Changes in the nuclear matrix of chicken erythrocytes that accompany maturation

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The protein composition and structure of nuclear matrices isolated from adult chicken immature and mature erythrocytes were analysed. Visualization of nuclear matrices by electron microscopy showed that immature-erythrocyte nuclear matrices had internal structures, while most mature-erythrocyte nuclear matrices did not. Both mature- and immature-erythrocyte nuclear matrices were surrounded by a fibrous network of intermediate filaments. Two-dimensional gel electrophoretic analysis of proteins obtained from fractionated nuclear matrices led to the assignment of the proteins as components of the nuclear pore-lamina, internal matrix, or cytoskeleton. Common and different proteins belonging to one of the three groups were identified in

nuclear matrices of immature and mature erythrocytes. Investigation of the partitioning of histone deacetylase activity, an enzyme associated with the internal matrix, among the erythroid nuclear matrix fractions provided evidence that mature- and immature-erythrocyte nuclear matrices have internal structures. However, the activity of histone deacetylase and level of internal matrix proteins from mature-erythrocyte nuclear matrices were less than those from immature-erythrocyte matrices. The low levels of nuclear RNA and internal matrix proteins may account for lack of visual evidence for an internal matrix in mature erythrocytes.

INTRODUCTION

Many nuclear metabolic processes are associated with the nuclear matrix, including RNA processing and transport [1–4], transcription [5–7], DNA replication [8,9], and dynamic histone acetylation [10–12]. This nuclear substructure is also involved in the organization of the eukaryotic genome into discrete chromatin domains [13–15]. The nuclear matrix consists of a nuclear pore-lamina complex, residual nucleoli and internal matrix [16–18]. The association of most, if not all, nuclear macromolecular metabolism with the nuclear matrix suggests that these processes contribute to the nuclear matrix structure. However, there is conflicting evidence about whether ‘transcriptionally silent’ adult chicken mature erythrocytes have an internal matrix.

Electron-microscopic observations of nuclear-matrix preparations have suggested that the presence of an internal matrix is dependent upon (or forms because of) the transcriptional activity of the nucleus [18–21]. Adult chicken mature erythrocytes, which are often considered as transcriptionally repressed, lack internal matrices [20]. Further, nuclear matrices from primitive erythrocytes of 5-day-old chick embryos, but not those from definitive erythrocytes of 11–14-day old chick embryos, have internal matrices [20]. The transcriptional activity of the 11–14-day embryonic definitive erythrocytes is less than that of primitive erythrocytes [22]. Transcriptional re-activation leads to the formation of internal structures in mature-erythrocyte nuclei [19]. The structure of the nuclear matrix of immature erythrocytes from anaemic adult birds has not yet been reported. In contrast with the electron-micrographic data, there is biochemical evidence that adult chicken mature-erythrocyte nuclei have an internal matrix. Biochemical studies have presented evidence for the organization of chromatin domains in mature erythrocytes [23,24], and *in vitro* nuclear matrix DNA binding assays have shown that the mature-erythrocyte nuclear matrix binds matrix

attachment regions (MARs) of various gene domains (e.g. α -globin and lysozyme gene domains) [24,25]. Further, ARBP, a MAR-binding protein that is a component of the internal matrix, is found in nuclear matrices of chicken mature erythrocytes [26].

In contrast with the view that mature erythrocytes are transcriptionally inert, there is evidence that these mature cells are transcriptionally active. Transcription run-on assays in permeabilized cells show the β -globin and histone H5 genes are transcribed in mature erythrocytes [27]. Furthermore, RNA synthesis by RNA polymerase II occurs in adult mature erythroid nuclei, but at a level 4-fold lower than that in adult immature erythroid (reticulocyte) nuclei [28]. Although transcription is occurring in mature erythroid nuclei, the RNA content in mature erythrocytes is minimal [29].

In the present study we compared the structure and composition of nuclear matrices isolated from mature and immature erythrocytes. We show that adult chicken immature-erythrocyte nuclear matrices had internal matrix structures, while most mature-erythrocyte nuclear matrices do not. However, a few mature-erythrocyte nuclear matrices had internal structures. Studies monitoring histone deacetylase activity from fractionated nuclear matrices provide evidence for internal matrix structures in mature- and immature-erythrocyte nuclei. Two-dimensional gel-electrophoretic analysis of nuclear matrix proteins from the erythroid cells reveals alterations in nuclear matrix protein composition that occur during maturation.

