Methods in Cell Biology

VOLUME XVIII

Chromatin and Chromosomal Protein Research. III

Edited by

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1978

ACADEMIC PRESS • New York San Francisco London
A Subsidiary of Harcourt Brace Jovanovich, Publishers

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 largely attributed 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 are attempting 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 the first volume (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. The second 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 fraction of chromatin, nucleoli, and chromosomes. This volume (Volume 18) focuses on approaches for examination of physical properties of chromatin as well as chromatin fractionation, and immunological and 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.

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Chapter 1

Fractionation of Chromatin into Template-Active and Template-Inactive Portions

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I. Introduction

In recent years several approaches have been made to separate transcriptionally repressed from transcriptionally active regions of chromatin. The earlier fractionation methods devised to separate the transcriptionally active from inactive regions were based on autoradiographic and cytological observations with isolated cells and nuclei (*1*). In these studies the transcriptionally inert regions are seen as highly condensed material (termed heterochromatin) while those regions active in RNA transcription (as judged by incorporation of radioactive RNA precursors) are visualized as extended diffuse fibers (called euchromatin). Other microscopic observations also indicate that chromatin is in an extended fibrous state when transcriptionally active. These observations include puffing of the *Drosophila* salivary gland chromosomes (*2*), the extended conformation of balbiani rings (*3*), as well as the extended configuration of rRNA genes in the act of producing ribosomal RNA (*4,5*).

The term chromatin fractionation states implicitly that one or more forms of chromatin are to be separated from one or more other forms. This goal has been approached in several different ways, all depending on the different structural and chemical properties of the varied kinds of chromatin. The first report concerning such fractionation involved the separation, by differential centrifugation, of euchromatin (less condensed)

from heterochromatin (more condensed) (*6*). Frenster's demonstration that the condensed heterochromatin was more easily sedimented than the extended euchromatin set the groundwork for the fractionation of sheared chromatin by sucrose density gradients. This approach, introduced by Chalkley and Jensen (*7*), has been used widely in studies of chromatin fractionation. We (*8*) have based our fractionation on the fact that expressed, template-active sequences are more readily attacked by nucleases than are nonexpressed sequences. McCarthy and his colleagues have made use of the fact that the DNA of expressed sequences melts at a lower temperature than does the DNA nonexpressed sequences. This procedure recovers the template-active DNA in denatured form. McCarthy *et al.* (*9*) have also based a fractionation on the fact that the template-active portions of the genome bind RNA polymerase molecules while the nonexpressed portions of the genome do not [confirmed by Marushige and Bonner (*10*)]. Finally, sheared chromatin has been separated into fractions by ion-exchange chromatography (*11,12*).

The regions which are active in RNA synthesis probably are interspersed between inactive, nontranscribed regions. To liberate template-active (expressed) sequences from inactive (nonexpressed) sequences, chromatin must be sheared or clipped into fragments smaller than the length of the whole template-active sequence. Statistical calculations by Davidson *et al.* (*13*) show that on the average the fragments liberated must be one-third or less of the length of the expressed sequence. The average length of the transcriptional unit in rat liver chromatin has been calculated by Davidson *et al.* (*13*) to be 6500 ± 500 base pairs. Thus the shearing method chosen for use in chromatin fractionation should produce fragments on the order of 2000 base pairs or less, with minimal alteration of the structural aspects of the chromatin. Such shearing or clipping may be done in a variety of ways which include sonication, hydrodynamic shear, and nuclease clipping. Although several investigators have used sonication for shearing of chromatin, this does not appear to be a recommended procedure. Sonication of chromatin to the size (a few hundred to a few thousand base pairs) required for separation of template-active from template-inactive fragments results in the liberation of histones from the fragments, and artifactually increases template activity (Marushige and Bonner, unpublished). The effect of mechanical shear on chromatin structure has also been examined in detail. Noll *et al.* (*14*) have shown that chromatin prepared by methods involving mechanical shear has staphylococcal nuclease and trypsin digestion patterns markedly different from those obtained with native chromatin or with chromatin sheared by brief incubations with nuclease. Doenecke and McCarthy (*15*) using restriction

modification methylases, have analyzed the movement of histones along the DNA strand during chromatin fragmentation. They find that histones move laterally along the DNA fiber during the mechanical shearing process. In conclusion, caution must be exercised to avoid artifacts arising due to rearrangement of chromosomal proteins during the preparation and shearing of chromatin prior to fractionation.

