NUCLEAR MATRIX GENERATION DURING REACTIVATION OF AVIAN ERYTHROCYTE NUCLEI: AN ANALYSIS OF THE PROTEIN TRAFFIC IN CYBRIDS

CHRISTOPHER L. F. WOODCOCK AND HILARY WOODCOCK

Department of Zoology, University of Massachusetts, Amherst, MA 01003, USA

SUMMARY

It has previously been shown that an internal nuclear matrix is generated during the reactivation of the chick erythrocyte nucleus in mouse L-cell cytoplasts. This experimental system has now been used to identify the major polypeptides that migrate into the nucleus during the reactivation process. Mouse L-cells were prelabelled with $^{35}$S methionine, enucleated using cytochalasin B, and fused with 14- to 17-day embryonic chick erythrocytes. Sixteen hours post-fusion, the redistribution of the labelled proteins was examined by electron microscopic autoradiography, and two-dimensional polyacrylamide gel fluorography of the isolated nuclei was used to identify the major imported species. After allowing for cytoplasmic contamination, 15 nucleus-associated polypeptides were identified, two of which also matched with counterparts in the L-cell nuclear preparation. Five of the nucleus-associated polypeptides were tentatively identified (on the basis of one-dimensional gel matches) as nuclear matrix proteins; these five included the two that had counterparts in L-cell nuclei. The autoradiographic results showed that 16 h post-fusion, the specific activity (silver grains/unit area) of the reactivated nucleus was about half that of the cytoplasm, with no evidence for an accumulation of labelled protein at the nuclear periphery. When well-reactivated nuclei were distinguished from poorly reactivated nuclei on the basis of the extent of chromatin decondensation, it was found that their specific activities were quite similar, but because of the difference in size, the well-reactivated nuclei contained about twice as much labelled protein. Estimates of the protein traffic based upon the autoradiographic data indicated that the nuclei had increased in mass by 10–20% during the 16 h reactivation period.

INTRODUCTION

The concept that the structure and function of the eukaryotic nucleus are centred upon a karyoskeleton, nuclear scaffold or nuclear matrix is an attractive one that has received considerable experimental support (reviewed by Shaper et al. 1980; Berezney, 1984). However, a major difficulty in studying the karyoskeleton is that it is typically revealed only after removal of the bulk of DNA, histones and other soluble nuclear components, which could promote the accretion of material that was not part of an in vivo structure. Most karyoskeletal preparations contain numerous polypeptides and, aside from the well-characterized components of the nuclear lamina, it is not clear which, if any, of these were part of an original intranuclear framework (for discussion, see Benavente et al. 1984). For example, ribonuclear

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proteins that are commonly found in the nuclear matrix (reviewed by Bouteille et al. 1983) might have a structural role, or it could be that in vivo they were simply in close proximity to a karyoskeleton. There is also the possibility that the karyoskeletal components were originally located in a protein-rich domain in the nucleus, but one that lacked the structural integrity exhibited by other cellular elements such as the cytoskeleton. This type of loose arrangement might well show the sensitivity to relatively minor changes in preparation conditions that is characteristic of karyoskeletal structures (e.g. see Kaufmann et al. 1981; Lewis et al. 1984). Experimentally, it is extremely difficult to distinguish between the many possibilities, but a system in which a karyoskeleton were generated de novo might enable the key structural components to be identified, isolated and subjected to further study.

One such system is the 'reactivation' of the chick erythrocyte nucleus in a host cytoplasm, a process that involves a decondensation of chromatin, uptake of proteins from the host, and the resumption of nucleic acid synthesis (Harris, 1970; Appels & Ringertz, 1975; Ege et al. 1975; Lipsich et al. 1978). Recently, we reported that the mature erythrocyte nucleus has no detectable internal nuclear matrix (LaFond & Woodcock, 1983), but that during reactivation in mouse L-cell cytoplasts a typical nuclear matrix is generated (LaFond et al. 1983). We have also shown that nuclear matrix formation, like many of the other changes that accompany reactivation (Ege et al. 1975; Lipsich et al. 1978), occurs in the absence of protein synthesis (Woodcock & Baldwin, unpublished data). So, the materials needed for matrix formation are apparently not synthesized in response to the fusion event. When these studies were begun, two further lines of evidence suggested that it is the influx of new protein into the nucleus, rather than the modification of existing nuclear protein, that gives rise to the matrix. Firstly, the erythrocyte nucleus has very little non-histone protein; and secondly, the uptake of protein was reported to be quite substantial, resulting in a threefold increase in nuclear mass (Ege et al. 1975). However, as will be discussed below, these arguments are no longer so persuasive.