MATERIALS AND METHODS

Isolation of adult chicken immature and mature erythrocytes

Immature erythrocytes (40 % early and mid-polychromatic erythrocytes and 55 % late polychromatic erythrocytes) were collected

Abbreviations used: IF, intermediate filament; NM, nuclear matrix; IM, internal matrix; NPL, nuclear pore-lamina; NM-IF, nuclear matrix with associated intermediate filaments; ARBP, attachment region binding protein; MAR, matrix attachment region.

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from adult White Leghorn chickens treated with phenylhydrazine hydrochloride as described previously [30]. Mature erythrocytes were collected either from adult White Leghorn chickens at a local slaughterhouse or from adult White Leghorn chickens used to prepare immature erythrocytes. The results of studies on mature-erythrocyte nuclear matrix composition and structure were identical with red blood cells obtained from either source of birds. Cells were collected directly into an equal volume of blood collection buffer [10 mM Tris/HCl (pH 7.5)/25 mM EDTA/75 mM NaCl]. The cells were harvested by centrifugation at 500 *g* for 4 min. They were then washed twice in blood collection buffer, and the packed erythrocyte cell pellet was frozen at -80°C until use.

Isolation of erythrocyte nuclei and nuclear matrices

Nuclei were isolated by lysing erythrocytes in CSK buffer [100 mM KCl/3 mM MgCl_2 /10 mM Pipes (pH 6.8)/1 mM EGTA/0.3 M sucrose/0.5% (v/v) thiodiglycol/1 mM PMSF] with 0.25% Triton X-100. The nuclei collected by centrifugation (1085 *g*, 10 min) were resuspended in CSK buffer. The nuclear suspension was passed three times through a 22-gauge needle and then layered on top of CSK buffer with 0.7 M sucrose. The nuclei were collected by centrifugation at 1935 *g* for 10 min. For some studies the nuclei were incubated for 10 min on ice in CSK buffer containing 1 mM CuCl_2 . Nuclear matrices were prepared using a procedure derived from the protocol of He et al. [16]. Briefly, purified nuclei were resuspended in digestion buffer [10 mM Pipes (pH 6.8)/50 mM NaCl/300 mM sucrose/3 mM MgCl_2 /1 mM EGTA/0.5% (v/v) Triton X-100] at 20 A_{260} units/ml. DNase I (D 5025; Sigma) was added to a final concentration of 100 $\mu\text{g}/\text{ml}$, and the nuclei were digested for approx. 60 min at 23°C . Ammonium sulphate was added dropwise from a 4 M stock to a final concentration of 0.25 M, and the 0.25 ammonium sulphate nuclear matrices (NM1-IF) were collected by centrifugation at 5100 *g* for 10 min. The supernatant S1 contained most of the chromatin. The NM1-IF nuclear matrices were resuspended in digestion buffer and re-extracted by slowly adding NaCl to a final concentration of 2.0 M from a 4.0 M stock solution while mixing. This was centrifuged at 2000 *g* for 10 min to produce a soluble fraction (S2) and an insoluble fraction (the 0.25 ammonium sulphate/2 M NaCl-extracted nuclear matrix, NM2-IF). Sometimes the NM2-IF nuclear matrices were extracted with 2 M NaCl and 1% (v/v) 2-mercaptoethanol for 30 min on ice. The soluble (S3) and insoluble (NM3-IF) fractions were separated by centrifugation at 5100 *g* for 10 min. Nuclear pore-lamina complexes (NPL) were isolated by the protocol described by Kaufmann and colleagues [31]. To separate nuclear matrix proteins from intermediate filaments, NM2-IF nuclear matrices were solubilized in disassembly buffer [8 M urea/20 mM Mes (pH 6.6)/1 mM EGTA/1 mM PMSF/0.1 mM MgCl_2 /1% (v/v) 2-mercaptoethanol] as described previously [32]. The samples were dialysed against assembly buffer [0.15 M KCl/25 mM imidazole hydrochloride (pH 7.1)/5 mM MgCl_2 /2 mM dithiothreitol/0.125 mM EGTA/0.2 mM PMSF]. The re-assembled intermediate filaments (IF) were removed by centrifugation (150000 *g*, 90 min). Both pellet (IF) and supernatant (NM) fractions were saved.

Histone deacetylase assay

Histone deacetylase assay was done in 25 mM sodium phosphate/citric acid buffer, pH 7.0, and 150000 d.p.m. (180 μg) total chicken erythrocyte histones at 37°C for 30 min as described previously [10]. After incubation, enzyme reaction was stopped by 0.12 M/0.72 M (final concentration) of acetic acid/HCl, and

two volumes of ethyl acetate were mixed with reaction solution. The samples were centrifuged at 9000 *g* for 1 min, and a half volume of added ethyl acetate was then counted for radioactivity in a liquid-scintillation counter to detect the amount of liberated [^3H]acetate.