II. Criteria for Fractionation

A. Template Activity

Template activity in support of DNA-dependent RNA polymerase is an obvious candidate as a criterion for fractionation of template-active from template-inactive chromatin. The expressed portion of the genome must by definition be capable of transcription, and this has generally been found. Template activity, the rate of transcription of native chromatin by a fixed amount of RNA polymerase relative to the rate of transcription of purified homologous DNA by the same amount of RNA polymerase, has been measured for several kinds of chromatin (*16*). The template activity correlates well with the metabolic activity of the tissue or cell type being examined. For example, rat liver chromatin, a tissue active in RNA synthesis, has a template activity of 18–20%, while chicken erythrocyte chromatin which is little transcribed has a template activity of 2%. Regardless of the fractionation method used, the amount of chromatin isolated in the template-active fraction should reflect the template activity of the isolated native chromatin. With a few exceptions (*17*), all methods of chromatin fractionation yield fractions more active and less active in RNA synthesis. The template-inactive fraction generally possesses some capacity to support DNA-dependent RNA synthesis. It appears that template activity of whole chromatin (as defined above) is the same whether the *Escherichia coli* or homologous RNA polymerase is used (*18* and Van den Broek, personal communication). However, the template activity of the putatively template-active fraction may be higher than that measured with *E. coli* RNA polymerase by as much as a factor of 30 when homologous form II RNA polymerase is used (*21*). This drastic difference may reflect differences in recognition elements in template-active chromatin, or it may merely indicate that the template-active fraction, as isolated, has a large proportion of single-stranded DNA, since eukaryotic RNA polymerase II prefers denatured to native DNA as a substrate.

B. Nascent RNA

The template-active fraction would be expected to bear nascent RNA. Chromatin pulse labeled with, for example, [^{3}H]uridine *in vivo*, should yield an active chromatin fraction bearing all the nascent RNA. This criterion has been used by several investigators. Thus, Billing and Bonner (*19*) found that the fraction of chromatin most readily attacked by nuclease (shown by other criteria, discussed below, to be the expressed fraction) bears the great bulk of the nascent RNA. The same is true of chromatin fractions separated by sucrose density gradient centrifugation (*18,20,21*). It is also true for the active and inactive fractions of chromatin as separated by ECTHAM–cellulose chromatography (*11,12,22*). This criterion is inconclusive, however, because the nascent RNA transcripts are very sensitive to clipping by cellular ribonucleases and by the hydrodynamic forces required to shear chromatin (see above). Therefore it is important to establish that the cofractionating nascent RNA is intimately associated with the template-active fraction, since the fractionation of free RNA would be coincident with the template-active chromatin in most fractionation schemes.

C. Endogenous RNA Polymerase

It might be expected that RNA polymerase would be found associated with the expressed portion of the genome. RNA polymerase molecules are clearly visible in the electron micrographs of template-active chromatin (*4*) and are not obvious in electron micrographs of template-inactive portions of chromatin. More compelling, it has been shown for the case of rat liver chromatin that all of the endogenous RNA polymerase activity is found in the putative template-active fraction as separated from the template-inactive by nuclease digestion (*10*). The same is true for chromatin fractionated by sucrose density gradient centrifugation (*9*). B. J. McCarthy (personal communication) has used RNA polymerase to purify the template-active portion of chromatin in a slightly different way. A large excess of *E. coli* RNA polymerase is added to sheared chromatin. The *E. coli* RNA polymerase binds to the template-active portion of chromatin, not to the template-inactive portion. The fragments containing a large number of bound RNA polymerase molecules are separated from the fragments that do not contain such RNA polymerase by exclusion chromatography.

In summary, ability to bind RNA polymerase at physiological ionic strength and/or presence of RNA polymerase in a fraction forms one additional criterion for a meaningful fractionation of chromatin into expressed and nonexpressed regions.

D. Sequence Complexity

Fractionation with respect to DNA sequences may be demonstrated in two ways. First, the inactive heterochromatin has been shown (*23*) to be enriched in the highly repetitive satellite DNAs. These satellite DNA sequences differ in base composition, and therefore band at a different buoyant density than the bulk of the nuclear DNA. Thus the finding that the satellite DNA is localized in the inactive regions indicates that fractionation has occurred (*24*).

The second, and more conclusive, type of sequence fractionation involves those sequences actively involved in transcription. These sequences should be preferentially localized in the template-active fraction. The expressed portion of the genome would be expected to include a subset of the single copy sequences of the entire genome. These sequences would be expected to be hybridizable by messenger RNA, and to be hybridizable to nuclear RNA as well. A study of the sequence structure of the template-active fraction of chromatin as compared to that of the template-inactive fraction of chromatin has been carried out by Gottesfeld *et al.* for rat liver chromatin (*25*). In this case it is shown, as will be discussed in more detail below, that the template-active fraction of rat liver chromatin as prepared by nuclease digestion contains 10% of the single copy sequences, and that these sequences are to a large degree different from the single copy sequences contained in the template-active portion of rat brain chromatin. Gottesfeld *et al.* (*26*) have also shown that template-active single copy DNA hybridizes to a much greater extent to whole cell RNA than does the single copy DNA of the template-inactive fraction of rat liver chromatin. Studies of the sequence structure of the putative template-active fraction as compared with whole genomal DNA provide a powerful tool for the verification of whether or not a fractionation has been achieved.

E. Use of Probes

Recently Hawk *et al.* (*27*) have investigated the validity of ECTHAM–cellulose (*11*) and glycerol (*18*) gradient fractionation using mouse cells infected with the Moloney strain of murine leukemia virus.

