Nuclear structures

Isolation and characterization

Edited by

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and

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Preface

During the decade since the publication of *Subnuclear Components*, as techniques for the isolation of molecular and macromolecular constituents of cell nuclei improved, the emphasis in research has shifted from individual molecular components to identifiable structures such as the nuclear envelope, ribonucleoprotein particles, etc. The present book is designed to reflect not only current interests in the structures now recognized to exist in the nuclei of cells, but also changes and advances in preparative techniques for nuclear components and certain macromolecules. We are of the opinion that, in view of the movement of interests and research, the publication of this book will be timely; moreover, to the best of our knowledge, no other text covers these aspects.

The overall objective of this book is to provide reasoned assessments of current techniques used to isolate and characterize nuclear structures, together with 'best buys' in recommended procedures. To achieve these ends we have persuaded each of our authors to use their experience and judgement in their field to review available procedures, to recommend particular methods, and to give reasons for their choices. It is regrettable that precise technical details (for example, conditions of centrifugation) are not always given in today's scientific papers and we have specifically asked for such essentials to be included. As a result, technical details are omitted from the text of this book only in exceptional circumstances, for example when a contributor, in making comparisons of experimental approaches, has had to rely solely on the information provided in an original article. In addition, each contributor has provided an indication of the evaluation of their end-product. Consequently, a degree of difference in balance between background material and detailed technical information exists throughout the book; this is merely a reflection of how different areas have progressed to date.

We appreciate that not all known nuclear structures are dealt with in the chapters to follow. Some are omitted simply because no particular advances have occurred in their preparation during the past ten years. The methodology to isolate others is not as yet at a stage that is suitable for discussion, for example the components of the mitotic apparatus other than the chromosomes themselves. The isolation of nuclei from cells is, of course, of prime importance, but again no major advances in these methodologies have appeared in recent years. Each author has, however, been asked to quote their preferred procedure for preparing nuclei. Although the chapters in part act as current reviews of specific areas of the biochemistry and molecular biology of the cell nucleus, the thrust of the book is a practical one. In

this, it is our belief that an approach based on critical comparisons between methodologies and detailed explanations of procedures recommended avoids the production of what would otherwise be little more than a compendium of recipes, lacking guidance as to what menu is appropriate to a particular situation.

We must express our gratitude to our authors for their cooperation and understanding, particularly during lengthy discussions over the final format of their contributions. We are also indebted to the staff of Butterworths Scientific Ltd for help, advice and encouragement throughout the preparation of the book.

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Contents

Chapter 1

Nuclear matrices

Shona A. Comerford, Paul S. Agutter and Alexander G. McLennan

More than 40 years ago, it was shown that a subfraction of nuclear proteins resisted extraction with buffers of high ionic strength (Mayer and Gulick, 1942). During the 1950s and 1960s, evidence accumulated that this salt-resistant fraction represented a definite intranuclear protein or ribonucleoprotein fibrillar network (for reviews see Berezney and Coffey, 1976; Agutter and Richardson, 1980). The first detailed isolation procedure for this structure, the nuclear matrix, was published by Berezney and Coffey (1974) and since then both the validity of this procedure and the physiological reality of the matrix have been controversial issues. The susceptibility of nucleoplasmic constituents to artefactual precipitation under a variety of conditions has been emphasized (*see* e.g. Skaer and Whytock, 1977; Kaufmann, Coffey and Shaper, 1981; Laemmli, Lewis and Lebkowski, 1981), and it is clear that while Berezney and Coffey (1974) isolated an intranuclear fibrillar structure, other workers using apparently similar techniques (nuclease digestion, extraction with high salt concentrations and treatment with non-ionic detergents) isolated only the peripheral pore-complex-lamina (Aaronson and Blobel, 1975; *see also* Chapter 3 in this volume).

We take the view that more recent electron microscopic evidence (e.g. Brasch, 1982; Guatelli *et al.*, 1982; Capco, Wan and Penman, 1982; Diaz de la Espina *et al.*, 1982), taken together with earlier studies, establish the physiological reality of the matrix beyond reasonable doubt, and that the immediate task is to obtain an isolated preparation that corresponds ultrastructurally to the *in situ* network described in these publications. Our view is not universally held, however, and other workers would consider that the controversy about the existence of the matrix is not yet settled.