In the present study, we have monitored the protein traffic from the host cytoplasm into the reactivating chick nuclei in 'cybrids' formed by fusing erythrocytes with enucleated L-929 mouse fibroblasts that had been prelabelled with $[^{35}S]$methionine. The redistribution of labelled protein was followed using autoradiography, which also provided an estimate of the amount of protein taken up during reactivation. In a second series of experiments, the reactivated nuclei were isolated, and the major imported proteins located on two-dimensional polyacrylamide gels. The general conclusion is that the major imported species are nucleus-specific in the sense that they represent a particular subset of cytoplasmic proteins that are concentrated to differing degrees in the nucleus. Some of these proteins appear to match counterparts in the host cell nucleus, and some are resistant to extraction with DNase I and high-salt solutions. These latter should be useful in further studies into the nature of karyoskeleton. Contrary to earlier reports, we find that the extent of the protein influx is quite small, resulting in a 10–20% increase in mass of the erythrocyte nucleus.
**MATERIALS AND METHODS**

**Cytoplast preparation**

Mouse L-929 fibroblasts were cultured as previously described (LaFond et al. 1983). For $^{35}$S-labelling, they were grown for 24 h in medium containing 10% of the normal amount of methionine and 0.5 $\mu$Ci ml$^{-1}$ of $[^{35}S]$methionine (1000 Ci mmol$^{-1}$, New England Nuclear). Enucleation was carried out using Ficoll gradients containing 10 $\mu$g ml$^{-1}$ cytochalasin B (Aldrich Chemical Co.) as described by Wigler & Weinstein (1975), after which the cytoplasts were washed in fresh medium, then resuspended in Earle’s balanced salt solution (EBSS). Erythrocytes from 14- to 17-day chick embryos were collected into and washed in EBSS, after which they were mixed with cytoplasts at a ratio of 20 to 50 erythrocytes per cytoplast. The mixture was then pelleted, and resuspended in ultraviolet light-inactivated Sendai virus at a concentration of 200 haemagglutinin units ml$^{-1}$ in EBSS at 4°C. The final concentration of cytoplasts in the fusion mixture was approximately 10$^7$ ml$^{-1}$. After 20-min incubation on ice, followed by 45 min at 37°C, the suspension was centrifuged, washed three times with complete medium, and placed in suspension culture in spinner flasks for 16 h. The atmosphere was maintained at 5% CO$_2$ during the reactivation period.

**Enrichment of cybrids containing reactivated nuclei**

A population of cytoplasts 60–80% of which contained reactivated nuclei was obtained by fractionation of the spinner culture mixture on discontinuous Percoll gradients. A 20–7% suspension of the Percoll stock (Pharmacia, 23% in water) was prepared using minimal essential medium (MEM) at 10 times its normal strength (M.A. Bioproducts), and subsequently diluted with normal MEM to give Percoll concentrations of 14, 9.75, 9.0 and 7%; 3 ml of each of the three solutions were layered in a 15 ml Corex centrifuge tube, the cybrid/erythrocyte mixture was resuspended in the 7% Percoll solution, and 3 ml was placed on top of the gradients, which were overlaid with MEM and centrifuged at 500 g for 15 min. After this time, the free erythrocytes had pelleted, while the fused cybrids formed two pink-coloured bands in the lower portion of the gradient.