PAGE

Proteins of the various nuclear matrix preparations were electrophoretically resolved on 10 or 15% polyacrylamide/SDS gels. Two-dimensional gel electrophoresis was done as described previously [33]. The isoelectrofocusing gel (first dimension) contained 1.6% (w/v) pH 3–10 Ampholines (Fisher Biotech). Molecular masses and isoelectric points were determined using two-dimensional SDS/PAGE standards (Bio-Rad), carbamylated carbonic anhydrase (Pharmacia Biotech) and high-molecular-mass standard mixture (Sigma or Bio-Rad). The gels were stained with Coomassie Blue and sometimes further stained with silver [34].

Preparation and analysis of isolated nuclear matrices by electron microscopy

The pelleted matrices were resuspended in digestion buffer and glutaraldehyde (2.5% v/v) or glutaraldehyde and formaldehyde (2% of each) were added. The nuclear matrices were then fixed in this solution by incubation at 4°C for 30 min to 2 h. After a brief wash, the samples were post-fixed for 1 h in a 1% OsO_4 solution buffered with Millonig's phosphate buffer. The specimens were then washed in water, passed through a graded ethanol series, and embedded in Spurr's resin. After polymerization, sections in the silver-to-gold region (60–90 nm in thickness) were collected, stained with uranyl acetate and lead citrate and examined in a Zeiss EM-902 electron microscope at zero energy loss operated at 80 kV.

RESULTS

Structure of the adult chicken immature- and mature-erythrocyte nuclear matrices

Most circulating erythroid cells from anaemic adult chickens are transcriptionally active mid- and late-polychromatic erythrocytes (called 'immature erythrocytes') [29,35]. Nuclear matrices were isolated from immature erythrocytes by a fractionation protocol that is effective in preserving the morphology of residual internal nuclear structures [16,17]. Isolated nuclei were digested with DNase I and then extracted with 0.25 M ammonium sulphate, generating the nuclear matrix NM1-IF. The NM1-IF nuclear matrices were further extracted with 2 M NaCl, yielding NM2-IF nuclear matrices. The NM1-IF and NM2-IF nuclear matrices were revealed by thin-section electron microscopy. Figure 1 shows that NM1-IF and NM2-IF nuclear matrices had extensive internal fibrogranular structures. The appearance of the internal matrix structures was similar to those described for transcriptionally re-activated mature erythrocytes and 5-day embryonic primitive erythrocytes [19,20]. Approx. 50% of the nuclear matrices lacked internal structures, and some matrices had remnants of internal structures attached to the periphery (see Figure 1A).

In contrast with nuclear matrices from immature erythrocytes, most mature-erythrocyte NM1-IF and NM2-IF nuclear matrices were without internal structures (Figures 2A–2C), and many nuclear matrices had collapsed (results not shown). There were a few exceptions where mature-erythrocyte NM1-IF and NM2-IF nuclear matrices had internal structures (Figures 2D–2E). How-

Figure 1 Electron-microscopic revelation of nuclear matrices isolated from chicken immature-erythrocyte nuclei

The 0.25 M ammonium sulphate (NM1-IF; **A–C**) and 0.25 M ammonium sulphate/2 M NaCl (NM2-IF; **D** and **E**)-extracted nuclear matrices were prepared from immature erythrocyte nuclei as described in the Materials and methods section. Individual nuclear matrices containing a fibrogranular network are shown. The arrows point to the nuclear periphery. The arrowheads in (**A**) and (**B**) point to the filaments attached to the nuclear periphery. The bar represents 1 μm .

ever, the internal structures of these mature-erythrocyte nuclear matrices were less extensive than those observed in immature-erythrocyte nuclear matrices. Fibres and clumps of fibres were seen surrounding the outside of the mature- and immature-erythrocyte nuclear matrices (see arrowheads in Figures 1A and 2A). These results confirm previous studies showing that adult chicken mature-erythrocyte nuclear matrices lack internal matrix structures [19,20], but show that adult chicken immature-erythrocyte nuclear matrices do have internal structures.