In vivo these cells produce abundant RNA homologous to the Moloney type C leukemia virus but not RNA homologous to the type B mouse mammary tumor virus or globin RNA. Fractionation of chromatin into expressed and nonexpressed fractions and hybridization with cDNA copies of the viral and globin RNAs showed that the sequences (genes) for production of type C and type B viral products were equally distributed between the two fractions of chromatin. They also found a random dis-

tribution of globin sequences in the template-active and inactive fractions. Thus although fractions differing in physical properties (suggesting valid fractionation) were obtained, they were unable to separate active from inactive genes by these methods.

III. Methods of Fractionation

A. Separation of Euchromatin and Heterochromatin

Separation of metabolically active euchromatin from metabolically inactive heterochromatin has been described by Frenster *et al.* (*6*). They lysed lymphocyte nuclei, briefly sonicated the nuclei, and then separated heavier from lighter fractions by differential centrifugation. That chromatin which pellets at 100 *g* is classified as heterochromatin and that which does not pellet at 3000 *g*, but does pellet at 78,000 *g*, is classified as euchromatin. The latter constitutes about 20% of the total. If the nuclear RNA had been labeled in life then the specific activity of the lighter fraction exceeded that of the heavy fraction by a factor of 3.

This method of fractionation has been applied to the fractionation of chromatins of rat liver and rat Novikoff ascites cells by DeBellis *et al.* (*17*). These investigators used template activity in support of RNA synthesis by *E. coli* RNA polymerase as their criterion. By this assay, euchromatin and heterochromatin of both sources possessed identical activities.

B. Fractionation by Sucrose Density Gradient Centrifugation

Following the early lead of Frenster (*6, 28*), many investigators have separated sheared chromatin by sucrose density gradient centrifugation (*29*). Chromatin is sheared either in the VirTis blender or by other means, such as sonication, loaded on a linear or isokinetic sucrose gradient, and centrifuged. Provided only that a suitably small size of fragment has been achieved (10,000 base pairs or less of DNA), two fractions result—a heavy fraction and a light fraction. The light fraction makes up about 10–20% of the total DNA. The first thorough study of this fractionation is that of Chalkley and Jensen (*7*). These workers showed that the heavy fraction can be converted to the light fraction by treatment with 4 *M* urea and that this conversion is irreversible after removal of the urea. They concluded that the heavy fraction consists of fragments similar to those of the light fraction, but linked by interfragment bands perhaps due to histone H1. Chalkley and Jensen found that the light fraction has a somewhat higher template activity than the heavy frac-

tion (about a factor of two). The template activity of the heavy fraction, after conversion to light fraction, is the same as that of material originally light, according to Chalkley and Jensen (*7*).

Typical of reports on the fractionation of chromatin by sucrose density gradient centrifugation is that of Berkowitz and Doty (*20*). These workers investigated chick embryo reticulocyte chromatin, which was sheared by sonication and applied to a sucrose density gradient; a slowly sedimenting fraction then was separated from a rapidly sedimenting fraction. The slowly sedimenting or light fraction constituted 12% of the total. The template activity of the slowly sedimenting fraction was twice that of the rapidly sedimenting fraction (determined with *E. coli* RNA polymerase) and, in addition, the concentration of nascent globin RNA transcripts as determined by hybridization of nascent mRNA to globin cDNA was 5 times more abundant in the transcripts of the light fraction than among the transcripts of the heavy fraction. The chromatin had been sheared to such a degree that the light fraction DNA was on the average 700 base pairs long while the template-inactive or heavy fraction DNA was on the average 1500 base pairs long. Similar results have been found by Rodriques and Becker (*30*) in the fractionation of rat liver chromatin by glycerol gradient centrifugation. They find similar histone composition in the light and heavy fractions, although more histone was associated with the heavy fraction. The nonhistone proteins show marked differences with unique species in the euchromatin and heterochromatin fractions.

In 1975 Doenecke and McCarthy described similar results with *Drosophila* chromatin sheared either to 2 kb (kilobase) or to 0.6 kb and fractionated on a sucrose gradient (*31,32*). This yields about 30% of the total chromatin as a light or slowly sedimenting fraction. This slowing sedimenting fraction possessed essentially all of the template activity for support of RNA synthesis by *E. coli* polymerase and also bore the bulk of the nascent RNA of the whole chromatin (*9*). Murphy *et al.* working with mouse myeloma chromatin sheared by VirTis shearing (or by the French press) and fractionated on sucrose or glycerol gradients find 11% of the total DNA in the light or slowly sedimenting fraction (*18*). This slowly sedimenting fraction has the bulk of the template activity as determined by transcription of the homologous RNA polymerase and also bears the bulk of the nascent RNA labeled *in vivo*.

Interestingly, many investigators of chromatin fractionation do not provide chemical analyses of either their starting material or the derived fractions. The data of Table I illustrate the histone/DNA and nonhistone chromosomal protein/DNA ratio for calf thymus chromatin and its fractions as separated by sucrose density gradient centrifugation and similar data for chick embryo reticulocyte chromatin. In both cases histone is marginally enriched in the heavy (template less active) fraction. Nonhistone proteins