**Autoradiography**

Cybrids were harvested from the Percoll gradients, washed with MEM, and fixed overnight at 4°C in 2% glutaraldehyde, 2.5% sucrose, 50 mM-PIPES, pH 7.2. After washing in buffer plus sucrose, the cells were post-fixed for 1 h with 1% OsO$_4$ in PIPES, then washed three times with water, and dehydrated through an ethanol series. All steps up until 100% ethanol were carried out in the cold, and the washing solutions were retained for radioactivity measurements. Some cybrids were taken through the nuclear isolation procedure up to and including the addition of lysing medium (see below) before being fixed. After dehydration, the preparations were embedded in an Epon/Araldite mixture, and gold sections were cut and collected on collodion-coated nickel grids. The sections were stained with uranyl acetate and lead citrate, and coated with carbon. L4 emulsion (Ilford Ltd) was applied by the loop method (Williams, 1977a), and exposure was for 3 weeks before developing with D19 developer (Kodak Corp.), rinsing with 2% acetic acid, and fixing with 25% sodium thiosulphate for 3 min. Grids were examined in a Siemens 102 electron microscope, photographed at ×5000 magnification, and printed at a final magnification of ×17000 for scoring. During the sectioning, care was taken to remove thick sections at regular intervals to avoid the scoring of serial sections of the same population of cybrids.

**Isolation of nuclei**

Nuclei were prepared from mouse L-929 cells grown in spinner cultures, or after trypsin release from monolayers. Cells were washed twice with cold NPB medium (10 mM-KCl, 0.5 mM-MgCl$_2$, 0.005% spermine, 1.0 mM-phenylmethylsulphonyl fluoride (PMSF), 10 mM-Tris-HCl, pH 7.5) and resuspended in NPB to a concentration of about 2×10$^6$ cells ml$^{-1}$. After 1 h at 0°C, the cells were lysed by the slow addition of lysing medium (20 mM-KCl, 300 mM-NaCl, 0.5 mM-sucrose, 2 mM-dithiothreitol, 0.002% spermine, 1.0 mM-PMSF, 0.5% Triton X-100, 20 mM-Tris-HCl, pH 7.4) with vortexing. After each addition and vortexing, the suspension was monitored by phase-contrast
microscopy for the release of nuclei. For complete nuclear release, it was usually necessary to add approximately 0.75 vol. of lysing medium to the cells. When nuclear release was judged complete, the suspension was passed through a 22-gauge needle, mixed with 2 vol. of 2-3 M-sucrose in 10 mM-KCl, 0.5 mM-MgCl₂, 1 mM-PMSE, 10 mM-Tris-HCl, pH 7.8 (TKLM), layered over 5 ml of the same sucrose solution, and centrifuged at 70,000 g in an SW41 rotor (Beckman Instruments) for 1 h. The pellet contained clean intact nuclei, as shown by phase-contrast microscopy.

Reactivated nuclei were prepared from cytoplasts in a similar way to that described for L-cell nuclei except that the lysed suspension was mixed with 8 vol. of sucrose solution, and it was found necessary to extend the centrifugation time to 3 h to pellet these nuclei through the sucrose cushion.

Nuclei were prepared from embryonic erythrocytes using a modification of the method of Zentgraf & Franke (1984). Blood was collected from 14- to 17-day embryos, washed in SSC solution (140 mM-NaCl, 15 mM-sodium citrate, 10 mM-Tris-HCl, pH 7.8), and resuspended in SSC to a final concentration of about 5 × 10⁷ erythrocytes per ml. After centrifugation at 250 g for 10 min, the pellet of erythrocytes was taken up in 100 mM-NaCl, 0.5 mM-MgCl₂, 10 mM-Tris-HCl, pH 7.4, and an equal volume of lysing medium was added slowly with vortexing between additions. The suspension was kept on ice for 5 min, with vortexing each minute. To remove the last traces of plasma membrane, NP-40 detergent was added to 0.2% with vortexing, and the nuclei kept on ice for a further 10 min before pelleting through 2.3 M-sucrose as described above.