Destabilization of the immature-erythrocyte internal matrix

Reducing agents have been found to destabilize the internal matrix [17,26,31,36]. To find out whether the internal matrix structures of erythrocyte nuclear matrix preparations could be disrupted with reducing agents, adult chicken immature-erythrocyte NM2-IF nuclear matrices were extracted with 2 M NaCl and 1% 2-mercaptoethanol, yielding NM3-IF. Figure 3 shows that most NM3-IF nuclear matrices lacked internal structures, and empty nuclear shells containing nuclear pore-lamina were readily detected. These observations show that reducing agents disrupt the internal matrix structures of immature-erythrocyte nuclear matrices.

Destabilization of erythrocyte internal matrices measured by histone deacetylase activity

We have observed that histone deacetylase resides in the internal matrix and, to a lesser extent, with the nuclear pore-lamina [10,11]. Thus the fate of internal matrix proteins following the treatment of nuclear matrices with agents that destabilize internal structures could be monitored by measuring histone deacetylase activity. Histone deacetylase specific activity in immature-erythrocyte NM2-IF nuclear matrices was approx. 11-fold greater than that of mature-erythrocyte NM2-IF nuclear matrices (Table 1). Previous studies have reported that histone deacetylase activity is lower in mature than in immature erythrocytes [37]. The mature- and immature-erythrocyte NM2-IF nuclear matrices were treated with 2-mercaptoethanol and 2 M NaCl. The specific activity of histone deacetylase in the nuclear-matrix extract (fraction S3) and residual insoluble nuclear pore-lamina (NM3-IF) was determined. Nuclear pore-lamina complexes (NPL), which lack internal structures, were also prepared by a method described by Kaufmann and colleagues [31]. The specific activity of histone deacetylase in fraction S3 from immature erythrocytes was 4–5-fold greater than that of fractions NM3-IF or NPL, providing biochemical evidence that internal matrix structures had been disrupted (Table 1). Similarly, the specific activity of

Figure 2 Electron-microscopic revelation of nuclear matrices isolated from chicken mature-erythrocyte nuclei

The 0.25 M ammonium sulphate (NM1-IF; **A**, **D** and **E**)- and 0.25 M ammonium sulphate/2 M NaCl (NM2-IF; **B**, **C** and **F**)-extracted nuclear matrices were isolated from mature-erythrocyte nuclei as described in the Materials and methods section. The arrows show the nuclear periphery. The arrowheads in (**A**), (**D**) and (**F**) point to the filaments attached to the nuclear periphery. The bar represents 1 μ m.

Figure 3 Electron-microscopic revelation of high-salt/2-mercaptoethanol-treated nuclear matrices

NM2-IF nuclear matrices of chicken immature-erythrocyte nuclei extracted with 2 M NaCl/1% (v/v) 2-mercaptoethanol to yield NM3-IF nuclear matrices are shown. The arrows show the nuclear periphery. The bar represents 1 μ m.

histone deacetylase in mature-erythrocyte S3 fraction was greater than that found in fractions NM3-IF or NPL (2–3-fold), but the level of increase was less than that observed for the S3 fraction

Table 1 Histone deacetylase activity in immature- and mature-erythrocyte nuclear matrix and nuclear pore-lamina

Nuclear-matrix fractions were assayed for histone deacetylase activity as described in the Materials and methods section. The fractions are 0.25 M ammonium sulphate/2 M NaCl-extracted nuclear matrices (NM2-IF), 2 M NaCl/1% (v/v) 2-mercaptoethanol extract of NM2-IF (S3), nuclear pore-lamina remaining after extraction of NM2-IF with high salt and reducing agent (NM3-IF) and nuclear pore-lamina (NPL) isolated by the protocol described by Kaufmann and colleagues [31]. Specific histone deacetylase activity (d.p.m./mg of protein/min) is shown. Each value represents the mean $\pm$ S.E.M. for three different assays.

Immature erythrocytes		Mature erythrocytes	
Nuclear-matrix fraction	Histone deacetylase (specific activity)	Nuclear-matrix fraction	Histone deacetylase (specific activity)
NM2-IF	744 $\pm$ 43	NM2-IF	62.2 $\pm$ 1.0
S3	1489 $\pm$ 75	S3	94.7 $\pm$ 8.0
NM3-IF	321 $\pm$ 21	NM3-IF	29.8 $\pm$ 1.2
NPL	353 $\pm$ 29	NPL	30.3 $\pm$ 0.6

of immature erythrocytes. However, fraction S3 of mature and immature cells had similar amounts (76 and 71 % respectively) of the histone deacetylase activity that was originally present in nuclear-matrix NM2-IF. The histone deacetylase specific activities of NM3-IF and NPL fractions from immature (or mature) erythrocytes were similar. These observations suggest that immature- and mature-erythrocyte nuclear matrices have internal structures.