The persistence of this controversy seems to us to have resulted from the poor characterization of the matrix to date. In contrast to the well-established components of the cytoskeleton, 'nuclear matrices' are ultrastructurally and biochemically ill-defined. Ultrastructurally, almost all preparations comprise irregular, heterogeneous, broad, rather amorphous fibres rather than regular, homogeneous, fine, well-formed ones. Biochemically, their main specific polypeptide components (other than lamins – *see* Chapter 3) have not been identified; antibodies that react only with the postulated intranuclear fibrils *in situ* have not been obtained, though recent findings by Chaly *et al.* (1983) may resolve this problem, and *in vitro* reconstitution has not been accomplished. We believe

that until these deficiencies are remedied, no real progress can be made with the potentially richly rewarding studies of the role of the matrix in a number of major nuclear functions such as DNA replication (Berezney and Coffey, 1975), RNA processing (Wunderlich, Berezney and Kleinig, 1976), steroid hormone binding (Agutter and Birchall, 1979; Barrack and Coffey, 1980), viral replication (Hodge *et al.*, 1977; Chin and Maizel, 1977) and carcinogenesis (Zbarsky, Dmitrieva and Yermolayeva, 1962; Hemminki and Vainio, 1979).

The confusion of the last decade has been exacerbated by attempts to pursue such studies despite the poor state of basic characterization of the structure, by the concomitant proliferation of 'isolation' procedures and by inconsistencies in nomenclature. (The term 'matrix' has been used specifically to describe an intranucleoplasmic fibrillar system, and more generally to refer to whole chromatin-depleted nuclear residues comprising pore-complex-laminae and nucleolar residues together with such fibrils. We shall use the term in the latter, general sense. Moreover, such phrases as 'nuclear skeleton', 'nuclear ghost' and 'nuclear cage' have been used to describe analogous preparations. We shall avoid these terms, but it should be emphasized that studies of the 'nuclear cage' have thrown valuable light on the mechanism of DNA replication. (*See* Chapter 2.)

In this chapter, we discuss types of isolation procedures for the matrix and also a series of studies which made an important contribution to clarifying the issues involved in matrix isolation. Other published procedures for matrix isolation are variants of those treated here. Discussion of these five major procedures and their derivatives seems to us to provide a reasonable overview of the evolution of matrix isolation techniques, and in doing so it throws some light on the development of the controversy surrounding the existence of the structure *in vivo*. We must emphasize that although a satisfactory isolation procedure might now be available, such detailed biochemical characterization as is necessary for further progress is still far from completed.

Isolation of nuclear matrices

The method of Berezney and Coffey (1974)

This method was originally designed for the preparation of rat liver nuclear matrices. Later it was used to isolate matrices from other mammalian tissues, including endometrium (Barrack *et al.*, 1977) and lung (Agutter and Birchall, 1979). Its importance lies partly in this apparent versatility, partly in its role in initiating the controversy about the matrix and partly in the fact that procedures that were developed subsequently were derived from it, or at least influenced by it.

Step 1 Rat liver (50 g) is minced and homogenized in 200 ml of STM (50 mM tris-HCl, 5 mM $MgCl_2$, 250 mM sucrose, pH 7.4 at 4°C) and filtered through cheesecloth. The homogenate is centrifuged at 780 × g_{max} (1600 rev/min in the 12 × 100 ml rotor of a MSE Coolspin centrifuge) for 10 min at 4°C and the pellet is resuspended in STM adjusted to 2.2 M sucrose. The nuclei obtained after centrifugation at 40 000 × g_{max} (16 500 rev/min in the 10 × 100 ml rotor of a MSE 50 centrifuge) for 90 min at 4°C are washed (*see* Note 1, p.9) twice in STM. All subsequent operations except nuclease digestion are performed at 0°C in 10 mM tris-HCl, pH 7.4 (adjusted at 0°C).

Step 2 The nuclei obtained by the above procedure are lysed by resuspension in 20 vol. of 10 mM tris-HCl, pH 7.4 buffer containing 0.2 mM $MgCl_2$, incubation for 10 min and centrifugation at 780 × g_{max} (as above) for 20 min. This step is repeated once.

Step 3 The pellet is resuspended in 10 mM tris-HCl, pH 7.4 buffer containing 0.2 mM $MgCl_2$, 2.0 M NaCl. After incubation for 10 min the suspension is centrifuged at 780 × g_{max} for 40 min. This step is repeated twice.

Step 4 The chromatin-depleted pellet is resuspended in 10 vol. of 1% (v/v) Triton X-100, 5 mM $MgCl_2$ in 10 mM tris-HCl, pH 7.4 buffer and incubated for 10 min. The membrane-depleted nuclei are recovered by centrifugation at 780 × g_{max} for 20 min. This step is not repeated.

