassay has been the method of choice for antigen quantitation for many years; however, until the recent development of solid phase methods it was not applicable to chromosomal proteins. Because of its dependence on antibody affinity this test is also able to distinguish between related antigens.

Immunocytochemical localization methods are widely used to study the subcellular localizations of antigenic proteins. However, these methods are difficult to control and do not easily provide for quantitation.

Immunodiffusion methods have limited applicability to chromosomal protein research because of the difficult solubility characteristics of most chromosomal proteins and the observation that antibodies to certain fractions often do not immunoprecipitate with antigen.[10]

Each of these methods and their applications to chromatin research is reviewed in this chapter. Certain of the methods, which we commonly use, are described in detail. Preparative methods vary greatly between different investigators and since this may have relevance to the observed results, those used by us are also described in detail.

II. IDENTIFICATION OF ANTIGENS SEPARATED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

Although these methods are among the most recently developed they are discussed first because they allow the detection of different antigens simultaneously in crude preparations. Some physical properties, such as apparent molecular weight in sodium dodecyl sulfate (SDS) or pI are immediately available for individual antigens. The ability to identify electrophoretically separated antigens is particularly valuable if one wishes to screen an antiserum against a crude preparation of proteins to determine if any activities display interesting correlations.

Recently, identification of antigenic proteins separated by polyacrylamide gel electrophoresis has been greatly simplified. Most of the older methods involved lengthy periods of diffusing antisera into and out of the gels. However, the newer methods involve replicate transfer of the separated proteins from gels onto nitrocellulose sheets[11] or diazobenzylomethy-paper[12] (DBM-paper) followed by immunochemical detection of antigenic species. Several variations of the "immunotransfer" methods exist; however, all of these greatly shorten the time required to identify antigenic proteins.

The immunotransfer method that we use to identify nonhistone protein antigens[13] is adapted from Towbin et al.[11] Proteins are separated by SDS-PAGE and transferred electrophoretically onto nitrocellulose sheets. Remaining protein binding capacity on the nitrocellulose is saturated with bovine serum albumin (BSA) and other serum proteins. Antigens are immunochemically localized by the peroxidase anti-peroxidase (PAP) method.[14] The preparative and immunological methods are described first and the applications follow. The other immunotransfer methods and their application to nonhistone protein research are then discussed. Finally, the older approaches to identifying antigens separated by polyacrylamide gel electrophoresis are briefly reviewed.

A. Preparative Methods

Our experience has been that clean purified nuclei are essential for obtaining tissue specific antisera to nuclear proteins. For this reason the methods used to prepare dehistonized chromatin for immunization of rabbits are detailed here.

1. Purification of Nuclei

The methods available for the purification of nuclei vary somewhat with cell type. However, the early methods of Blobel and Potter[15] and Chauvea et al.[16] developed for the isolation of rat liver nuclei can be adapted, with minor changes, to prepare nuclei from most cell types with the exception of some blood cells.[17-20] The changes are primarily in the conditions required for disrupting cells.

a. Materials

The apparatus used for homogenizations consists of a 200 m*l* glass-Teflon® homogenizer (Glenco Scientific, Inc.) powered by a 3/8 in. electric drill mounted on a drill press (Sears Craftsman® Model No. 335.25926). The press is secured to the bench with one or more lead bricks. The drill speed is regulated with a variable transformer. The clearance of the tight pestle should be 0.01 in. and that of the loose pestle 0.03 in. The homogenizer should be cooled by placing it into a plastic container of ice and water.