Nuclear matrix preparation (Shaper et al. 1980)

After harvesting from the sucrose centrifugation step, nuclei were taken up in 5 mM-MgCl₂, 1 mM-CaCl₂, 10 mM-Tris·HCl, pH 7.4, containing 1 mg ml⁻¹ DNase I (Worthington Corp., Freehold, NJ), and stirred gently for 30 min at 20°C in a siliconized tube. Nine volumes of high-salt buffer consisting of 2.2 M-NaCl, 0.2 mM-MgCl₂, 1 mM-PMSE, 10 mM-Tris·HCl, pH 7.4, were then added, and the nuclei were extracted with stirring for 1 h at 4°C. After this time, 0.5 ml of TKLM buffer containing 2.3 M-sucrose was laid under the suspension, and the extracted nuclei were centrifuged onto the sucrose layer (500 g, 10 min). The supernatant above the sucrose was withdrawn, and the nuclei and sucrose pad were resuspended in high-salt solution and extracted as before. Finally, the residual nuclei were given two low-salt washes in 0.2 mM-MgCl₂, 1 mM-PMSE, 10 mM-Tris·HCl, pH 7.4.

Electrophoresis

One-dimensional polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) was carried out in 18% gels as described (LaFond & Woodcock, 1983), except that the mercaptoethanol concentration in the freshly made sample buffer was raised to 5%, and the samples were sonicated in this buffer, heated to 95°C for 5 min, and clarified in an Eppendorf centrifuge (5 min) before applying to the gel. Two-dimensional non-equilibrium pH-gradient electrophoresis was performed using the method of O’Farrell et al. (1977), with the following modifications: the first-dimension gel contained pH 3–10 ampholytes (Pharmacia), and an additional 2.5 μg ml⁻¹ riboflavin, and polymerization occurred overnight in the presence of ultraviolet light. The proteins were solubilized as described by Peters & Comings (1980), and were focused using 500 V for 1880 volt-hours. For the second dimension, the same 18% polyacrylamide system was used as for the one-dimensional gels.

Gels were stained with Coomassie Brilliant Blue, and photographed before treating with FluoroHance (Research Products International, Mount Prospect, IL) according to the manufacturer’s directions. The dried gels were exposed to Kodak X-Omat X-ray film at −80°C, and the fluorograms developed in D19 developer (Kodak).

Protein content of cytoplasts

Mouse L-cell cytoplasts were prepared as described, washed in EBSS, and a suspension was counted using a haemocytometer. A known volume was then pelleted, dissolved in 150 μg ml⁻¹ sodium deoxycholate solution, precipitated with 7% trichloroacetic acid (TCA), redissolved in Lowry reagent containing SDS (Peterson, 1977), and colour was developed with Folin and Ciocalteu’s phenol reagent. This protein assay was obtained as a kit (P5656) from Sigma Chemical Co., St Louis, MO. Standard curves were obtained with bovine serum albumin and a mixture of
Nuclear matrix proteins

high molecular weight proteins (SDS-6H, Sigma), both of which gave similar results. To estimate
the dry mass of L-cells, a known number of cells was washed with EBSS, fixed with 4%
glutaraldehyde in EBSS for 2h, washed extensively with water, and then dried in vacuo over
CaCl₂.

RESULTS

Experimental system

In order to study the migration of protein from the mouse L-cell cytoplast into the
chick erythrocyte nucleus during the reactivation process, it was necessary to use a
large-scale experimental system for enucleation and fusion, and a method of isolating
reactivated nuclei from the 'cybrids'. L-cell enucleation was carried out in Ficoll
gradients containing cytochalasin B, as described by Wigler & Weinstein (1975), and
after fusion with 17-day chick erythrocytes the products were maintained in sus-
pension culture for 16h. To monitor the efficiency of enucleation, fusion and
reactivation, samples were removed and allowed to settle on Petri dishes after which
they were stained with Giemsa's stain. In a typical experiment, 10⁸ cytoplasts
(>99% enucleated) yielded 20–40% cybrids containing 4×10⁷ to 8×10⁷ erythrocyte
nuclei. The cybrids used for nuclear isolation were separated from unfused eryth-
rocytes and cytoplasts on a Percoll gradient, and after homogenization in the
presence of Triton X-100, the reactivated nuclei were purified by sedimentation
through 2–3 M-sucrose. Light-microscopic analysis indicated that the isolated nuclei
were mostly clean and free of whole cells (Fig. 1). Occasional nuclei had what
appeared to be attached cytoplasmic tags, and some small particulate matter was also
present (arrowheads, Fig. 1).