Step 5 Residual nucleic acids are removed by incubation with 200 μg DNAase I, 200 μg RNAase A in 1 ml of 5 mM $MgCl_2$ at 22 °C for 1 h. The matrices are then sedimented by centrifugation at 780 × g_{max} and are washed twice in 5 mM $MgCl_2$. (For discussion of the most important features of this method, *see* Notes 2 and 3, p.10.)

The method of Wunderlich and Herlan (1977)

This method was designed to isolate matrices from the macronuclei of *Tetrahymena pyriformis*. The Ca^{2+}/Mg^{2+} ratios used are apparently appropriate to the internal environment of this organism; as the total concentrations of these cations increase and decrease, the matrix respectively contracts and expands (Herlan, Quevedo and Wunderlich, 1978).

Step 1 The isolated nuclei from 10^9–10^{10} cells are incubated for 10 min, 4 °C in 4–5 vol. of 20 mM tris-HCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 0.1 mM ATP, 0.3% (v/v) Triton X-100, 1% (w/v) polyvinylpyrrolidine K90, pH 7.4, and centrifuged at 900 × g_{max} (1700 rev/min in the 12 × 100 ml rotor of a MSE Coolspin centrifuge) for 10 min at 4 °C.

Step 2 After washing (*see* Note 1, p.9) in LCMT (20 mM tris-HCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 0.1 mM ATP, pH 7.4) they are incubated with 100 μg DNAase I, 100 μg RNAase A in 5 ml of HCMT (i.e. LCMT adjusted to 50 mM $MgCl_2$ and 75 mM $CaCl_2$) for 75 min at 20 °C.

Step 3 The nuclease action is terminated by the addition of 9 vol. (45 ml) of LCMT followed by centrifugation at 1300 × g_{max} (2000 rev/min in the 12 × 100 ml rotor of an MSE Coolspin centrifuge) for 20 min at 4 °C.

Step 4 The nuclease digestion is repeated under the same conditions for 30 min in HCMT and is terminated as before. Finally, the matrices are washed (*see* Note 1, p.9) twice in HCMT. The yield of matrix material obtained by this method is discussed in Note 4, p.10, and implications for the universality of the matrix are discussed in Note 5, p.10).

The method of Long, Huang and Pogo (1979)

At the time of this publication, the interests of Pogo and his co-workers centred on the organization of intranuclear ribonucleoprotein in cultured cells. The method is designed specifically for isolating RNA-rich matrices from Friend erythroleukaemic cells, but was derived from earlier studies by the same group (*see* e.g. Faiferman and Pogo, 1975; Miller, Huang and Pogo, 1978). In Note 6 (p.10) and in the following text, the implications of the RNA content of the isolated structure are discussed. In Note 7 (p.11) the possibility of applying the same or a very similar procedure to other cell types is discussed; specifically, the work of van Venrooij and his colleagues (van Eekelen and van Venrooij, 1981) on HeLa cell matrices is examined.

Step 1 The cells are washed twice with 10 mM PIPES, 100 mM KCl, 1.5 mM $MgCl_2$, 146 mM sucrose, pH 7.0 (adjusted at 23 °C). In this context, 'washed' is to be understood not as in Note 1, but as replacement of the culture medium by an equal volume of the buffer.

Step 2 Lysis of the cells is achieved using the same medium supplemented with 0.5 mM phenylmethylsulphonyl fluoride (PMSF) (*see* Note 6, p.10), 0.25% (v/v) Triton X-100 and 0.25% (w/v) saponin. To facilitate lysis, repeated passage (10–20 times) through a plastic pipette is recommended by the authors (Note 8, p.11).

Step 3 Differentiated nuclei are sedimented at 250 × g_{max} (900 rev/min in the 12 × 100 ml rotor of a MSE Coolspin centrifuge), and undifferentiated nuclei at 500 × g_{max} (1250 rev/min in the same rotor), for 10 min at 4 °C.

Step 4 After washing (Note 1) twice in 10 mM tris-HCl, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 146 mM sucrose, 0.5 mM PMSF, pH 7.7 (adjusted at 23 °C), the nuclei are resuspended to a concentration of 1.5 × 10^8/ml in the same buffer supplemented with 500 µg/ml of DNAase I (*see* Note 9, p.11) and are incubated at 10 °C for 60 min (undifferentiated) or 120 min (differentiated nuclei).

