# Methods in Cell Biology

## VOLUME XVII

*Chromatin and Chromosomal Protein Research. II*

*Edited by*

GARY STEIN and JANET STEIN

LEWIS J. KLEINSMITH

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# PREFACE

During the past several years considerable attention has been focused on examining the regulation of gene expression in eukaryotic cells with emphasis on the involvement of chromatin and chromosomal proteins. The rapid progress that has been made in this area can be attributed largely to development and implementation of new, high-resolution techniques and technologies. Our increased ability to probe the eukaryotic genome has far-reaching implications, and it is reasonable to anticipate that future progress in this field will be even more dramatic.

We have attempted to present, in four volumes of *Methods in Cell Biology*, a collection of biochemical, biophysical, and histochemical procedures that constitute the principal tools for studying eukaryotic gene expression. Contained in Volume 16 are methods for isolation of nuclei, preparation and fractionation of chromatin, fractionation and characterization of histones and nonhistone chromosomal proteins, and approaches for examining the nuclear-cytoplasmic exchange of macromolecules. This volume (Volume 17) deals with further methods for fractionation and characterization of chromosomal proteins, including DNA affinity techniques. Also contained in this volume are methods for isolation and fractionation of chromatin, nucleoli, and chromosomes. Volume 18 focuses on approaches for chromatin fractionation, examination of physical properties of chromatin, and immunological as well as sequence analysis of chromosomal proteins. In the fourth volume (Volume 19) enzymic components of nuclear proteins, chromatin transcription, and chromatin reconstitution are described. Volume 19 also contains a section on methods for studying histone gene expression.

In compiling these four volumes we have attempted to be as inclusive as possible. However, the field is in a state of rapid growth, prohibiting us from being complete in our coverage.

The format generally followed includes a brief survey of the area, a presentation of specific techniques with emphasis on rationales for various steps, and a consideration of potential pitfalls. The articles also contain discussions of applications for the procedures. We hope that the collection of techniques presented in these volumes will be helpful to workers in the area of chromatin and chromosomal protein research, as well as to those who are just entering the field.

We want to express our sincere appreciation to the numerous investigators who have contributed to these volumes. Additionally, we are indebted to Bonnie Cooper, Linda Green, Leslie Banks-Ginn, and the staff at Academic Press for their editorial assistance.

GARY S. STEIN
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# CONTENTS

# Part A. Isolation of Nuclei and Preparation of Chromatin. I I

## Chapter 1

## *Procedures for Minimizing Protease Activity during Isolation of Nuclei, Chromatin, and the Histones*

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### I. Introduction

Of the many proteins in the eukaryotic cell nucleus, the highly basic proteins associated with DNA, the histones, rank among the most intensively studied. Early work (*1–3*) indicated that there might be a large number of different histones, which led to the idea that they may act as gene repressors (*4*). However, it was later realized that there were only five major classes of histones (*5–9*) and that the erroneous earlier estimates, based on the number of bands observed on a polyacrylamide gel, were the consequence of proteolytic degradation.

Preparation of nucleoprotein has followed one of two principles: (1) purification from disrupted whole tissue or (2) preparation from purified nuclei. The method described by Bonner *et al.* (*10*), based on the procedure of Zubay and Doty (*11*), illustrates the first principle and is still widely used. Although Zubay and Doty specifically designed their system to minimize nucleolytic degradation by removal of metal ions with ethylenediaminetetraacetic acid (EDTA) and operation at pH 8 (*11*), these conditions, un-

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fortunately, afford proteolysis of the histones. The second principle is typified by a rigorous purification of nuclei for the removal of cytoplasmic contaminants, which have been considered as a probable source of proteolytic activity. The use of sucrose solutions for the isolation of intact nuclei (*12*) and the washing of the nuclei with Triton X-100 for removal of the outer aspect of the nuclear membrane and adhering cytoplasmic components (*13*) are the main features of the method. An equally important inclusion has been sodium bisulfite as an inhibitor of histone proteolysis. There is no obvious difference between the two methods when histones are prepared from calf thymus, but when applied to tissues having a greater ratio of cytoplasm to nucleus, such as liver, the method of Bonner *et al.* (*10*) can yield histones which can be significantly degraded. The concerns of cytoplasmic protease contamination (*14*) have been substantiated (*15,16*) and one study (*16*) indicated that a "neutral chromatin-associated protease" (*17*) is, in fact, of mitochondrial origin.