Fig. 1. Phase-contrast image of isolated reactivated nuclei after sedimentation through
2–3 M-sucrose. Some adhering material and small particles are seen (arrowheads). Bar,
10 μm.
In order to study the transfer of proteins from cytoplasm to nucleus, L-cells were prelabelled with $[^{35}S]$methionine for approximately one cell cycle prior to enucleation, fused with chick erythrocytes and reactivation was allowed to proceed for 16 h. After this time, the cybrids were fixed and prepared for autoradiography. The results enabled the transfer of protein to be quantified and also gave a low-resolution indication of its distribution. Autoradiography was also used to determine the extent to which the low-salt and detergent treatments used in the isolation of the nuclei were extracting nuclear proteins.

As seen in Fig. 2, both the cytoplasm and nuclei of the cybrids had silver grains over them, confirming the mass movement of protein from cytoplasts to reactivating nuclei (Appels & Ringertz, 1975; Ege et al. 1975). For quantification, silver grains were scored as cytoplasmic, nuclear interior or nuclear periphery. Grains within 400 nm (Williams, 1977b) of the nuclear envelope were considered separately, since they could have arisen from cytoplasmic proteins, intranuclear proteins, or from the nuclear envelope and its associated structures. It was also possible that an accumulation of labelled protein occurred at the nuclear periphery. The results,

Fig. 2. Autoradiograph of 16 h cybrid in which the L-cell cytoplasm was prelabelled with $[^{35}S]$methionine. Portions of two erythrocyte nuclei are shown, both of which have the dispersed chromatin characteristic of well-reactivated nuclei. Silver grains are present over cytoplasm, nuclear interior and nuclear periphery. The circle over one peripheral grain illustrates the 400 nm half-distance used to identify the envelope-associated label. Bar, 1 \( \mu \text{m} \).
Nuclear matrix proteins

### Table 1. Silver grain counts from autoradiographs of 16 h cybrids prepared from mouse L-cells prelabelled with $^{35}$S]methionine

<table>
<thead>
<tr>
<th>Sample area</th>
<th>Number of silver grains</th>
<th>Total area ($\mu$m$^2$)</th>
<th>Grains $\mu$m$^{-2}$ (−background)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>343</td>
<td>10,044</td>
<td>(0.034)</td>
</tr>
<tr>
<td>Cybrid cytoplasm</td>
<td>5878</td>
<td>7,540</td>
<td>0.746</td>
</tr>
<tr>
<td>L-cell nuclei ($n = 20$)</td>
<td>489</td>
<td>642</td>
<td>0.728</td>
</tr>
<tr>
<td>All reactivated nuclei, excluding periphery ($n = 115$)</td>
<td>87</td>
<td>154</td>
<td>0.531</td>
</tr>
<tr>
<td>Periphery of reactivated nuclei ($n = 115$)*</td>
<td>362</td>
<td>626</td>
<td>0.544</td>
</tr>
<tr>
<td>Poorly reactivated nuclei, internal + peripheral ($n = 41$)$†$</td>
<td>80</td>
<td>139</td>
<td>0.542</td>
</tr>
<tr>
<td>Well-reactivated nuclei, internal + peripheral ($n = 74$)$†$</td>
<td>152</td>
<td>282</td>
<td>0.505</td>
</tr>
<tr>
<td>Reactivated nuclei treated with nuclear isolation solutions ($n = 42$)</td>
<td>47</td>
<td>147</td>
<td>0.320</td>
</tr>
</tbody>
</table>

* Grains within 400 nm of the nuclear envelope.
† Peripheral grains internal to the nuclear envelope, estimated as described in the text.

