

## Interphase Nuclear Matrix and Metaphase Scaffolding Structures

LEWIS, Catherine, *et al.*

### Abstract

The protein compositions of purified metaphase chromosomes, nuclei and their residual scaffold and matrix structures, are reported. The protein pattern of nuclei on sodium dodecyl sulphate/polyacrylamide gels is considerably more complex and rich in non-histone proteins than that of chromosomes. Nuclei contain about three to four times more non-histone proteins relative to their histones than chromosomes. Besides the protein components of the peripheral lamina, several protein bands are specific or at least highly enriched in nuclei. Conversely, two proteins X0 ( $33 \times 10^3$  Mr) and X1 ( $37 \times 10^3$  Mr) are highly enriched in the pattern of metaphase chromosomes. We have compared morphologically the previously defined nuclear matrices type I and II. The type I nuclear matrix is composed of the known lamina proteins, which form the peripheral lamina structure, and a complex series of proteins that form the internal network of the matrix as observed by electron microscopy. This internal network is stabilized similarly to the metaphase scaffolding by metalloprotein interaction. Both the scaffolding and the internal network of the [...]

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## INTERPHASE NUCLEAR MATRIX AND METAPHASE SCAFFOLDING STRUCTURES

CATHERINE D. LEWIS, JANE S. LEBKOWSKI, ANN K. DALY  
AND ULRICH K. LAEMMLI

*Departments of Biochemistry and Molecular Biology, University of Geneva,  
30, quai Ernest-Ansermet, Geneva, Switzerland*

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### SUMMARY

The protein compositions of purified metaphase chromosomes, nuclei and their residual scaffold and matrix structures, are reported. The protein pattern of nuclei on sodium dodecyl sulphate/polyacrylamide gels is considerably more complex and rich in non-histone proteins than that of chromosomes. Nuclei contain about three to four times more non-histone proteins relative to their histones than chromosomes. Besides the protein components of the peripheral lamina, several protein bands are specific or at least highly enriched in nuclei. Conversely, two proteins X0 ( $33 \times 10^3 M_r$ ) and X1 ( $37 \times 10^3 M_r$ ) are highly enriched in the pattern of metaphase chromosomes.

We have compared morphologically the previously defined nuclear matrices type I and II. The type I nuclear matrix is composed of the known lamina proteins, which form the peripheral lamina structure, and a complex series of proteins that form the internal network of the matrix as observed by electron microscopy. This internal network is stabilized similarly to the metaphase scaffolding by metalloprotein interaction. Both the scaffolding and the internal network of the matrix dissociate if thiols or certain metal chelators are used in the extraction buffer. Under these conditions the resulting nuclear structure, called matrix type II, appears empty in the electron microscope, with the exception of some residual nucleolar material. This latter material can be extracted from the internal network by exhaustive treatment of the nuclei with RNase before extraction with high salt.

Immunoblotting and activity studies show RNA polymerase II to be tightly bound to the type I, but not to the type II matrix, or to the scaffolding structure. No polymerase II enzyme was detected in isolated metaphase chromosomes. Another nuclear enzyme, poly(ADP-ribose) polymerase is not bound to either of the residual nuclear matrices or to the scaffolding structures. The association of RNA polymerase with the internal network of the nuclear matrix is consistent with the idea that transcription occurs in close association with this structure.

### INTRODUCTION

Recent observations have provided evidence that the chromatin fibre is organized into domains or loops, which are constrained by a residual framework both in metaphase chromosomes (Paulson & Laemmli, 1977; Adolph, Cheng & Laemmli, 1977a; Adolph, Cheng, Paulson & Laemmli, 1977b) and in interphase nuclei (Benyajati & Worcel, 1976; Cook & Brazell, 1976; Igo-Kemenes & Zachau, 1978; Adolph, 1980; Lebkowski & Laemmli, 1982a,b). The residual framework of metaphase chromosomes has been called a 'scaffolding' (Laemmli *et al.* 1978) since this structure retains some of the morphological features of metaphase chromosomes (Paulson & Laemmli, 1977; Earnshaw & Laemmli, 1983). Recently, we have found that the scaffolding is composed of a subset of non-histone proteins, which include two prominent high molecular weight species, Sc1 and Sc2, of about 170 000 and 135 000  $M_r$  (Lewis & Laemmli, 1982).

The protein framework involved in the organization of the interphase chromatin is thought to correspond to the nuclear matrix, which can be isolated following extraction of nuclei with high salt (Berezney & Coffey, 1974; Comings & Okada, 1976; Herman, Weymouth & Penman, 1978; Long, Huang & Pogo, 1979; van Eekelen & van Venrooij, 1981). Morphologically, this structure is composed of three elements: (1) a peripheral nuclear lamina, (2) an internal protein network, and (3) a residual nucleolar structure. The peripheral lamina is the best-studied component and is composed of three major proteins of 60 000–70 000  $M_r$  (Aaronson & Blobel, 1975; Gerace & Blobel, 1980; Jost & Johnson, 1981).

We have recently been able to distinguish two levels of folding of the chromatin fibre in nuclei (Lebkowski & Laemmli, 1982*a,b*). One level of organization is thought to result from an attachment of the DNA to the peripheral nuclear lamina structure. We propose that the DNA is stabilized at a second level by a complex set of proteins, which forms the residual network in the interior of the nuclear matrix. A series of recent reports suggest that the nuclear matrix has more than a structural role, and may be involved in DNA replication (Vogelstein, Pardoll & Coffey, 1980; Pardoll, Vogelstein & Coffey, 1980; Dijkwel, Mullenders & Wanka, 1979), transcription (Jackson, McCready & Cook, 1981), and RNA processing (Ciejek, Nordstrom, Tsai & O'Malley, 1982).

## MATERIALS AND METHODS

### *Isolation of nuclei and metaphase chromosomes*

HeLa S3 cells were maintained in suspension culture at 37 °C in RPMI-1640 medium supplemented with 5% (w/v) newborn calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. For autoradiography, the cells were labelled with [<sup>35</sup>S]methionine at 1–2 µCi/ml for 48 h before isolation. Metaphase chromosomes were isolated from cells blocked in metaphase at a concentration of  $2 \times 10^5$  cells/ml with Colcemid at a final concentration of 0.06 µg/ml for 12–16 h. The isolation of metaphase chromosomes in an aqueous, reticulocyte standard buffer (RSB) or in a polyamine buffer system was performed as described by Lewis & Laemmli (1982). Isolation of interphase nuclei by the RSB method was performed as described by Lebkowski & Laemmli (1982*b*). Isolation of interphase nuclei in a polyamine buffer system was performed as follows. HeLa cells (500 ml) growing exponentially at a concentration of  $2 \times 10^5$  cells/ml were collected in four tubes (1400 g, 5 min), and washed three times at room temperature with 50 ml/pellet in a buffer containing 20 mM-KCl, 0.05 M-spermine, 0.13 M-spermidine, 0.5 mM-EDTA, 3.7 mM-Tris·HCl (pH 7.4), (solution I). The pellets were resuspended at 4 °C in 30 ml of solution I containing 0.1% digitonin and 50 µg/ml RNase A, and homogenized using a type A pestle. Nuclei were pelleted (5 min at 1000 g) in the Clay Adams Dynac centrifuge. The nuclei were vortexed and rehomogenized 7–8 times before a final wash and re-suspension in solution I containing 0.1% digitonin but no EDTA. All solutions contained 0.1 mM-phenylmethylsulphonyl fluoride (PMSF), 10 KIU/ml Trasylol (Mobay Chemical Co.), 1% thiodiglycol (Pierce), except during homogenization of the cells when 1 mM-PMSF and 100 KIU/ml Trasylol were used. We will refer to chromosomes and nuclei isolated either in the reticulocyte standard buffer or the polyamine-containing buffer as RSB or polyamine chromosomes and nuclei, respectively.