The choice of buffer varies with cell type. The buffer chosen depends on the relative fragilities of the whole cells compared to their nuclei. Normal rat livers (also applicable to most other organs) are minced and homogenized in 0.25 *M*, 10 m*M* Tris-HCl (pH 7.5), 25 m*M* KCl, 5 m*M* $MgCl_2$ termed STKM buffer. 13762 MAT-B cells (and many other tissue culture cell lines) yield intact nuclei in 0.25 *M* sucrose, 10 m*M* Tris-HCl (pH 7.5). Novikoff ascites hepatoma cells, and many other tumor cells, are particularly resistant to disruption and so require homogenization in 10 m*M* Tris-HCl (pH 7.5). Immediately before use, 100 μ*l* of 100 m*M* phenylmethylsulfonyl fluoride (PMSF) in isopropanol is added to each 100 m*l* of the homogenization buffers.

Heavy sucrose is prepared by dissolving sucrose and salts with low heat on a magnetic stirrer to give 2.3 *M* sucrose, 50 m*M* Tris-HCl (pH 7.5), 25 m*M* KCl, and 5 m*M* $MgCl_2$.To obtain intact nuclei, free of debris, most cell types, particularly Novikoff hepatoma cells, must be fresh (never frozen). Further, preparation of nuclei needs to be monitored continuously by phase contrast microscopy to achieve good yields of purified nuclei.

b. Procedure

Cells or minced tissues are washed in 10 m*M* sodium phosphate (pH 7.2) buffered saline (PBS). The washed cells are disrupted in 10 volumes of the appropriate homogenization buffer by several passes of the tight pestle in the homogenizer. Extent of cell disruption is determined by phase contrast microscopy; homogenizations are continued until at least 80% (routinely greater than 95%) of the nuclei are released from the cells. Homogenizations should be discontinued if nuclei begin to break or clump to a significant degree. Tissue homogenates are filtered through several layers of cheesecloth to remove connective tissue. The homogenates are centrifuged at 1000 × g for 10 min. Pellets from tissues homogenized in STKM are homogenized directly into heavy sucrose; pellets prepared in other buffers are generally resuspended in STKM and pelleted again. The pellets are homogenized in about five times the original packed cell volume (or tissue weight) of heavy sucrose with a loose pestle. Homogenizations should be continued until nuclei and cells are well dispersed. The homogenates are poured into cellulose nitrate centrifuge tubes to about two thirds total volume. Formation of sharp interfaces, which could trap nuclei, are avoided by pouring fresh heavy sucrose

through the homogenates to fill and balance the tubes. The purified nuclear pellets are obtained by centrifugation in a swinging bucket rotor (SW27 or SW28, Beckman Instruments) at 100,000 × g for 1 hr.

Some tissues or cells require special treatments to obtain good yields of clean nuclei. In general, tissues of more fibrous nature require an initial homogenization with blades to mince the connective tissue and break the cells. When this is done in 10 volumes of STKM, the nuclei usually remain intact. We have found the Virtis® homogenizer the most effective blade type instrument. Although conditions vary with material, usually about half maximal power output for 1 to 3 min is sufficient treatment. The extent of breakage is monitored in the phase contrast microscope after every 15 sec of homogenization. The treatment is stopped once the majority of nuclei are freed from cells and before the nuclei show signs of damage.

The homogenate is filtered and nuclei purified by centrifugation through heavy sucrose as described above.

Tissue culture cells that grow in suspension require some modifications to achieve good levels of cell breakage without disrupting the nuclei. Homogenization in STKM was not found to be effective in breaking the cells as it is in the case of liver tissue. We have found that these cells are easily lysed in 0.25 *M* sucrose 10 m*M* Tris-HCl pH 7.5. However, the nuclei are very fragile and also break along with the cells. Once this occurs the chromatin and cytoplasm are not easily separated. A more efficient method involves washing (vortex mixing) the cells two times with 60 volumes of STKM or 0.25 *M* sucrose, 5 m*M* $MgCl_2$. These cells are then suspended in 30 volumes of 10 m*M* Tris-HCl pH 7.5, 1.0 m*M* $MgCl_2$ by vortex mixing. Many cells usually break at this stage and nearly complete breakage is achieved with additional incubation on ice (30 min) combined with more vortex mixing. Under these conditions, nuclei and cytoplasmic materials are well dispersed. Nuclei should remain intact, and if whole cells or nuclei with contamination remain, the material can be mechanically homogenized with a loose fitting pestle. If nuclei tend to break during the procedure, the Mg^{++} level can be increased slightly, but it has never required more than 2 m*M* $MgCl_2$. Once nuclei are clean, as determined by phase contrast microscopy, an equal volume of 2.2 *M* sucrose, 5 m*M* $MgCl_2$ is mixed into the homogenate. The nuclei are then purified by centrifugation through fresh heavy sucrose as described above. Nuclei should not be concentrated by low speed centrifugation while they are in 10 m*M* Tris-HCl, 1 m*M* $MgCl_2$ as this causes them to break and allows the chromatin to aggregate with cytoplasmic materials. If this occurs it is not possible to resuspend the material well enough to achieve a good separation in a subsequent heavy sucrose step.