Since the nature of the problem of proteolysis of nuclear proteins is currently best understood with regard to the histones, we have purposely limited the scope of this article to those proteins. However, there is no reason to believe that the nonhistone proteins are any less susceptible to proteolytic degradation, and in one report this has been shown to be so (*18*). The methods and comments outlined below are derived largely from extensive experience using calf thymus and rat liver. These two tissues are used almost exclusively for studies on the structure and function of chromatin. However, we suggest that the following method for minimizing proteolysis can be employed with some confidence, at least as a starting point, for the isolation of nuclei (*19,20*) and nucleoprotein from any source, with the proviso that the integrity of the nucleoproteins is assayed at every step.

## II. Methods

### A. Assay for Proteolytic Degradation of Nuclear Proteins by Polyacrylamide Gel Electrophoresis

The histones may be conveniently assayed on acid-urea gels (*6,7*). The loss of any of the major fractions and appearances of degradation products are readily apparent when compared to a standard of acid-extracted whole histone from calf thymus. A sodium dodecyle sulfate (SDS)–polyacrylamide gel system has been used to assay for proteolysis of the nonhistone proteins (*18*).

## B. Purification of Nuclei and the Isolation of Chromatin (*8,9*)

### 1. Stock Solutions

The solutions required are: (a) "Grinding medium" containing 0.25 *M* sucrose–10 m*M* $MgCl_2$–10 m*M* Tris-HCl–50 m*M* $NaHSO_3$. The sucrose–$MgCl_2$–Tris can be prepared as a 5-fold concentrated stock at pH 8.0 and stored at 4°. The $NaHSO_3$ is added as the solid just prior to use because of its hydrolysis to $H_2SO_3$ in solution. After addition of solid $NaHSO_3$, the medium is used without readjustment of the pH. (b) "Washing medium" contains all the components of grinding medium and in addition 0.2–1.0% Triton X–100. The concentration of Triton X–100 used depends on the fragility of the nuclei and must be determined for a particular tissue. For calf thymus and rat liver 1.0% Triton X–100 is used. This solution can also be prepared as a 5-fold concentrated stock (pH 8.0). Solid $NaHSO_3$ is again added just prior to use, without readjustment of the pH. (c) "Tris–EDTA–bisulfite" contains 10 m*M* Tris–HCl–20 m*M* EDTA–50 m*M* $NaHSO_3$. The Tris and EDTA are dissolved, and the solution is adjusted to pH 8.0. Solid $NaHSO_3$ is then added, and the solution is used immediately without further adjustment of the pH.

### 2. Disruption of Tissue

All operations are performed at 4°. Isolation and purification of nuclei should be monitored with a light microscope and staining with acetocarmine. Fresh tissue is cut into small pieces and frozen at −15°C (also for storage until required). Freezing is necessary to cause cell breakage. The frozen tissue is added to ice-cold grinding medium (approximately 20 ml/gm tissue) and blended at maximum speed in a Waring blender for 3 minutes. The suspension is filtered through four layers of cheese cloth and then two layers of Miracloth (Chicopee Manufacturing Company, New Jersey). The filtrate is centrifuged at 480 *g* for 10 minutes, and the supernatant is discarded. Disruption of tissue is carried out in the absence of Triton X-100 (which would otherwise cause frothing) when maximum blending speed is used. For the isolation of nuclei from cultured normal or tumor cells, frozen cells are simply suspended directly into washing medium using a Thomas tissue homogenizer or, for larger quantities, a VirTis homogenizer at low speed (10–15 V).

### 3. Purification of Nuclei

The pellet of nuclei is washed by suspension in washing medium (10 ml or greater per gram of tissue) using either a Waring blender at low speed (20 V) for 3 minutes or a Thomas tissue homogenizer. The suspension is centrifuged