### *Isolation of nuclear matrices and metaphase scaffolds*

Metaphase scaffolds were isolated from RSB or polyamine chromosomes as described by Lewis & Laemmli (1982). Nuclear matrices types I and II were isolated from RSB nuclei as described by Lebkowski & Laemmli (1982*b*). Type I and II nuclear matrices from polyamine nuclei were

obtained as follows. Isolated polyamine nuclei in 200  $\mu$ l (10 o.d.<sub>260</sub> units/ml), were digested with 40  $\mu$ g/ml of DNase I for 1 h at 4 °C in solution I containing 0.1 % digitonin, 10 mM-MgCl<sub>2</sub>, but no EDTA. For type I matrices, 0.1 mM-CuSO<sub>4</sub> or 0.5 mM-CaCl<sub>2</sub> was added, under an N<sub>2</sub> atmosphere, to the nuclease-digested nuclei for 10 min at 4 °C or 10 min at 37 °C, respectively. The addition of 5 mM-EDTA was used to stop the reaction. The addition of Cu<sup>2+</sup> or Ca<sup>2+</sup> is necessary to stabilize the type I matrix structure if isolated from polyamine nuclei that have been metal-depleted (Lewis & Laemmli, 1982). Histones were extracted from the nuclei by the addition of 4.5 ml of a lysis buffer containing 2 M-NaCl, 10 mM-Tris·HCl (pH 7.4), 10 mM-EDTA, 0.1 % digitonin at 4 °C for 20 min. The lysis mixture containing the nuclei was spun at 15 000 rev./min for 30 min at 4 °C in the SW50.1 rotor (Beckman). Type II matrices were isolated in an identical manner except that the addition of Cu<sup>2+</sup> was omitted. All solutions contained 0.1 mM-PMSF, 1 % thiodiglycol and 10 KIU/ml Trasylol.

### Detection of RNA polymerase II by immunoblotting

Nuclear and chromosomal samples to be tested for RNA polymerase II antibody binding were isolated without thiodiglycol or RNase treatment, electrophoresed on sodium dodecyl sulphate (SDS)/4 M-urea/polyacrylamide gels, and transferred to nitrocellulose filters as described for DNA-protein blotting (Bowen, Steinberg, Laemmli & Weintraub, 1980). The filters with the blotted proteins were processed as described by Stick & Krohne (1982). A monoclonal antibody to *Drosophila* RNA polymerase II, provided by E. Bautz (undiluted mouse ascites fluid, 1.5 mg/ml protein), was used at a dilution of 1:500. Antibody binding was visualized by <sup>125</sup>I-labelled rabbit anti-mouse antibody (Amersham, 1  $\mu$ Ci/ml, sp. act. 1  $\mu$ Ci/ $\mu$ g) as the secondary antibody.

### In vitro transcription assays

Polyamine or RSB chromosomes and nuclei were isolated without thiodiglycol or RNase treatment and used either directly or frozen at -80 °C in 20 mM-Tris·HCl (pH 7.9), 75 mM-NaCl, 0.5 mM-EDTA, 8.5 mM-dithiothreitol, 0.125 mM-PMSF and 50 % glycerol. Immediately before use, samples to be tested were washed twice and resuspended in RSB, 0.1 mM-PMSF containing no detergent, at a concentration of 150 o.d. units/ml (*A*<sub>260</sub>) at 4 °C. The buffer system of Cox (1976) was routinely used for the assay. Reaction mixtures (0.3–0.5 ml) contained 25 mM-Tris·HCl (pH 7.9), 1 mM-MnCl<sub>2</sub>, 0.5 mM-dithiothreitol, 0.5 mM of each ATP, CTP and GTP, 30  $\mu$ M-UTP, 250 mM-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10  $\mu$ M-[<sup>3</sup>H]UTP (20  $\mu$ Ci). Where appropriate, Sarkosyl (0.6 %) or heparin (1 mg/ml) were included. Mixtures were preincubated at 32 °C for 5 min before the addition of nuclei or chromosomes (100–200  $\mu$ g of DNA in 20  $\mu$ l or RSB). Samples (50  $\mu$ l) were removed at 15- to 30-min intervals, and the reaction was stopped in each sample by the addition of 5 mM-EDTA, followed by 200  $\mu$ g/ml DNase I, 40  $\mu$ g *Escherichia coli* transfer RNA, 10 mM-MgCl<sub>2</sub>. The samples were spotted in duplicate onto DE81 filters (Whatman), which were washed three times in 10 % Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 0.5 % Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 0.1 % SDS (Gariglio, Llopis, Oudet & Chambon, 1979), three times in water and twice in ethanol. The filters were dried and counted.

### Poly(ADP-ribose) polymerase assays

Poly(ADP-ribose) polymerase was assayed by a modification of the method of Ogata, Ueda, Kawaichi & Hayashi (1981). Assays were performed with 1  $\mu$ M-<sup>32</sup>P-labelled NAD (850 Ci/mmol, Amersham) at 22 °C in 0.1 mM-Tris·HCl (pH 8.0) 10 mM-MgCl<sub>2</sub>, 1.25 mM-dithiothreitol. For each assay, 1–2 *A*<sub>260</sub> units of nuclei or chromosomes in a volume of 30  $\mu$ l were added to give a final reaction volume of 100  $\mu$ l. The reaction was terminated by the addition of 10  $\mu$ l of reaction mixture to 0.5 ml of absolute ethanol at -20 °C using 25  $\mu$ l cytochrome *c* (1  $\mu$ g/ml) as carrier. After 2 h at -20 °C, the solution was centrifuged at 10 000 rev./min for 5 min in the microfuge (Eppendorf). The pellet was resuspended in final sample buffer and electrophoresed as described by Laemmli (1970) with the modifications outlined by Lewis & Laemmli (1982).

For metaphase scaffolds and nuclear matrices, nuclei and chromosomes were digested with DNase I and treated with CuSO<sub>4</sub>, as described above, before the assay. In this case the polymerase reaction was terminated by the addition of 20 mM-nicotinamide after 10 min of incubation with the <sup>32</sup>P-labelled NAD. Samples (10  $\mu$ l) were treated with 1 ml lysis buffer (2 M-NaCl, 10 mM-Tris·HCl,

pH 9.0, 10 mM-EDTA, 0.1 % digitonin) for 20 min, and then pelleted in the microfuge. The supernatants were precipitated with trichloroacetic acid.

### *Electron microscopy of nuclear matrices*

Nuclei isolated the RSB method were resuspended in 10 mM-cacodylate (pH 7.4), 10 mM-NaCl, 5 mM-MgCl<sub>2</sub>, 0.1 % digitonin, 1.0 % thiodiglycol, 10 KIU/ml Trasylol, 0.1 mM-PMSF, and digested with 40 µg/ml DNase I for 1 h at 4 °C. For type I nuclear matrices, 1 ml of nuclei (5 o.d. units A<sub>260</sub>) were mixed with 8 ml of 2 M-NaCl, 10 mM-cacodylate (pH 9.0), 10 mM-EDTA, 0.1 % digitonin, 1 % thiodiglycol, 10 KIU/ml Trasylol, 0.1 mM-PMSF and were extracted for 20 min at 4 °C.

Following extraction, glutaraldehyde (Fluka EM grade) was added to each sample to a concentration of 0.2 %, and fixation was allowed to proceed for 60 min at 4 °C. All operations were carried out in glass conical tubes. After glutaraldehyde fixation, the samples were spun at 600 g for 20 min in the Clay Adams Dynac centrifuge. The pellets were resuspended gently in 100 mM-cacodylate (pH 7.4), 1.0 % glutaraldehyde and again fixed for 3 h at 4 °C. After this second fixation, the samples were pelleted (600 g, 10 min), washed three times with 100 mM-cacodylate (pH 7.4), and were post-fixed with 1 % OsO<sub>4</sub> in 100 mM-cacodylate (pH 7.4), overnight at 4 °C. Following post-fixation, the samples were washed three times with 100 mM-cacodylate (pH 7.4) and dehydrated in the following ethanol series (2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 95 %, and three times at 100 %). Each dehydration step was for 15 min at 4 °C. The samples were then kept at room temperature and allowed to be penetrated with increasing concentrations of Epon in 100 % ethanol (10, 25, 50, 75 and 100 %). For each change of Epon, the sample was mixed with the resin and allowed to stand at room temperature until the sample sank to the bottom of the tube. After penetration with 75 % Epon, the samples were centrifuged at 2000 g for 10 min in the Clay Adams centrifuge to compact the pellets. The tubes were drained well and the pellets were resuspended in 200 µl of 100 % Epon. The samples in 100 % Epon were transferred to beam capsules and the capsules were filled with 100 % Epon. The samples were allowed to be penetrated with 100 % Epon overnight at 45 °C followed by polymerization for 4 days at 60 °C. The blocks were trimmed with glass knives, and pale gold sections were cut with a diamond knife on the Sorvall Porter Blum microtome. The sections were collected on 200 mesh copper grids that were coated with parlodion and carbon. The sections were stained for 15 min with 0.5 % (w/v) uranyl acetate in 50 % (v/v) methanol and 5 min with 0.5 % uranyl acetate in 50 % methanol and 5 min with lead citrate. The sections were viewed in a Zeiss EM109 electron microscope at 40 kV.