Step 5 After this incubation, the suspension is layered over 20–25 ml of 1 M sucrose in 10 mM tris-HCl, 100 mM KCl, 1.5 mM $MgCl_2$, pH 7.7 (adjusted at 23 °C) and centrifuged at 6000 × g_{max} (6000 rev/min in the 6 × 14 ml rotor of a MSE Europa 50 centrifuge) for 10 min at 4 °C. The matrices pellet.

Step 5 of this procedure can be modified in two ways. It can be replaced by simple sedimentation as described for the nuclei, in which case the pellet is rich in chromatin fragments (this might be useful if interactions between the matrix and DNA or histones is being studied). Alternatively, the KCl concentration can be increased to 0.5–1.5 M, in which case the extent to which DNA and histones are extracted is increased correspondingly.

In either case, however, the recovery of nuclear RNA in the matrices is close to 100%. This raises an interesting and important issue because ribonuclease treatment has been found to disrupt the intranuclear matrix fibrils. Thus, while the findings from early applications of the methods described on p.2 *et seq.* of this chapter seemed to point to a more or less wholly proteinaceous fibrillar matrix, the results obtained by Long, Huang and Pogo (1979) recalled earlier indications that the intranuclear ground substance is a network comprising both protein and RNA

(Smetana, Steele and Busch, 1963; Narayan *et al.*, 1967; Faiferman and Pogo, 1975). This emphasized an additional aspect of the controversy surrounding the reality and nature of the matrix: how dependent is the integrity of the structure on RNA?

The studies of Kaufmann, Coffey and Shaper (1981)

Although the work of Shaper and his colleagues was not intended to lead to a new method for isolating matrices, it elucidated the mechanisms underlying the procedures previously used and has played a crucial part in initiating subsequent methodological developments. Therefore, we summarize its principal conclusions here and urge the reader who is interested in matrix isolation to study this key paper in the field with particular care.

Kaufmann, Coffey and Shaper began with the question: why did Berezney and Coffey (1974) and Aaronson and Blobel (1975), using apparently identical techniques and the same starting material, obtain such markedly different results? (*See* p.2 *et seq.* and Note 3, p.10), for details.) When they attempted to shorten the operation time of the Berezney–Coffey procedure, treating the nuclei initially with DNAase and RNAase to avoid overnight incubation (cf. Berezney and Coffey, 1977) but using the same buffers as Berezney and Coffey, supplemented with PMSF (*see* p.2), Kaufmann, Coffey and Shaper obtained a far less extensive intranuclear network and no identifiable nucleolar residues. Further investigation revealed that structures of very different compositions and morphologies could be isolated from the same batch of nuclei, if the order and speed of the extraction steps were varied slightly.

The main conclusions were first, when RNAase treatment is used *after* high-ionic-strength extraction, intranuclear matrix fibrils and nucleolar residues can be retained. When it is used *before* such extraction, intranuclear structure is largely absent and only empty spheres of nuclear envelope persist. (*See* Note 6, p.10).

Secondly, the stability of the intranuclear fibrils is dependent on the oxidative crosslinking of proteins with endogenous sulphydryl groups. If such crosslinking is made extensive at the pure-nuclei stage, for example by overnight incubation, by treatment with sodium tetrathionate or by cationic detergents, then even RNAase treatment before high-salt extraction does not disrupt the matrix. If matrices are obtained by, for instance, the method of Berezney and Coffey (1977), then subsequent treatment of them with 1% (v/v) 2-mercaptoethanol in high-salt buffer destroys the internal structure and leaves only hollow spheres of nuclear envelope (*see* Notes 2 and 3, p.10).

Thirdly, reproducibility of the findings, including recovery of nuclear protein and RNA, depends on the inclusion of PMSF in all the buffers used, including those used for isolating nuclei. Other serine protease inhibitors are less satisfactory. (In our hands, phenylmethylsulphonylchloride (PMSC) is as effective as PMSF; cf. Agutter, 1983). (*See* Notes 2 and 7, pp.10,11.)

Fourthly, extraction with solutions containing Triton X-100 leads to aggregation of the material, and therefore more satisfactory matrix preparations are obtained if the nuclear membranes are not removed by detergent treatment during isolation.

On the basis of these findings, it seems appropriate to use PMSF or PMSC throughout the isolation procedure and to avoid detergent extraction. Moreover, the stability of the matrix structure seems to depend both on the integrity of the