Figure 4 Proteins of chicken immature- and mature-erythrocyte nuclear matrices

Nuclear proteins were isolated from nuclei (N), soluble (S) and insoluble nuclear matrix (NM) fractions obtained from the sequential extraction of DNase I-digested nuclei with 0.25 M ammonium sulphate (S1, NM1-IF), 2 M NaCl (S2, NM2-IF) and 2 M NaCl/2-mercaptoethanol (S3, NM3-IF). A 10 μ g portion of protein was electrophoretically resolved on a 15% polyacrylamide/SDS gel (A). For the 10% polyacrylamide/SDS gel (B), 10, 13 and 16 μ g of S2 protein, 10 μ g of S3 protein and 8, 10 and 13 μ g of NM3-IF protein were loaded on to the gel. 'CH' is the core histones. 'L' shows the position of lamins A, B and C. Lanes containing the molecular-mass markers (M; Bio-Rad high-molecular-mass gel standards) are shown. The gels were stained with Coomassie Blue.

Protein composition of the NM1-IF and NM2-IF nuclear matrices of adult chicken mature and immature erythrocytes

The percentage of nuclear protein in NM1-IF and NM2-IF nuclear matrices from immature erythrocytes was $24.5 \pm 2.1\%$ and $16.7 \pm 1.7\%$ ($n = 12$) respectively, while corresponding nuclear matrices from mature cells had $18.7 \pm 1.1\%$ and $11.0 \pm 0.9\%$ ($n = 6$) respectively of the nuclear protein. Extraction of the immature- or mature-erythrocyte NM2-IF nuclear matrices with 2 M NaCl and 1% 2-mercaptoethanol (nuclear-matrix extract) released $44.6 \pm 2.9\%$ (immature) or $53.6 \pm 3.4\%$ (mature) ($n = 8$) of the NM2-IF protein.

The proteins of the soluble and insoluble fractions produced by the sequential 0.25 M ammonium sulphate, 2 M NaCl and 2 M NaCl/2-mercaptoethanol extraction of DNase I-digested (23 °C) immature- and mature-erythrocyte nuclei were analysed by SDS/PAGE. Figure 4(A) shows the protein composition of the nuclear fractions resolved on a 15% polyacrylamide/SDS gel. The 0.25 M ammonium sulphate-soluble fraction (S1), which contained approx. 76% (immature erythrocytes) or 81% (mature erythrocytes) of the total nuclear protein, contained significant amounts of the core histones H2A, H2B, H3, and H4 and the linker histones H1 and H5. This is consistent with most of chromatin being localized in this fraction. Most NM1-IF nuclear-matrix proteins of immature and mature cells had molecular masses greater than 30 kDa; very little histone was present in these fractions. The histones remaining in fraction NM1-IF were extracted with 2.0 M NaCl (see fraction S2). Most of the proteins of the NM2-IF and 2 M NaCl/2-mercaptoethanol-soluble (S3) and -insoluble (NM3-IF; nuclear pore-lamina) nuclear fractions had molecular masses greater than 30000.

Figure 4(B) shows a 10% polyacrylamide/SDS gel of the nuclear fractions S2, S3 and NM3-IF. The spectrum of proteins in fractions S2 and S3 were similar, but different from those of the insoluble nuclear fractions NM1-IF, NM2-IF and NM3-IF (Figure 4). The 55, 58, 65, 67, 200 and 220 kDa proteins were prominent in the nuclear pore-lamina NM3-IF fraction, with the

65 and 67 kDa proteins probably being the lamins A, B and C [38]. Fractions S2 and S3 had proteins of 80, 99 and 105 kDa that were more prominent in these fractions than in fractions NM1-IF, NM2-IF and NM3-IF. Fraction S3 contained proteins of approx. 65, 156, 200 and 220 kDa that were at lower relative levels in fraction S2. The spectrum of proteins in the respective nuclear fractions from immature- and mature-erythrocyte nuclei was similar, but differences were noted. A 67 kDa protein of immature-erythrocyte fractions S2, S3 and NM3-IF was present at higher levels than the corresponding mature-erythrocyte nuclear fractions (see the arrowhead in Figure 4B).