## RESULTS

### *The proteins associated with metaphase chromosomes, nuclei and their residual structures*

We have compared the protein pattern of highly purified metaphase chromosomes and nuclei by SDS/polyacrylamide gel electrophoresis. A striking general difference regarding the composition of the non-histone proteins of these two structures is evident. This difference is particularly clear if the samples are loaded so as to have a nearly identical staining intensity of both the core and the H1 histones (Fig. 1). The non-histone protein pattern of nuclei in lane a is considerably more complex and rich compared to that of chromosomes in lane b. We have roughly determined the relative enrichment of the non-histone proteins in nuclei by densitometric scanning of the stained patterns. We find that nuclei are enriched in non-histone proteins by a factor of 3–4 as compared to chromosomes. Examination of the gel patterns permits the identification of many proteins that appear to be specific to nuclei, a representative set of such proteins is observed in the 32 to 42 × 10<sup>3</sup> M<sub>r</sub> range indicated in Fig. 1 by dots. Also evident are the well-known major structural components of the peripheral lamina

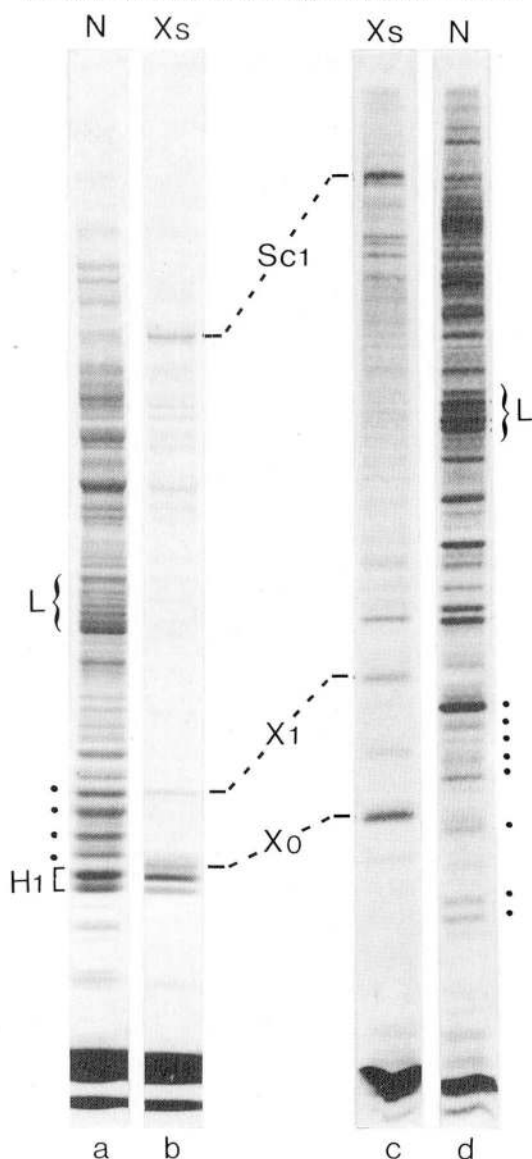


Fig. 1. Protein patterns of nuclei and metaphase chromosomes. Nuclei (N) and metaphase chromosomes (Xs) were isolated from HeLa cells in buffers containing polyamines, treated with DNase I (see Materials and Methods) and the protein patterns were analysed by SDS/polyacrylamide gel electrophoresis. The stained patterns of nuclei (lane a) and chromosomes (lane b) were obtained on linear 5 % to 15 % gradient gel. The autoradiograph of [ $^{35}\text{S}$ ]methionine-labelled chromosomes (lane c) and nuclei (lane d) was obtained on an 11 % gel. L, the cluster of lamina proteins; dots, proteins enriched in preparations of nuclei; H1, histone; Sc1, scaffold protein 1.

structure in a complex cluster of bands labelled L. Many other proteins bands that are enriched in nuclei are observed in Fig. 1.

Conversely, we observe many fewer proteins that are specifically enriched in

chromosomes, but two bands X1 ( $37 \times 10^3 M_r$ ) and X0 ( $33 \times 10^3 M_r$ ) are proteins that are characteristic of the pattern of metaphase chromosomes. The protein X0 migrates slightly above histone H1a in a 5 % to 15 % linear gradient gel (Fig. 1, lane b) and it is sometimes difficult to distinguish it from histone H1a in stained gels. However, the autoradiographic pattern derived from [ $^{35}\text{S}$ ]methionine-labelled chromosomes permits easy identification of proteins X0 and X1 (see Fig. 1, lane c). Nuclei, and to a lesser extent chromosomes, are usually contaminated by cytoplasmic components, such as actin and the various intermediate filaments. Comparison of the patterns permits the conclusion that the major differences in protein observed between nuclei and chromosomes are not due to different extents of cytoplasmic contamination.

In Fig. 2 the gel patterns of the residual scaffold structures and the nuclear matrices derived from chromosomes and nuclei, respectively, are shown for comparison. We have previously suggested the existence of metalloprotein interactions important for the structural stability of the metaphase scaffold and part of the nuclear matrices. The metals involved were identified to be  $\text{Ca}^{2+}$  and or  $\text{Cu}^{2+}$  (Lewis & Laemmli, 1982; Lebkowski & Laemmli, 1982*a,b*). The biological roles of these metals, if any, are not understood, but they are important parameters, which need to be dealt with since exposure of nuclei to these metals affects the stability and composition of the residual structures. The metaphase scaffold derived from chromosomes stabilized with  $\text{Cu}^{2+}$  contains 3–4 % of the total chromosomal proteins, the major ones being the proteins Sc1 ( $170 \times 10^3 M_r$ ) and Sc2 ( $135 \times 10^3 M_r$ ) (Lewis & Laemmli, 1982). This pattern is shown in lane f of Fig. 2 for comparison, next to the pattern of unextracted chromosomes in lane e. Included in Fig. 1 is the protein pattern of scaffolds derived from chromosomes exposed to  $\text{Ca}^{2+}$ , this structure contains about 10 % of the total chromosomal proteins. This latter pattern contains the same major proteins Sc1 and Sc2 but it is more complex regarding the minor proteins (Lewis & Laemmli, 1982). The relatively simple pattern of the chromosomal scaffold needs to be compared to the much more complex pattern of the nuclear matrices. We have defined two types of matrices: the type I matrix derived from metal-containing nuclei, and the type II matrix derived from metal-depleted nuclei (Lebkowski & Laemmli, 1982). The protein pattern of the type I matrix is complex and contains about 10–15 % of the total nuclear proteins (Fig. 2, lane c) if derived from nuclei exposed to  $\text{Ca}^{2+}$ . Among the most prominent bands are those of the nuclear lamina structure (L) and many high molecular weight proteins. We have included in Fig. 2 (lane b) the matrix type I pattern derived from nuclei exposed to  $\text{Cu}^{2+}$ ; this pattern is even more complex, containing 20–25 % of the total nuclear proteins. A series of prominent nuclear proteins in the  $32$  to  $42 \times 10^3 M_r$  range is particularly evident in the latter pattern.