The blood cells have required completely different approaches to obtain good yields of clean nuclei. Chicken erythroid cell nuclei have been especially difficult to isolate free from physical cytoplasmic contamination. The most effective approach involves the use of nitrogen cavitation.[17] About 80 m*l* of blood can be drawn from a normal chicken by heart puncture into a syringe containing 20 m*l* of SSC, 0.01% heparin. When the drawing is completed, the blood is diluted to 200 m*l* with SSC, 0.01% heparin. After centrifugation (700 × g, 10 min) the buffy coat is removed by aspiration and discarded.

The erythroid cells are washed two additional times in saline, 5 m*M* KCl, 7.5 m*M* $MgCl_2$ and additional buffy coat aspirated off. The erythroid cells are diluted to 400 m*l* in saline, 1 m*M* $CaCl_2$ and placed in a cell disruption bomb (Parr Instruments Co., Moline, Ill.). All operations are performed at 4°C and the cavitation should be done in a cold room. The cells are allowed to equilibrate at 1250 psi for 30 min with continuous stirring. The cell suspension is released from the bomb through the outlet, and nuclei and whole cells are collected by centrugation in a conical tube (700 × g, 10 min). The whole cells form a red layer on the bottom of the pellet and the nuclei form a

white band above it. The nuclei can be aspirated off from the whole cell layer. Washing in saline, 5 m*M* KCl, 7.5 m*M* $MgCl_2$ and aspiration of the nuclear layer is continued until no whole cell contamination is visible before proceeding to the wash with Triton® X-100 as described below. It should be noted that we have had little success in applying this method to cell types other than chicken erythroid.

The human neutrophilic peripheral blood granulocyte was an unusually difficult cell nucleus to isolate. In this case, the main source of contamination was not microscopically visible as in the chicken erythrocyte, but originated from soluble proteins binding to nuclear materials during isolation. With these cells it was not only necessary to isolate clean nuclei, but the cells required pretreatments with particular fixatives in order to obtain nuclei free from cytoplasmic proteins.[20] The granulocytes were isolated from units of freshly collected citrate anticoagulated whole blood by dextran sedimentation of red cells and removal of the mononuclear cells by Isopaque-Ficoll separation. The granulocytes were washed in PBS and collected by low speed centrifugation (700 × g, 10 min). The cell pellets were freeze-dried and then treated with 5 volumes of formaldehyde(4%)-acetone for 1 min. The fixative was poured off and cells dispersed in PBS using a loose fitting Teflon® pestle in a glass homogenizer. The cells were collected by centrifugation (200 × g, 10 min) and this wash was repeated. The cell pellets were washed twice in 0.25 *M* sucrose, 10 m*M* Tris-HCl, pH 7.5 and then dispersed in 200 volumes of 0.2% CPC (cetylpyridinium chloride). Normally at this point, nuclei are freed intact from an unfixed cell during the process of being dispersed in this solution of cationic detergent. However, cells that are fixed required sonication (usually 30 to 60 sec of sonication, administered at 10-sec intervals) to achieve release of clean intact nuclei. The CPC method has been successfully employed with many different cells that grow in a suspension (suspension cultures, ascites cells and blood cells). One limitation to more general application of this method in our laboratory has been the inability to use the complement fixation test with nuclear materials exposed to CPC. Chromatins prepared from CPC isolated nuclei consistently exhibit strong anticomplementarity activity. Our use of this cationic detergent with the fixed granulocytes was out of necessity since no other aqueous method was suitable with the fixed cell. The nonaqueous methods were attempted, but nuclei could not be separated from cytoplasmic materials.