Internal matrix structures in nuclear-matrix preparations from chicken erythroblasts isolated from the bone marrow of anaemic birds were stabilized when nuclei were incubated with Cu^{2+} [39]. Immature-erythrocyte nuclei were incubated in the presence of Cu^{2+} , and nuclear matrices isolated. The protein pattern of the NM2-IF proteins from Cu^{2+} -treated and non-treated nuclei were identical. Further, the percentage of nuclear protein and activity of histone deacetylase in nuclear-matrix preparations NM1-IF and NM2-IF isolated from Cu^{2+} -treated or non-treated nuclei were similar (results not shown). Similarly, Lafond et al. [19] found that incubation of mature-erythrocyte nuclei with sodium tetrathionate, an agent that stabilizes nuclear matrices [17], yielded nuclear-matrix preparations without internal matrix structures.

Two-dimensional electrophoretic separation of nuclear-matrix and intermediate-filament proteins

Intermediate filament proteins (IF) were reassembled and removed from the NM2-IF proteins (see the Materials and methods section). The nuclear-matrix proteins (NM fraction) contained $15.7 \pm 2.3\%$ and $20.7 \pm 4.7\%$ ($n = 3$) of the protein in the immature and mature NM2-IF fraction respectively. These results suggest that most proteins in the NM2-IF fraction were cytoskeletal proteins.

Figure 5 Two-dimensional electrophoretic gel patterns of nuclear matrix proteins from mature and immature chicken erythrocytes

NM2-IF nuclear matrix proteins (120 μ g) were electrophoretically resolved on two-dimensional gels as described in the Materials and methods section. The molecular masses of the marker proteins run in the same gel are shown in kDa. The carbamoylated carbonic anhydrase ('ca') shows the pI range from 6.6 to 4.5 (left to right). 'LA', 'LB' and 'LC' are lamins A, B and C respectively. 'V', 'S' and 'A' are vimentin, spectrin and actin respectively. The gels were stained with Coomassie Blue.

Table 2 Proteins in immature- and/or mature-erythrocyte nuclear-matrix fractions

Protein and group	Molecular mass (kDa)	pI
Intermediate filament (IF)		
Vimentin (V)	52	5.2
Spectrin (S)	285	5.2
Actin (A)	41	5.3
1IF	190	4.95
2IF	50	5.25
3IF	46.5	5.15
4IF*	55.5	5.05
5IF*	52.5	5.0
6IF*	20	5.3
Nuclear pore-lamina (NPL)		
NPL group	43–48	4.9–5.0
1NPL	180	5.35
2NPL*	67	5.48
Internal matrix (IM)		
1IM	32	4.6
2IM	35	4.55
3IM	38	5.05
4IM	38	4.95
5IM*	90	5.2
6IM*	70	5.0
7IM*	54	4.6
8IM*	56	5.75
9IM*	55	5.8
10IM†	37	4.55

*Protein is in greater amounts in immature-erythrocyte nuclear-matrix proteins.

†Protein is in greater amounts in mature-erythrocyte nuclear-matrix proteins.

proteins of the internal matrix and nuclear pore-lamina. Fraction S3 should be enriched in proteins of the internal matrix (for example histone deacetylase), while fraction NM3-IF has proteins of the cytoskeleton and nuclear pore-lamina. On the basis of the partitioning of a protein into the various fractions, a protein was assigned as a component of the IF, NPL or IM (Table 2). For example, a protein with a molecular mass of 180 kDa (pI 5.35) partitioned primarily to the NM fraction, suggesting that this protein was a component of the nuclear matrix (see the spot identified as '1NPL' in Figures 6E, 6F, 7A and 7B). The 180 kDa protein did not selectively partition into fraction S3, but was present in fraction NM3-IF (Figure 6). Thus this protein was assigned as a component of the nuclear pore-lamina. Among the IF protein was vimentin (marked as 'V' in Figures 5–7). In contrast with the nuclear pore-lamina proteins, vimentin was found in the IF and NM3-IF, but not in the NM and S3, fractions. Lamins A, B and C, which are components of the nuclear pore-lamina and internal matrix [40], were enriched in the NM fraction, depleted in the IF fraction, and present in both the S3 and NM3-IF fractions.

Comparison of the IF electrophoretic protein pattern (Figure 7) with that of NM2-IF (Figure 5) shows that the bulk of the abundant proteins in the NM2-IF fraction were intermediate-filament/cytoskeletal proteins. Besides vimentin, two proteins with molecular masses and pI values of spectrin ('S' in Figures 7E and 7F) and actin ('A' in Figures 7E and 7F) were abundant proteins in the IF fraction. Several common proteins were found in the IF fractions from mature and immature erythrocytes, but differences were noted (Table 2). Three of these proteins were assigned to the intermediate-filament/cytoskeletal group (4IF, 5IF and 6IF in Figures 6F and 7F), and one was assigned to the nuclear pore-lamina group (2NPL in Figures 6F and 7B). The protein 2NPL was prominent in the NM and NM3-IF fractions, but less so in the S3 fraction. Thus this protein was assigned to the NPL group.