Fig. 2 shows the pattern of the type II matrix in lane d. This structure is obtained from nuclei by the addition of metal chelators or thiols to the extraction buffers, which results in the solubilization of most of the proteins associated with the type I matrix, with the exception of the lamina proteins (Lebkowski & Laemmli, 1982). The type II matrix is almost exclusively composed of the three lamina proteins (L), a few minor

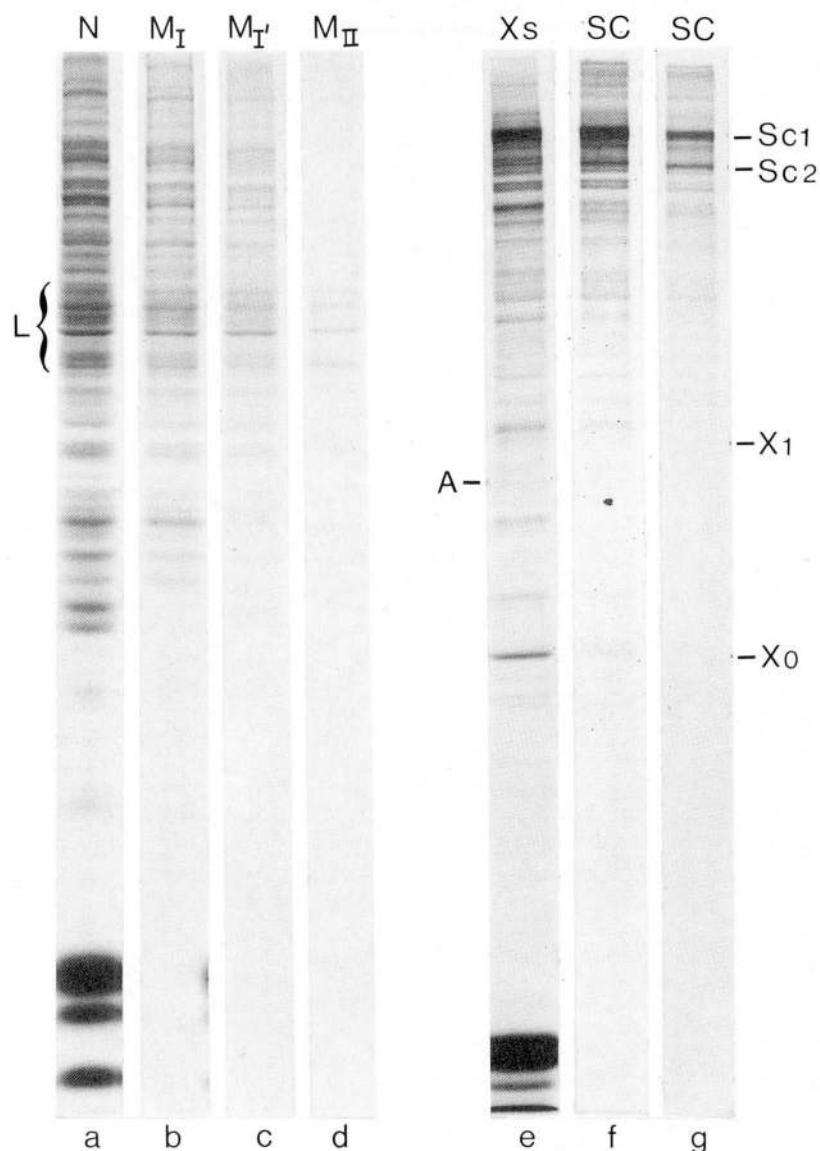


Fig. 2. Comparison of the protein patterns of nuclear matrices types I and II and of chromosomal scaffolds. Nuclear matrices (types I and II) were isolated from nuclei and chromosomal scaffolds were isolated from chromosomes, respectively, as described in Materials and Methods. The stained patterns were obtained by SDS/polyacrylamide gel electrophoresis on a 12.5% (w/v) gel. Lanes a, unextracted nuclei; b, type I matrices derived from nuclei exposed to  $\text{Cu}^{2+}$ ; c, type I matrices derived from nuclei exposed to  $\text{Ca}^{2+}$ ; d, type II matrices derived from metal-depleted nuclei. Lanes e, chromosomes; f, scaffolds derived from chromosomes exposed to  $\text{Cu}^{2+}$ ; g, scaffolds derived from chromosomes exposed to  $\text{Ca}^{2+}$ ; A, actin; L, lamina protein.

bands and a varying amount of contamination by cytoskeletal proteins, actin and the intermediate filaments. Only 3–5 % of the total nuclear proteins are recovered in the type II structure (Lebkowski & Laemmli, 1982*b*). These and our previous studies have demonstrated that the composition and morphology of the residual structures isolated from chromosomes and nuclei can differ depending on the method of isolation

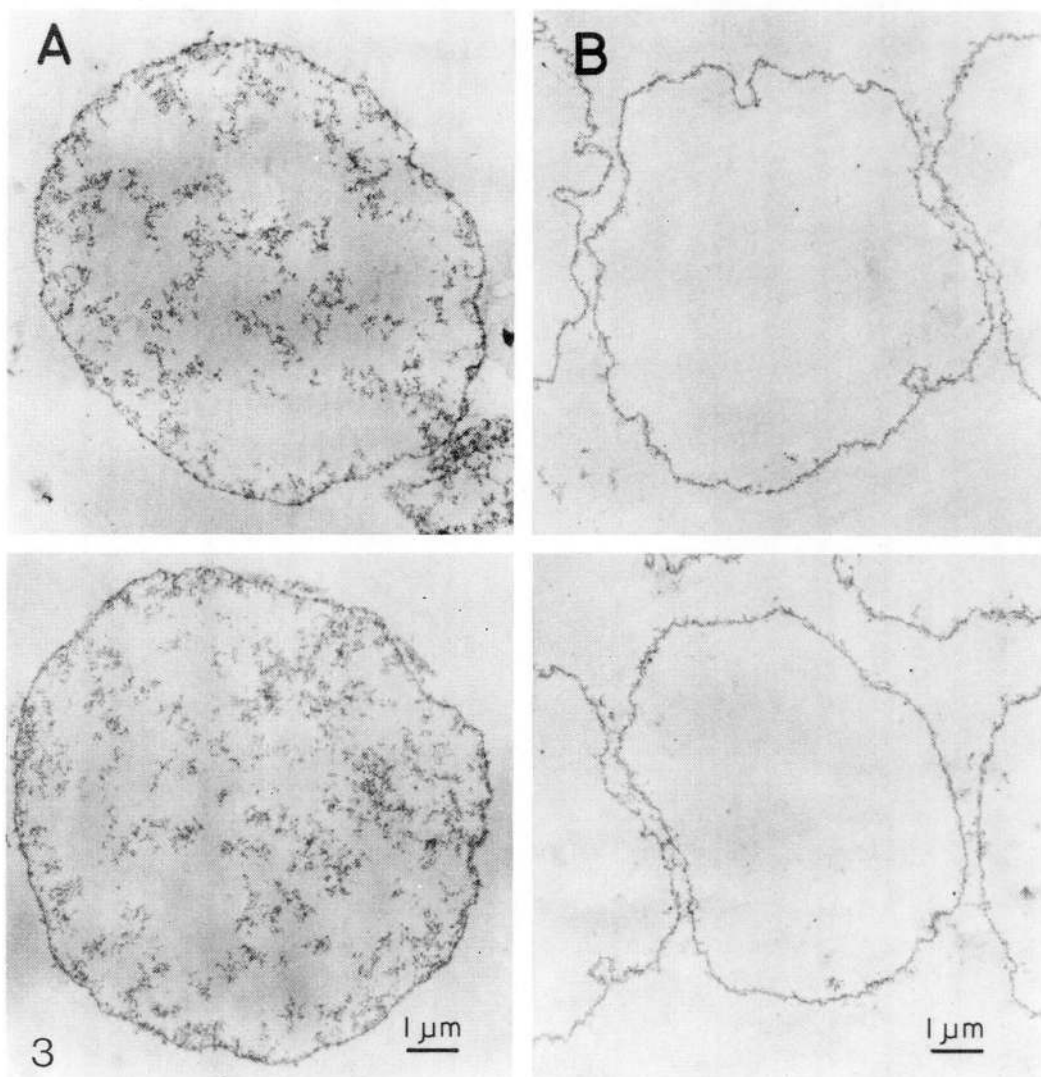


Fig. 3. Electron micrographs of type I and type II nuclear matrices. Interphase HeLa nuclei were isolated, digested with DNase I, extracted with the 2 M-NaCl lysis mixture and fixed for electron microscopy as described in Materials and Methods. During the cell homogenization step of nuclear isolation, the nuclei were digested with 50  $\mu\text{g}/\text{ml}$  RNase A at 4°C. A. Type I matrices after extraction with the 2 M-NaCl lysis mixture; B, type II matrices after extraction in the 2 M-NaCl lysis mixture containing 0.1 %  $\beta$ -mercaptoethanol.  $\times 4400$ .