together with those from L-cell nuclei (in these experiments, enucleation efficiency was deliberately lowered in order to provide a few L-cell nuclei as internal controls), are shown in Table 1. The number of silver grains per unit area was very similar in the L-cell nuclei and cytoplasts, and about 40% lower in both the interior and periphery of the reactivated nuclei. Thus, there was no evidence for an accumulation of labelled protein at the nuclear envelope: on the contrary, in terms of the specific activity, the periphery closely matched the nuclear interior. Therefore, in most of the subsequent analyses, that proportion of the peripheral grains corresponding (on the basis of area) to the nuclear interior was included in the data for the reactivated nuclei. In all populations of reactivated nuclei there is a considerable range of size from those that have hardly increased in size and contain mostly compact chromatin to those showing a several-fold increase in volume and a marked decondensation of the chromatin. Using the state of the chromatin as the criterion, the reactivated nuclei were divided into two groups, those showing little evidence of reactivation and those showing substantial changes. When the autoradiographic results are analysed in this way (Table 1), it is seen that the well-reactivated nuclei have a slightly lower specific activity than the poorly reactivated ones. However, as discussed below, when the increase in volume of the well-reactivated nuclei is taken into account, it is seen that they have imported about twice as much protein as the poorly reactivated ones.

We have used these autoradiographic data to estimate the amount of protein involved in the transfer. Since both cytoplasts and nuclei are approximately spherical in suspension culture, the mean diameter of each component can be estimated from their diameters in random sections using the relationship:

$$D = \frac{\pi N}{2(1/d_1 + 1/d_2 \ldots + 1/d_N)}$$
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where $D$ is the mean diameter, $N$ the number of measurements, and $d$ the observed diameter in the section (Williams, 1977c). In cases where the profiles of cytoplasm or nucleus were not completely circular, $d$ was taken as the mean of the major and minor diameters. The total radioactivity associated with each sphere is then directly proportional to $GV$, where $V$ is the volume, and $G$ the number of silver grains per unit area (Table 2). Thus, the ratio of total radioactive protein in the two-cell compartments is given by $GV(\text{cytoplasm})/GV(\text{nucleus})$. This ratio has been calculated for the poorly and fully reactivated subpopulations of erythrocyte nuclei as defined above (Table 2). From these values, we estimate that the cytoplasm donates from 0.60–1.2% of its protein to the alien nucleus.

If the total protein content of a cytoplasm is known, the protein traffic can be quantified (assuming that the cytoplasm proteins are uniformly labelled after one cell cycle in $[35S]$methionine), and used to determine the % gain by the erythrocyte nucleus. Cytoplasm protein was determined by a modified Lowry method (see Materials and Methods) using a mixture of high $M_r$ standards as reference. The result, $8.75 \times 10^{-8}$ mg per cytoplasm, was consistent with the experimentally determined value of $1.5 \times 10^{-7}$ mg dry weight of whole L-cells. Thus, the amount of protein donated to the erythrocyte nuclei ranged from $5.2 \times 10^{-10}$ mg per nucleus to $10^{-9}$ mg per nucleus. For conversion to % gain by the erythrocyte, the values of $3.0 \times 10^{-9}$ mg for the protein content and $4.8 \times 10^{-9}$ mg for the dry weight of the erythrocyte nucleus reported by Zentgraf et al. (1971) were used (Table 2). These calculations indicate that under our conditions, the maximal gain in dry weight due to protein uptake is about 30%. It should be noted that the values for erythrocyte DNA content determined by Zentgraf et al. (1971) are considerably lower than other values in the literature (e.g. see Sober, 1970), so these calculations of % gain by the erythrocyte nucleus are likely to be high rather than low estimates.