The intermediate-filament/cytoskeletal proteins partitioned with the NM3-IF fraction and less so with fraction S3. Vimentin, for example, was at very low levels in fraction S3. However, spectrin, actin and proteins, thought to be components of the cytoskeleton and/or nuclear pore-lamina, were found in this

The proteins of fractions NM2-IF, S3, NM3-IF, NM, and IF isolated from immature and mature erythrocytes, were resolved by two-dimensional gel electrophoresis. The fractions S3, NM3-IF, NM and IF contained a subset of the proteins present in NM2-IF (Figure 5). Fraction IF has the cytoskeletal proteins, and fraction NM contains nuclear-matrix proteins, including

Figure 6 Comparative analysis of nuclear matrix proteins isolated from mature and immature erythrocytes

Proteins of fraction S3 (2 M NaCl/2-mercaptoethanol extract of NM2-IF; 77 μ g) and fraction NM3-IF (nuclear pore-lamina remaining after extraction of NM2-IF with high salt and reducing agent; 120 μ g) were electrophoretically resolved by two-dimensional gel electrophoresis as described in the Materials and methods section. 'LA', 'LB' and 'LC' are lamins A, B and C respectively. 'V', 'S' and 'A' are vimentin, spectrin and actin respectively. 'IM', 'NPL' and 'IF' are proteins assigned as components of the internal matrix, nuclear pore-lamina and intermediate filament/cytoskeleton respectively. For further details, see the legend to Figure 5. Gels shown in (A), (B), (E) and (F) were stained with Coomassie Blue and then with silver.

fraction. Besides these proteins, lamins and two internal matrix proteins (1IM and 2IM in Figures 6A and 6B) were prominent in proteins in the S3 fraction from mature and immature nuclear matrices (NM2-IF).

Several proteins were more abundant in immature-erythrocyte fractions S3 and NM than in the corresponding fractions from mature erythrocytes (Table 2). Protein 5IM and 6IM were among the more abundant internal matrix proteins that were at higher levels in immature-erythrocyte S3 and NM fractions (Figures 6B and 7B). Silver-stained two-dimensional gel patterns of S3 and NM proteins accentuated the differences between the immature- and mature-erythrocyte fractions. The abundance of several proteins with pI values from 5.5 to 7.0 and molecular masses from 40 to 180 kDa was greater in immature-erythrocyte fractions S3 and NM (e.g., see proteins 8IM and 9IM in Figure 6D, cf. Figure 6C; Figure 7D, cf. Figure 7C). There was one exception where the level of a protein (marked as '10IM' in Figures 6A, 6C and 7A) was higher in mature than in immature S3 and NM fractions (Table 2).

DISCUSSION

The most abundant proteins in the nuclear-matrix preparations (NM2-IF) from immature and mature erythrocytes are cytoskeletal proteins. The intermediate-filament protein vimentin was prominent among the nuclear-matrix proteins. Proteins with the molecular masses and pI values of actin and spectrin were also present. It has been shown previously that the vimentin

filaments are associated with the lamins and are associated with the nuclear pore-lamina of chicken erythrocytes [41,42]. In agreement with the reports of others, these results show that filaments surrounding the nuclei are intermediate filaments and cytoskeletal remnants [32,41,43].

Electron-microscopic analysis of nuclear-matrix preparations reveals that approximately half of the immature-erythrocyte, but only a few of the mature-erythrocyte, nuclear matrices have an internal matrix. Our results examining mature-erythrocyte nuclear matrices agree with those of Lafond and Woodcock [20]. These investigators did not observe internal structures in the nuclear matrices of definitive erythrocytes of 11–14-day-old chick embryos [20], cells that are transcriptionally active [22]. The expectation was that nuclear matrices of red blood cells (definitive erythroid cells) from anaemic adult birds would also lack an internal matrix. However, this is not what we observed. A possible explanation for the discrepancy in observations may be the temperature used to digest the nuclei. Lafond and Woodcock [20] digested the erythroid nuclei at 4 °C with DNase I, while we did our digestions at 23 °C. It has been shown that incubating nuclei at 37 °C before nuclease digestion stabilizes internal matrix structures [17]. We observed that immature-erythrocyte nuclear-matrix preparations from nuclei digested at 4 °C had very low levels of histone deacetylase activity, consistent with the idea that these nuclear-matrix preparations did not have internal matrix structures [11].