(Lebkowski & Laemmli, 1982*a,b*; Lewis & Laemmli, 1982; Earnshaw & Laemmli, 1983). We have identified the metals  $\text{Ca}^{2+}$  and/or  $\text{Cu}^{2+}$  as the major parameters that affect the composition and morphology of the residual structures.

*Selective dissociation of the internal structures of the nuclear matrix*

We have previously examined the morphology of the metaphase scaffold in some detail (Earnshaw & Laemmli, 1983). The residual nuclear matrix has been examined in the electron microscope by various workers (Berezney & Coffey, 1974; Comings

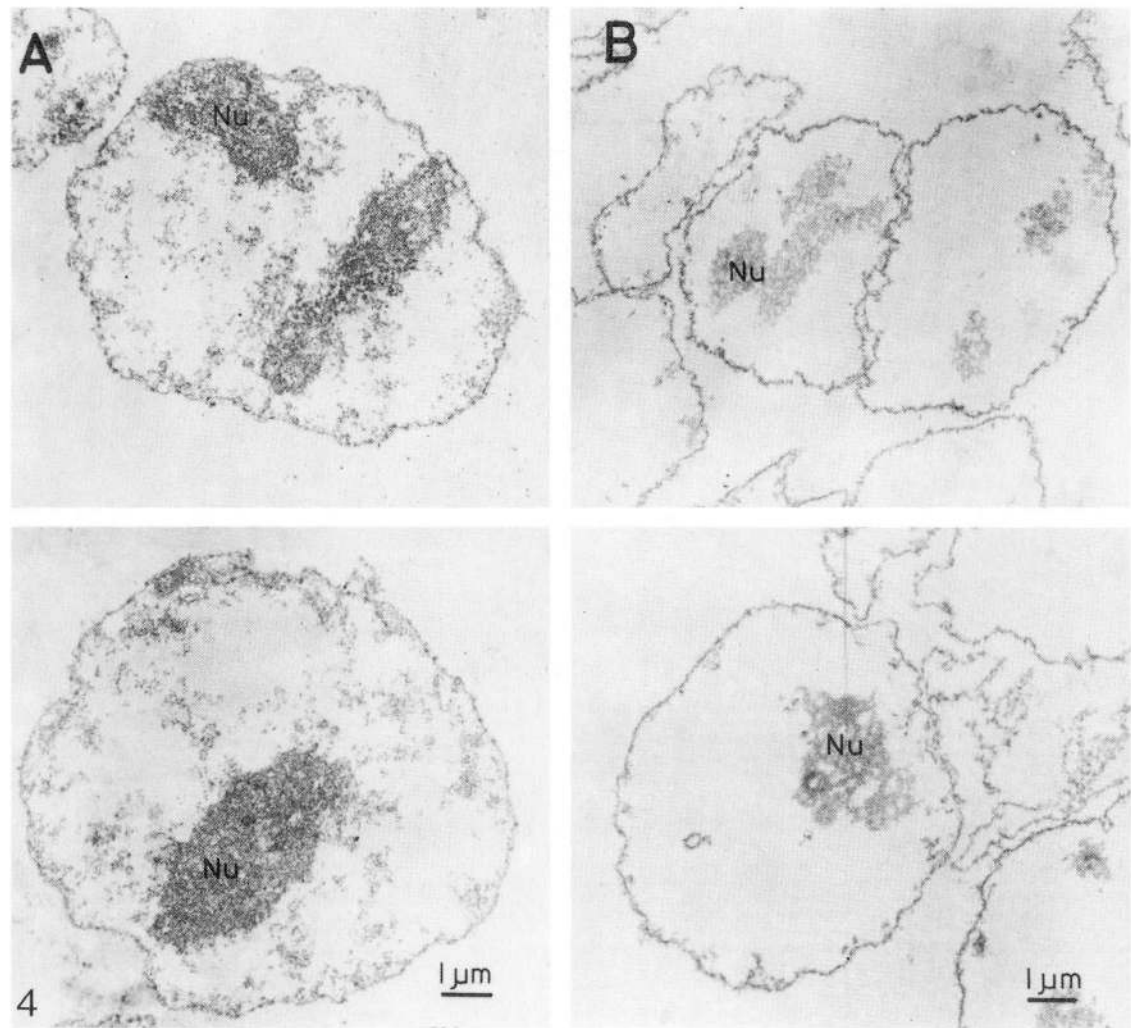


Fig. 4. Electron micrographs of type I and type II nuclear matrices from nuclei not treated with RNase A. Type I and II matrices were isolated from nuclei that were not digested with RNase A during isolation of the nuclei. A. Type I matrices isolated in the 2 M-NaCl lysis mixture; B, type II matrices isolated in the 2 M-NaCl lysis mixture containing 0.1%  $\beta$ -mercaptoethanol. Nu, residual nucleolar material.  $\times 4400$ .

& Okada, 1976; Herman *et al.* 1978; Long *et al.* 1979; van Eckelen & van Venrooij, 1981), yet controversy exists regarding the morphology and composition of these substructures (see Kaufman, Coffey & Shaper, 1981; Lebkowski & Laemmli, 1982). Comparison of the nuclear matrices types I and II prepared by our procedure in the light microscope suggests that most of the proteins lost during extraction in the absence of metals are derived from the interior of extracted nuclei. To study the morphology of the nuclear matrices in some detail we have prepared thin sections for the electron microscope. These structures are typically composed of three structural parts: a peripheral nuclear lamina, a residual nucleolar material and an internal network, which spans the space between the nucleolar material and the peripheral lamina. It is possible to extract selectively either the residual nucleolar material or the internal network. Extensive RNase digestion of the nuclei before extraction eliminates the residual nucleolar material (compare Figs 3 and 4). Metal chelation, on the other hand, by thiols before high salt extraction dissociates the internal network. The matrix type II obtained under these conditions is composed of the peripheral lamina and the nucleolar material and contains no visible internal network of protein (Figs 3B, 4B). RNase treatment eliminates the residual nucleolar structure from matrix type II; but is not required to solubilize the internal (non-nucleolar) network. This observation is consistent with our biochemical studies, which show that RNase digestion is not essential for the preparation of either matrix type (Lebkowski & Laemmli, 1982a,b).

A comparison of the proteins associated with nuclear matrices types I and II demonstrates that the disappearance of the internal network of the matrix coincides with the loss of many proteins (Fig. 2). We were unable to identify proteins that might be specific to residual nucleolar structure by comparing the gel pattern derived from extracted nuclei that were either treated or not treated with RNase (Laemmli & Lebkowski, 1983b). It is of importance to point out that the selective dissociation of either the internal network or the residual nucleolar structure is only reproducibly possible if the isolated nuclei are not exposed to  $\text{CaCl}_2$  at  $37^\circ\text{C}$ . We have previously demonstrated that incubation of nuclei with  $\text{CaCl}_2$  at  $37^\circ\text{C}$ , but not at  $4^\circ\text{C}$ , leads to a 'toughening' of the nuclei, which prevents solubilization of the internal network to form matrix type II (Lebkowski & Laemmli, 1983a,b). We have confirmed the biochemical observation by electron microscopy, finding that nuclei toughened with  $\text{CaCl}_2$  generate a structure similar to that shown in Fig. 4A. They contain both the internal network and the residual nucleolar structure despite the presence of thiols and/or metal chelators during extraction (data not shown).