Even taking into account their limited accuracy, these values are significantly different from the 327% gain in mass reported by Ege et al. (1975) for L-cell/erythrocyte cybrids at 15 h post-fusion. However, other evidence also leads to the conclusion that our low values are not seriously in error. If, as discussed in more detail below, the reactivated erythrocytes are isolated, the proteins solubilized, separated on SDS/polyacrylamide gels, and stained with Coomassie Blue, we find that the protein pattern is indistinguishable from that of unreactivated nuclei. A threefold increase in mass due to protein influx would be clearly evident as a marked increase in staining in the non-histone proteins. A major difference between the autoradiographic results and the gel pattern could be caused by the loss of imported protein during the nuclear isolation procedure. Substantial losses of soluble protein from isolated nuclei have been reported (e.g. see Peters & Comings, 1980; Kellermeyer, 1981). To examine this question, autoradiographic studies were carried out on prelabelled cybrids that had been exposed to the nuclear isolation conditions up to and including the addition of detergent (see Materials and Methods). After this treatment, the radioactivity in the reactivated nuclei was reduced by about 60% (Tables 1, 2), indicating that there was indeed a substantial
### Table 2. Analysis of silver grains from Fig. 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean diameter (μm)</th>
<th>Silver grains μm²/μm³ (G)</th>
<th>CV(Cytoplasma)</th>
<th>Protein loss (%)</th>
<th>Protein equivalent of label (mg/unit)</th>
<th>Mass Protein erythrocyte nucleus (mg/unit)</th>
<th>Gain by protein equivalent (mg/unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasma</td>
<td>13.5</td>
<td>1270</td>
<td>7.46</td>
<td>950</td>
<td>8.7×10⁻⁵</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>Poorly resorbed W.: activated</td>
<td>2.70</td>
<td>10.5</td>
<td>5.42</td>
<td>60</td>
<td>0.002×10⁻⁸</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>Nuclei</td>
<td>3.48</td>
<td>2.2</td>
<td>0.505</td>
<td>11</td>
<td>0×10⁻⁸</td>
<td>0×10⁻⁸</td>
<td>21</td>
</tr>
<tr>
<td>Nuclei after treatment with isolation solutions</td>
<td>2.62</td>
<td>9.2</td>
<td>0.320</td>
<td>2.9</td>
<td>Using the values of 0.30×10⁻⁸ mg and 62%, for the protein content and % of protein of the erythrocyte nucleus (Zentgraf et al. 1971).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
leaching of labelled protein. Direct scintillation counting of the supernatant fractions obtained during the isolation and fixation processes indicated that the whole cybrids lost approximately 20% of their TCA-precipitable radioactivity during these procedures. Thus, the reactivated nuclei lost proportionately more protein than the cytoplasm. Even allowing for a loss of this magnitude, however, a threefold increase in protein content would have been clearly evident from the protein gels.

Identification of imported proteins

Since $^{35}$S-methionine was to be used in these experiments, preliminary tests were made to establish the protein patterns of labelled and unlabelled L-cell cytoplasts and L-cell nuclei. Fig. 3 shows an SDS/polyacrylamide gel, which was first stained with Coomassie Blue, and then prepared for fluorography. For ease of comparison, the stain pattern and fluorograms are presented side-by-side for total cytoplasm proteins (lanes b,c), and L-cell nuclear proteins (lanes d,e). Most of the stained polypeptides have a counterpart in the fluorogram, although there are changes in the proportions of some bands. For example, the strongly staining cytoplasm material at about
55 000M_r is under-represented in the fluorogram, and the reverse is observed at about 43 000 M_r, while for L-cell nuclei the stained lane (Fig. 3, lane d) is dominated by the core histones, but in the fluorogram (lane e) histones show comparatively weak labelling compared with the non-histone proteins.

For the identification of the proteins imported from the L-cell cytoplast into the reactivating chick nucleus, prelabelled L-cells were fused with chick erythrocytes, reactivation was allowed to proceed for 16 h, and the nuclei were isolated and purified as described (Fig. 1). When these nuclei were solubilized and the polypeptides separated on SDS/polyacrylamide gels, the bulk protein pattern revealed by Coomassie Blue staining was very similar to that of unreactivated erythrocyte nuclei (Fig. 3, lane f). Histones dominated, and compared with L-cell nuclei there was very little non-histone protein. The fluorogram prepared from the same gel (lane g) shows that the labelled proteins were quite different from the bulk proteins: labelled core histones were absent (confirming that there were no contaminating L-cell nuclei), but there were major labelled bands in the 40 000–70 000M_r region of the gel. The fluorogram prepared from 16 h reactivated nuclei in another experiment is presented in lane i. The main difference between the preparations of lanes g and i is that unlabelled carrier protein was included in i, and the sample was precipitated with TCA and resolubilized prior to electrophoresis. This produces a much cleaner separation of the higher M_r species, but at the expense of the smaller polypeptides.