With the exception of the chicken erythroid cells and human granulocytes the sucrose method has been generally applicable in our research for isolating clean nuclei in good yields. The main attractive feature of this approach is the ability to use a heavy sucrose step to separate cytoplasmic components, and whole cells from the isolated nuclei. The separation of whole cells from nuclei is not possible when detergent methods have been used for breaking cells. This type of contamination is especially important to avoid in immunological studies of chromatin.

2. Preparation of Chromatin

The procedure for preparing chromatin is based on methods previously described.[21] Although the extents to which some preparations need to be homogenized may vary, the procedure is the same for all cell types studied.

a. Materials

A smaller glass-Teflon® homogenizer is used to prepare chromatins instead of the 200 m*l* one used to prepare nuclei. The pestle clearances should be 0.008 and 0.02 in. Buffers are 0.25 *M* sucrose, 10 m*M* Tris-HCl (pH 7.5), 0.5% Triton® X-100 (STT); 10 m*M* Tris-HCl (pH 7.5); 80 m*M* NaCl, 20 m*M* EDTA at pH 6.3 (SE); and 1.4 m*M* NaCl, 0.14 m*M* Na_3 citrate at pH 7.0 (1/100 SSC). Each buffer should contain 0.1 m*M* PMSF added just before use.

b. Procedure

Purified nuclei are suspended in 5 volumes (volume, milliliter, per original tissue volume, milliliter, or weight, gram) of STT and homogenized with a tight pestle until well dispersed. The Triton® X-100-extracted nuclei are centrifuged at 100 × g for 10 min and suspended in the same volume with 10 m*M* Tris-HCl (pH 7.5). The nuclei should be homogenized until they begin to rupture releasing chromatin. The number of passes of the pestle is highly variable with the source of the nuclei. The swollen, broken, and clumped nuclei are centrifuged at 3000 × g. The pellets are suspended in SE buffer to a volume equal to that of STT buffer used before, and thoroughly homogenized. In this buffer nuclei become much less swollen and usually begin to break and clump extensively. The nuclei are centrifuged at 3000 × g for 10 min and resuspended in SE buffer (one half the previous volume) and thoroughly homogenized again. After centrifugation at 7000 × g for 10 min the pellets are once more resuspended in SE buffer (with a further reduction of volume by one half), homogenized, and pelleted at 15,000 × g for 10 min. Chromatins are suspended in 1/100 SSC to a volume equal to the first SE wash and allowed to hydrate on ice for 10 min. The hydrated chromatins are homogenized by several passes of the tighter pestle and centrifuged at 30,000 × g for 20 min. Chromatins should be resuspended to concentrations of about 1 mg/m*l* as DNA. This can be estimated by dissolving 0.1 m*l* of chromatin into 1.9 m*l* of 5 *M* urea and determining the absorbance at 260 nm (chromatin at 1 mg DNA/m*l* under these conditions would give an absorbance reading of 1.0).