*RNA polymerase II but not poly(ADP-ribose) polymerase is bound to nuclear matrix type I*

The eukaryotic class II RNA polymerases are large enzymes, composed of 10–15 distinct polypeptides, including two high molecular weight proteins in the range of 130 to  $200 \times 10^3 M_r$  (e.g. see Paule, 1981). The engaged RNA polymerase forms very tight complexes with DNA, resisting extraction conditions that solubilize the histones. It was important to determine whether the two scaffolding proteins Sc1 and

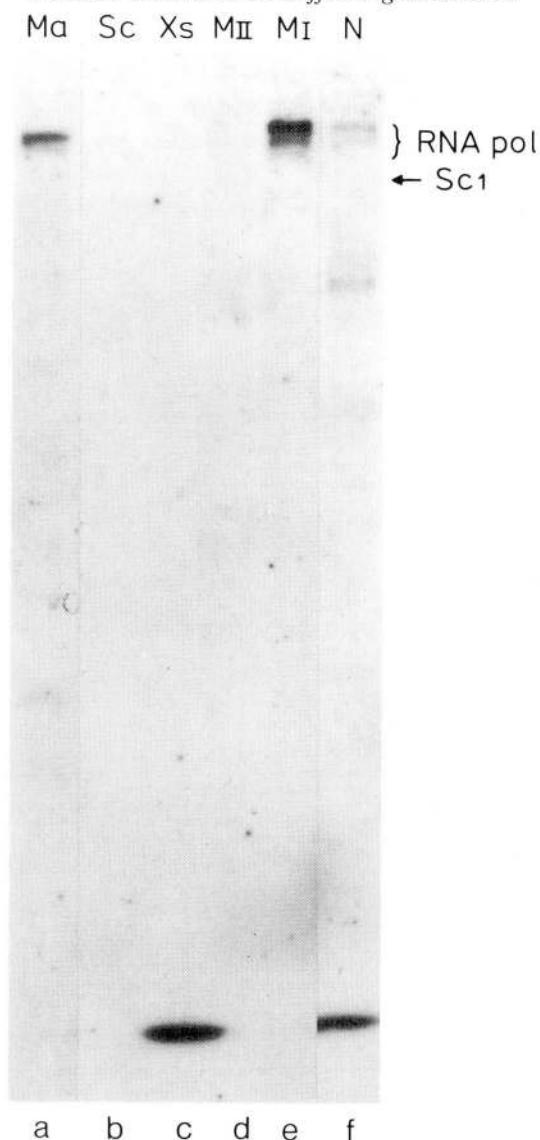


Fig. 5. Detection of RNA polymerase II in nuclei and type I nuclear matrices. Polyamine chromosomes, metaphase scaffolds, RSB nuclei and interphase matrices types I and II were isolated as described by Lewis & Laemmli (1982) and Lebkowski & Laemmli (1982b). RNase treatment and thiodiglycol were omitted from the isolation procedures. An extract was isolated from HeLa cells as described by Manley *et al.* (1980). Immunoblotting was carried out as described in Materials and Methods, using a monoclonal antibody directed against the two largest subunits of RNA polymerase II from *Drosophila*, as described by Kramer *et al.* (1980). Lanes a, 'Manley' extract; b, scaffold; c, chromosomes; d, nuclear matrix type II; e, nuclear matrix type I; f, whole RSB nuclei. The nuclear matrices types I and II are overloaded by a factor of about 3 relative to the nuclei. The relative positions of the Sc1 protein and the two RNA polymerase II subunits are indicated.

Sc2 are components of the polymerase. Furthermore, it has recently been suggested that the active transcription complexes are associated with the nuclear matrix (Jackson *et al.* 1981; Ciejek *et al.* 1982). If this is correct, polymerase molecules might be found preferentially bound to the nuclear matrix. Finally, the questions of the cellular location of the polymerase during mitosis, as well the physical basis for the absence of transcriptional activity during mitosis, have not been resolved (Matsui, Weinfeld & Sandberg, 1979; Gariglio, Buss & Green, 1974). For these reasons, we have tested metaphase chromosomes and scaffolds, nuclei and nuclear matrices for the binding of RNA polymerase II. Monoclonal antibodies directed against the two largest subunits of RNA polymerase II of *Drosophila melanogaster* were used (kindly supplied by E. Bautz) as probes against various nuclear substructures isolated from HeLa cells. This antibody cross-reacts with the two largest subunits of RNA polymerase II isolated from various organisms (Kramer & Bautz, 1981). The immunoblot in Fig. 5 identifies the two large subunits of the RNA polymerase II in whole nuclei (lane f) and in nuclear matrix type I (lane e), but not matrix type II (lane d). In both nuclei and matrix type I, a doublet of proteins is observed. As a control, we have included in the immunoblot (Fig. 5, lane a) an extract rich in RNA polymerase II prepared from HeLa cells according to the procedure of Manley *et al.* (1980). The binding of the RNA polymerase II subunits to the matrix I structure appears quantitative. We have loaded (in Fig. 5, lane e) three times as many matrices as we have loaded nuclei (in lane f). The immunoreaction is more intense by about this factor in the matrix sample as compared to that in nuclei. In contrast to the detection of the proteins of the large subunit of polymerase II in nuclei, matrix type I and the Manley extract, we detected no reaction in metaphase chromosomes and scaffolds (Fig. 5, lanes b and c). This result indicates that the Sc1 and Sc2 proteins are not the two largest subunits of RNA polymerase II. These data are not affected by the method of isolation of chromosomes or nuclei, since the same results are obtained using chromosomes and nuclei isolated in either aqueous or polyamine buffer systems (data not shown).

From the experiment shown in Fig. 5 it appears that no RNA polymerase II is observed in metaphase chromosomes. In order to verify this result by an alternative method, chromosomes and nuclei were used for *in vitro* transcription assays. In this assay system, engaged polymerase molecules will continue but not reinitiate transcription (Groner, Monroy, Jacquet & Hurwitz, 1975).

Time-course studies of transcriptional activities in both chromosomes and nuclei are presented in Fig. 6. For nuclei, the incorporation of [ $^3\text{H}$ ]UTP/ $\mu\text{g}$  DNA is shown to be highly dependent upon the inclusion of either the anionic detergent Sarkosyl or the polyanion heparin in the assay buffer. Transcriptional activity is stimulated in polyamine nuclei by as much as factors of 10 and 13 using Sarkosyl and heparin, respectively. These agents have been shown previously to stimulate RNA polymerase activity, presumably by releasing proteins from DNA that repress the polymerase (Gariglio *et al.* 1974; Cox, 1976).

In contrast to nuclei, the incorporation of [ $^3\text{H}$ ]UTP/ $\mu\text{g}$  DNA into chromosomes (Fig. 6, panel b) indicates a very low level of activity, which is never more than 6%

of that observed for nuclei. There is some marginal stimulation due to heparin and Sarkosyl. Transcriptional assays carried out with nuclei and chromosomes purified by either aqueous or polyamine buffers gave similar results (data not shown). These results confirm the immunoblotting data shown above. It is clear from both types of assay, *in vitro* transcription and immunoblotting, that very little, if any, polymerase is bound to purified metaphase chromosomes.

The drug  $\alpha$ -amanitin, which preferentially inhibits RNA polymerase II activity at