In contrast with the electron-micrographic analysis of mature-erythrocyte nuclear matrices, our results monitoring histone

Figure 7 Comparative analysis of nuclear-matrix and nuclear-attached cytoskeletal proteins isolated from mature and immature erythrocytes

Proteins isolated from fraction NM (nuclear matrix proteins; 96 μ g) and fraction IF (intermediate filament/cytoskeletal proteins; 120 μ g) were electrophoretically resolved by two-dimensional gel electrophoresis. For details, see the legends to Figures 5 and 6. Gels shown in panels (A), (B), (E) and (F) were stained with Coomassie Blue, and gels shown in panels (C) and (D) were stained with Coomassie Blue and then silver.

deacetylase activity in nuclear matrix fractions provide evidence that mature-erythrocytes nuclear matrices have internal matrix structures. Histone deacetylase activity is associated with the internal matrix and less so with the nuclear pore-lamina ([10,11]; the present study). Electron micrographs show that immature-erythrocyte nuclear matrices extracted with 2 M NaCl and 1 % 2-mercaptoethanol lack internal structures, suggesting that internal matrix proteins will be present in the extract (fraction S3). Accordingly, more than 70 % of the histone deacetylase activity present in the NM2-IF nuclear matrices of mature and immature erythrocytes was released into fraction S3. If mature-erythrocyte nuclear matrices lacked an internal matrix, then the histone deacetylase activity in the NM2-IF nuclear matrix and the nuclear pore-lamina preparations (NM3-IF or NPL) would be expected to be the same. This was not found. Instead the results argue that mature-erythrocyte nuclear matrices have internal matrix structures.

Disruption of internal matrices with reducing agents is used as a method to enrich for the components of the internal matrix [36,44,45]. A key feature of this procedure is that the extraction conditions are sufficiently gentle to preserve the biological activity of the constituents of this fraction. We have used this protocol to solubilize several components of the chicken erythrocyte internal matrix, including nuclear factor 1, a sequence-specific DNA binding transcription/replication factor, histone deacetylase, and histone acetyltransferase [45,46]. These proteins retained their DNA-binding or enzymic activities following extraction from the nuclear matrix. However, in the analysis of proteins in fraction S3, cytoskeletal proteins were found in the extract. Thus caution

should be exercised when using this procedure solely to identify proteins of the internal matrix.

The composition of the nuclear matrix distinguishes one cell type from another [32,47]. Accordingly several proteins designated as components of the internal matrix and nuclear pore-lamina are present in immature-, but not mature-, erythrocyte nuclear matrices. Several proteins more abundant in fraction S3 and NM from immature nuclear matrices with pI values similar to, but with molecular masses less than, those of lamins may be ribonucleoproteins [32].

There was a marked decline in histone deacetylase activity in mature-erythrocyte nuclear matrices. A comparative electrophoretic analysis of proteins in fraction S3 from immature and mature erythrocytes shows a general reduction in proteins of the internal matrix of mature cells. Correspondingly, the content of an internal matrix protein, ARBP, which binds to MARs, was lower in mature-erythrocyte nuclear matrices than in nuclear matrices isolated from chicken kidney or oviduct [26]. Thus the results with histone deacetylase and ARBP are consistent with the idea that mature cells have an internal matrix, albeit less stable or less developed than that present in nuclear matrices of other cell types.

The observed differences with immature- and mature-erythrocyte nuclear matrices may be a consequence of a reduction in transcriptional activity and decline in nuclear RNA in mature cells [20]. There is evidence that transcription is occurring in mature erythrocytes, but the levels of nuclear RNA are very low, suggesting most nuclear RNA synthesized is rapidly degraded [27–29]. A correlation exists between the presence of the fibro-

granular component of the internal matrix and transcription-related metabolism. This relationship is further supported by the observation of Lafond et al. [19] that re-activation of transcription in the mature-erythrocyte nucleus by cell fusion results in the reformation of a fibrogranular internal matrix. Many aspects of mRNA processing localize to the nuclear matrix [48]. Approx. 70 % of the total heterogeneous nuclear RNA is retained in nuclear matrix preparations [16]. Several studies have shown that removal of nuclear RNA leads to instability of the internal matrix [17,38,49]. It is likely that the machinery involved in processing the genetic information contributes to the morphology and stability of the internal matrix observed by electron microscopy.

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