3. Dehistonization of Chromatin

Histones and some of the nonhistone proteins are extracted from chromatin with 5 *M* urea, 2.5 *M* NaCl, at either pH 5.0 or 6.0. The buffers that have been employed include: 0.05 *M* sodium acetate pH 6.0,[21] 10 m*M* potassium phosphate pH 6.0,[22] 50 m*M* sodium phosphate buffer pH 6.0,[23] sodium succinate buffer, pH 5.0.[24] The buffer most commonly employed for these studies is 10 m*M* K H_2PO_4 pH 6.0 with the addition of 0.067 m*M* $MgCl_2$.[22] A certain major group of nonhistone proteins is not dissociated from chromatin[24] under these conditions. This may either be due to precipitation of some proteins at this reduced pH or to a tight DNA binding mechanism operating directly on DNA or through other DNA bound proteins.

a. Materials

To dehistonize chromatin a magnetic stirrer and a glass homogenizer with a loose fitting Teflon® pestle (as described above) are used. Stock dehistonization buffer[22] is 3.0 *M* NaCl, 7.5 *M* urea, 0.1 m*M* $MgCl_2$, 15 m*M* KH_2PO_4 at pH 6.0.

b. Procedure

Chromatins are diluted to 0.5 or 0.6 mg/m*l* as DNA with deionized water and mixed with 2 volumes of stock dehistonization buffer. After stirring for 2 to 3 hr at 4°C the dehistonized chromatins are pelleted by centrifugation at 100,000 × g for 36 hr. The supernatants are poured off and the pellets are gently rinsed with deionized water. The pellets are suspended to 1 to 2 mg/m*l* in 2 m*M* Tris-HCl pH 7.5 by homogenization with a loose pestle and stirring overnight at 4°C. Undissolved materials are removed by centrifugation at 1000 × g for 5 min.

B. Immunological Methods

Although the immunological methods detailed in this chapter are applied to a specific case, that of antibody to dehistonized chromatin preparations, the general approach of raising antisera to crude fractions has been used with many other cell or nuclear fractions. Particularly fruitful has been studies on plasma membrane antigens.

Table 1
IMMUNIZATION SCHEDULES

WK	Adjuvant	Mode	Total dose (μg DNA)	Bleeding
Rabbits A & B				
0	—	—	—	Preimmune
0	Complete	ID,TP	300	—
1	Complete	ID,TP	300	—
2	—	—	—	First immune
2	Incomplete	ID	300	—
3	Incomplete	ID	300	—
4	—	—	—	Second immune
4	Incomplete	ID	300	—
5	Incomplete	IM	300	—
6	—	—	—	Third immune
Rabbits C & D[a]				
0	—	—	—	Preimmune
0	Complete	ID,TP	300	—
1	Complete	ID,TP	300	—
2	Incomplete	ID	300	—
3	Incomplete	ID	300	—
4	—	—	—	First immune
4	Incomplete	IM	300	—
8	None	IV	300	—
9	—	—	—	Second immune
12	None	IV	300	—
13	—	—	—	Third immune

Note: Immunizations were administered according to the above schedule. Intradermal (ID) and intramuscular (IM) injections were delivered as small doses at multiple sites. Toe pad (TP) injections were administered to each of the toes on only one hind foot in a given week. Intravenous injections (IV) were prepared by mixing dehistonized chromatin in deionized water with a 10X saline solution.

[a] Rabbit D was injected according to the same schedule except that the total dose was in each case 600 g of dehistonized chromatin.

1. Immunizations

Schedules followed in immunizing rabbits with dehistonized chromatin are shown in Table 1. Injections were prepared by thoroughly emulsifying dehistonized chromatin (1 mg/mℓ) with complete or incomplete adjuvant. Intravenous injections were prepared by adding 10X saline to dehistonized cbromatin to give solutions 1X in saline. Modes of injection included intradermal, toepads of the hind feet, intramuscular, as well as intravenous. Antisera were collected by ear bleeding unless rabbits were to be sacrificed, in which case blood was collected by heart puncture. Sera were separated from cells by allowing coagulation to proceed at room temperature for 30 min then at 4°C overnight followed by centrifugation at 1000 × g for 10 min.

2. Electrophoretic Separation of the Proteins

The discontinuous SDS-PAGE method of Laemmli[25] with samples prepared as described previously[13] or by deoxyribonuclease I (DNase I) digestion provides high resolution separation of chromosomal proteins without extraction of DNA.