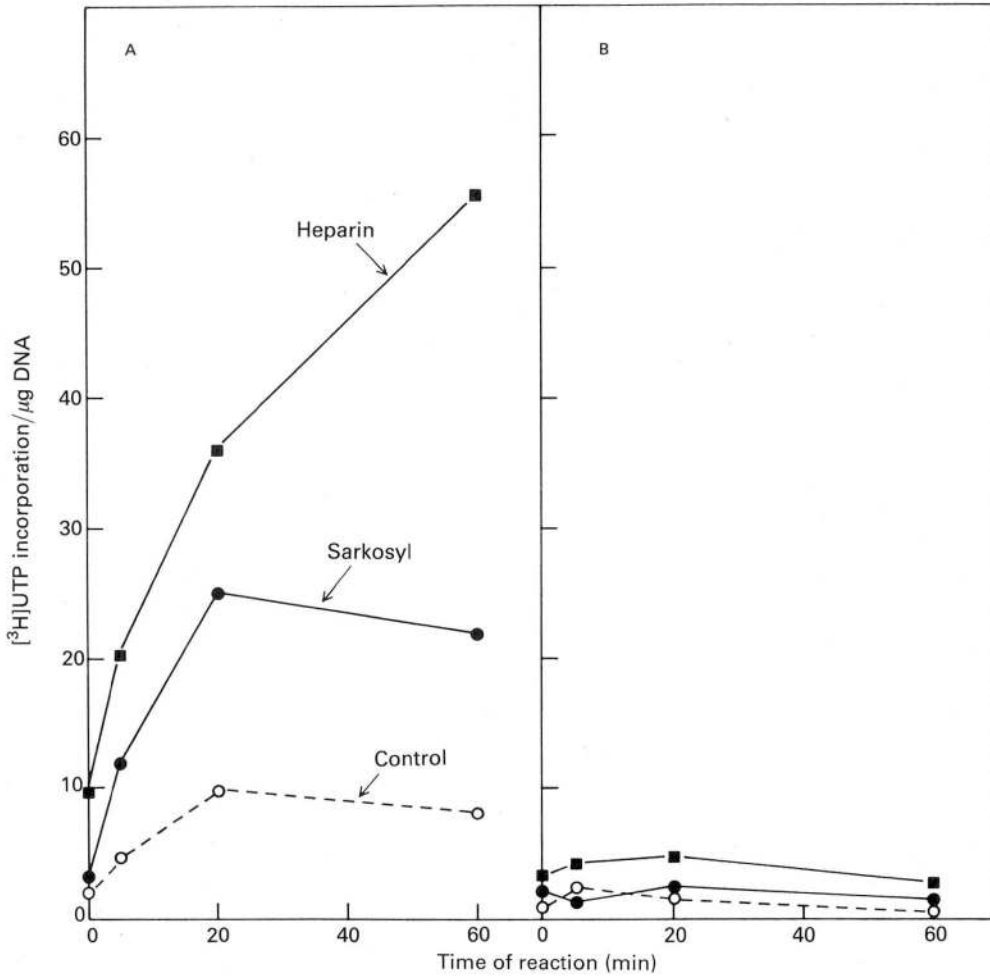


Fig. 6. *In vitro* transcription from chromosomes and nuclei. Polyamine chromosomes and RSB nuclei were isolated as described by Lewis & Laemmli (1982) and Lebkowski & Laemmli (1982b), but without RNase treatment and in the absence of thiodiglycol. Transcription assays were carried out according to the method of Cox (1976) and as described in Materials and Methods. A and B show transcriptional activity in nuclei and chromosomes, respectively, in intact nuclei or chromosomes (○---○), or following addition of Sarkosyl (●—●), or 1 mg/ml of heparin (■—■).

a concentration of  $0.5 \mu\text{g/ml}$  (Weinmann & Roeder, 1974), reduces the overall [ $^3\text{H}$ ]UTP incorporation in nuclei by 85–90%. In the presence of  $1 \mu\text{g/ml}$  of actinomycin D (Roberts & Newmann, 1966), all transcriptional activity is inhibited. At a concentration of  $0.04 \mu\text{g/ml}$  of actinomycin D, 10–15% activity due to RNA polymerase I is inhibited (data not shown).

It has been suggested that the negligible polymerase activity observed in metaphase chromosomes is due to the highly condensed state of the chromatin in these structures, and that interphase polymerase levels can be obtained from metaphase chromosomes if the enzyme is solubilized by sonication (Matsui *et al.* 1979). In order to determine the validity of this suggestion, transcription assays were performed with supernatants of sonicated nuclei and chromosomes as described (Matsui *et al.* 1979). The levels of [ $^3\text{H}$ ]UTP incorporation that were obtained (data not shown) indicate that sonication does not release equal amounts of polymerase activity from nuclei and chromosomes; rather, the activities are equivalent to those in the intact structures, respectively.

In order to compare the localization of RNA polymerase II with another nuclear enzyme, we have assayed for poly(ADP-ribose) polymerase activity in nuclei, matrices, chromosomes and scaffolds. Poly(ADP-ribose) polymerase is a nuclear enzyme (Chambon, Weill & Mandel, 1963), which is automodified by the repeated addition of ADP-ribose from the substrate NAD (Yoshinara *et al.* 1977; Ogata *et al.* 1981). The enzyme has also been shown to be present in metaphase chromosomes (Hotlund, Kristensen, Ostvold & Laland, 1980). Studies on partially purified and purified preparations of the enzyme have shown that DNA and histones are necessary for its activity (Jump & Smulson, 1980). In addition, nicking of DNA by DNase I has been shown to activate the enzyme in permeabilized cells (Benjamin & Gill, 1980). Automodification enables the enzyme to be detected by autoradiography of SDS/polyacrylamide gels following incubation of nuclei with  $^{32}\text{P}$ -labelled NAD. The major ADP-ribose acceptor in nuclei is a polypeptide of molecular weight  $105 \times 10^3$  (Fig. 7, lane 1), which corresponds to the molecular weight of poly(ADP-ribose) polymerase (Jump & Smulson, 1980). When longer reaction times are used (lane 2) up to 20 other peptides are also labelled. The nuclei used in this study were treated with micrococcal nuclease, which gives a three to four-fold increase in enzyme activity (data not shown). An identical pattern is obtained for metaphase chromosomes (data not shown).

To determine if poly(ADP-ribose) polymerase is present on metaphase scaffolds and nuclear matrices, micrococcal nuclease-treated nuclei and chromosomes were labelled with [ $^{32}\text{P}$ ]NAD and extracted with lysis buffers containing a range of salt concentrations (50 mM to 2 M-NaCl). Although the same results were obtained for both chromosomes and nuclei, only those for nuclei are shown (Fig. 7, lanes 2–18). It is clear that salt extraction releases the enzyme from the matrix. Dissociation of poly(ADP-ribose) polymerase is almost complete at a concentration of 0.5 M-NaCl. This extraction condition also solubilizes most of the other ADP-ribose acceptor proteins. The extraction of the poly(ADP-ribose) polymerase and its acceptor proteins is complete following extraction with 1 M-NaCl (lane 7). Thus type I nuclear matrices, which are prepared in a lysis buffer containing 2 M-NaCl (lane 8), contain

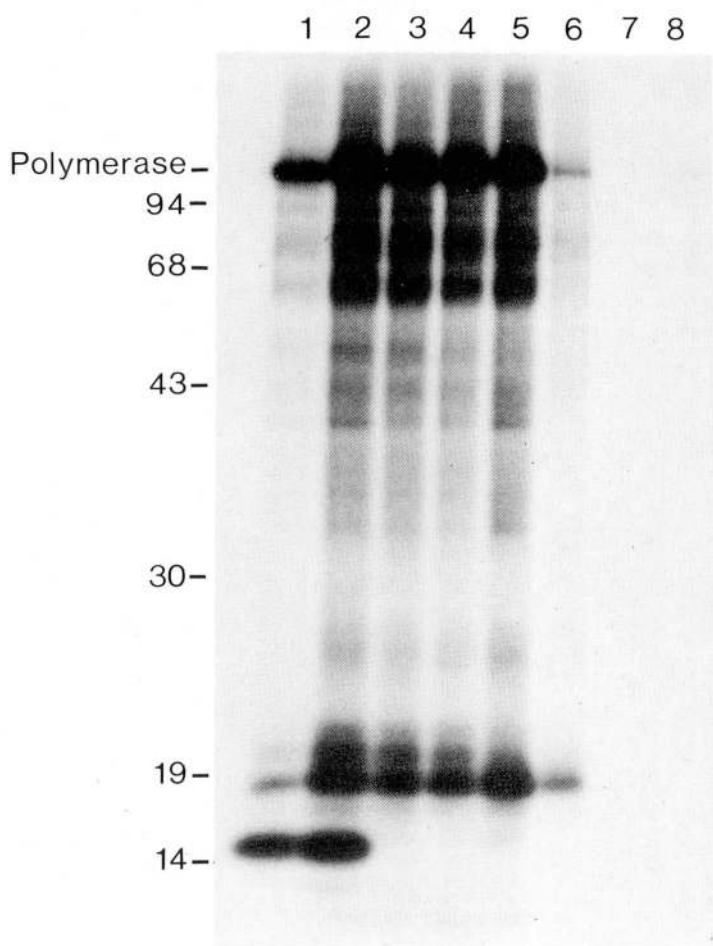


Fig. 7. Salt-dependent dissociation of poly(ADP-ribose) polymerase from nuclei. Polyamine nuclei were assayed for poly(ADP-ribose) polymerase after treatment with micrococcal nuclease. After a 10-min incubation with  $1\ \mu\text{M}$ - $[^{32}\text{P}]$ labelled NAD, samples were extracted with lysis buffers containing 50 mM to 2 M-NaCl. The resulting matrices were pelleted. Lanes 1 and 2 show nuclei that were incubated for 30 s and 10 min, respectively. Lanes 3–8 show residual nuclear structures prepared following extraction with 50 mM, 100 mM, 200 mM, 500 mM and 2 M-NaCl, respectively.

no poly(ADP-ribose) polymerase. Similar results were obtained for chromosomal scaffolds and for histone-depleted nuclei and chromosomes.

#### DISCUSSION

Comparison of the total gel pattern of chromosomes and nuclei permitted the identification of protein bands that are specific to or at least highly enriched in either of these structures. Proteins X0 ( $33 \times 10^3 M_r$ ) and X1 ( $37 \times 10^3 M_r$ ) are examples of typical chromosomal proteins while the proteins indicated by dots in Fig. 1 are

prominent nuclear proteins. The most characteristic set of proteins of the latter class are observed in the  $32$  to  $42 \times 10^3 M_r$  range, it is tempting to speculate that these proteins may be the structural components of the hnRNP particles (Beyer, Christen, Walter & Stourgeon, 1977). This set of proteins is also observed in matrix type I (Fig. 1), possibly supporting the finding that hnRNA is intimately linked to the nuclear matrix (Herman *et al.* 1978; Miller, Huang & Pogo, 1978; van Eekelen & van Venrooij, 1981).

Nuclei are considerably richer in the content and complexity of their non-histone proteins. Nuclei contain, relative to their histones in weight, three to four times more non-histone proteins than metaphase chromosomes according to our estimate. This finding is not entirely surprising since metaphase chromosomes are inert in terms of transcription and replication. Thus many of the proteins involved in these processes are expected to be released at mitosis. In addition, the nuclear lamina structure dissociates (Gerace & Blobel, 1980) and the hnRNP particles (Martin & Okamura, 1981) are known to be dispersed at mitosis. Our results also show that RNA polymerase is present in nuclei but not in metaphase chromosomes, whereas poly(ADP-ribose) polymerase is present in an active form in both.

We have defined two types of nuclear matrices. Most of the complex set of non-histone proteins observed in the type I but not in the type II structure must be associated with the internal network, which fills the space between the peripheral lamina structure and the residual nucleolar material. Electron micrographs confirm our biochemical observations, showing an internal network in the type I but not in the type II matrix. Residual nucleolar material can be selectively extracted by treatment of nuclei before extraction with RNase A. Thus metal chelation selectively allows solubilization of the internal network, while RNase treatment solubilizes the residual nucleolar material but not the former structure. The classification of the nuclear matrices into types I and II is no doubt an oversimplification. We have observed more subtle compositional differences between matrices depending on various parameters like pH, ionic strength, type of detergent, etc. . . These parameters need to be studied in more detail.

There has been some controversy concerning the composition of the nuclear matrix. Some investigators have found the internal network of fibres to be absent in preparations of nuclear substructures (Aaronson & Blobel, 1975; Dwyer & Blobel, 1978). While others find an internal network and residual nucleolar material (Berezney & Coffey, 1974; Comings & Okada, 1976; Herman *et al.* 1978; Long *et al.* 1979; van Eekelen & van Venrooij, 1981). This and our previous reports (Lebkowski & Laemmli, 1982*a,b*) define conditions under which the filled matrix type I or empty matrix type II structures are obtained. Other reports have also dealt with the questions of the biochemical composition and the morphology of the subnuclear structures (Fisher, Berrios & Blobel, 1982; Kaufmann *et al.* 1981; Adolph, 1980).

Some discrepancy regarding the effect of RNase treatment and the stability of the internal network remains. Kaufmann *et al.* (1981) have recently suggested that the internal network of the nuclear matrix is absent when nuclei are treated with DNase and RNase, and isolated under conditions that minimize formation of disulphide

bonds. Under our conditions RNase treatment of nuclei before extraction eliminates only the residual nucleolar material but not the internal network, an observation that is clearly supported by our biochemical studies. We have also suggested that the internal network is stabilized by metalloprotein interaction (Lebkowski & Laemmli, 1982) rather than by extensive disulphide bonding (Kaufmann *et al.* 1981). Additional work is under way, to study these questions in more detail.

RNA polymerase II is bound to the nuclear matrix type I but not to the type II structure, and it is absent from metaphase chromosomes. In contrast, poly(ADP-ribose) polymerase is present in nuclei and chromosomes, but absent in type I and II nuclear matrices. This enzyme is dissociated completely from nuclei and chromosomes following extraction at intermediate salt concentration of about 0.5 M-NaCl.

One of the biochemical events that distinguishes mitosis from the  $G_1$ ,  $S$  and  $G_2$  phases of the cell cycle is the absence in mitosis of any transcriptional activity of RNA polymerase I or II (Johnson & Holland, 1965; Morcillo, de la Torre & Gimenez-Martin, 1976), while continued synthesis of small mitochondrial RNAs by polymerase III has been detected in metaphase cells (Zylber & Penman, 1971). The physical basis for the absence of polymerase II activity has not been explained, but it has been suggested that inactivation of the enzyme might be caused by the dense packing of the chromatin in metaphase chromosomes or by the dissociation of the enzyme from the chromosome as the cell enters mitosis. The results presented here strongly suggest that the second alternative is the correct one.

What is the structural and functional relationship of the metaphase scaffolding and the nuclear matrix? The latter structure is compositionally and structurally much more complex, being composed of the peripheral lamina, a residual nucleolar network and an internal network. The peripheral lamina dissociates during mitosis and is consequently expected to have no major role in metaphase chromosome structure. The internal network of the matrix shares some properties with metaphase scaffolding. The structural stability of both is due to metalloprotein interactions, at least one of the major structural component of the scaffolding, Sc1, is also part of the internal network (J. van Ness, C. Lewis & U. K. Laemmli, unpublished data). Both structures are involved in the folding of the DNA of histone-depleted chromosomes and nuclei, respectively. We have recently presented sedimentation studies, which permitted the identification of two different levels of folding of the DNA in histone-depleted nuclei. One of these levels of DNA folding is due to the interaction of the DNA with the peripheral lamina while a second level is brought about by the internal network (Lebkowski & Laemmli, 1982a).

This structural role cannot be the only function of the internal nuclear network; the protein complexity alone suggests additional biological roles. Indeed various reports suggest the possible involvement of the matrix in DNA replication (Berezney & Coffey, 1975; Vogelstein *et al.* 1980), transcription (Jackson *et al.* 1981) and RNA processing (Ciejek *et al.* 1982). Our finding regarding the association of RNA polymerase II with the matrix is consistent with a role in transcription.

As a possible explanation for the attachment of these processes to the nuclear

matrix, one could propose that the matrix is involved in subdividing the nucleus into compartments, which are microenvironments containing and maintaining the various factors, proteins and enzymes needed to carry out each of these biological processes. The elements needed in a given compartment might have a high affinity for the matrix, thus preventing general diffusion and destruction of the compartment.

The association of RNA polymerase II with the nuclear matrix also could be due to an artefactual precipitation of the transcription complexes by the high salt concentration used in these experiments. These transcription complexes might be close to but not directly attached to a subnuclear structural element.

We have recently made progress in studying the DNA sequence organization of the higher-order chromatin loops. We find that the DNA loops are highly organized and the attachment of the DNA to the nuclear matrix occurs at highly specific sites. This specific loop organization is only observed following preparations of nuclear scaffolds by a newly developed method using low salt conditions (J. Mirkovitch, M.-E. Mirault & U. K. Laemmli, unpublished). Conversely, a loss of this specific organization of the loops is observed following exposure of the nuclei to high salt, possibly due to sliding of the DNA along the attachment points. Interestingly, we find that the transcribed regions of active genes are not attached to the matrix under the low salt conditions, whereas attachment of these transcribed regions is found if high salt conditions are used. These observations do not inspire confidence in the hypothesis that all interactions observed in high-salt-extracted matrices are of biological significance.

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