Subsequent work showed that most of the major labelled bands present in lanes g and i were largely attributable to cytoplasmic contaminants that copurified with the reactivated nuclei. The clearest indication of this came from experiments in which labelled cytoplasts were prepared and subjected to a mock fusion in which the erythrocytes were omitted. The nuclear isolation procedure was then followed, and the material that pelleted through 2.3 M-sucrose was separated on SDS/polyacrylamide gels as before. Most of the major labelled polypeptides found in the reactivated nuclei were also present in these 'no nuclei' preparations (not shown). We can therefore conclude that the major form of contamination of the isolated nuclei is not adhering cytoplasmic material but a particulate fraction that resists solubilization in detergent and sediments through 2.3 M-sucrose. Examination of this fraction by electron microscopy failed to reveal any regular structures such as cytoskeletal elements. It should be noted that these cytoplasmic contaminants are seen only after much longer exposure times (2–3 months) than those needed for 35S-labelled L-cell nuclei (≈7 days). Thus, although similar cytoplasmic contaminants are probably present in the L-cell nuclear preparations, in this case they are masked by the more highly labelled nuclear proteins.

In order to identify labelled polypeptides specific to the reactivated nuclei, the increased resolution of two-dimensional polyacrylamide gel electrophoresis was necessary. Since many nuclear proteins cannot be brought to focus in an electrofocusing gel, a non-equilibrium system was used (O’Farrell et al. 1977; Peters & Comings, 1980), and trial experiments were conducted with [35S]methionine-labelled L-cell nuclei to select conditions for the maximal retention of nuclear proteins. From these results (not shown), the relatively short electrofocusing time of
1880 volt-hours was chosen, even though spot sharpness was sacrificed to some extent.

Using these conditions, two-dimensional fluorograms were prepared from 16 h reactivated nuclei (Fig. 4) and from the 2.3 M-sucrose pellet prepared from 'no nuclei' cytoplasts (Fig. 5). A total of 24 species was identified on the fluorogram of reactivated nuclei (Fig. 4), of which nine (marked with rectangles) were also present in the preparation made in the absence of nuclei (Fig. 5). These nine cytoplasmic contaminants accounted for more than 80% of the radioactivity in the 16 h reactivated nuclei. To facilitate comparison, two-dimensional gels of total cytoplast proteins, total L-cell nuclear proteins, and total erythrocyte nuclear (unreactivated)

![Two-dimensional NEPHGE fluorogram of proteins associated with 16 h reactivated erythrocyte nuclei.](image)

Fig. 4. Two-dimensional NEPHGE fluorogram of proteins associated with 16 h reactivated erythrocyte nuclei. Mr standards were the same as in Fig. 3 with the addition of β-galactosidase (116 000). Rectangles enclose species that also appeared on the 'no nuclei' controls (Fig. 5), while circles denote the remaining labelled spots, which were checked for matches with other cellular compartments. Asterisks indicate species found in L-cell nuclei. Only the portion of the gel containing label is shown.
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Fig. 5. Two-dimensional fluorogram prepared from mock fusion experiments in which erythrocyte nuclei were omitted. Most of the major labelled polypeptides that are present in Fig. 4 are present.

proteins were prepared (Figs 6, 7). On the basis of their similar mobilities in both dimensions, a number of polypeptides were found to be common to two or more cell compartments. These homologies are described in detail in Table 3 and can be summarized as follows:

1. Fifteen polypeptides in the 16 h reactivated nuclei (encircled on Fig. 4) were not part of the gross cytoplasmic contamination identified by the 'no nuclei' controls. It is likely, however, that some of these proteins are also contaminants that adhere to the outside of the nuclei during the preparation.

2. Six of the spots in Fig. 4 (numbered 5, 7, 12, 13, 15, 17) had counterparts in the gel of total L-cell nuclear protein (Fig. 6), but four of these (marked with rectangles on the figures) were also present in the 'no nuclei' control (Fig. 5). It is probable that these latter are cytoplasmic contaminants common to both nuclear preparations: contamination of nuclear preparations by cytoskeletal material is
frequently observed with cultured cells (Staufenbiel & Deppert, 1982). The other two, of $M_r, 48 000$ (no. 7) and 57 000 (no. 15) are probably bona fide nuclear proteins. Their absence from the gel of total cytoplast proteins (Fig. 7) indicates that they must be present in relatively minor amounts in the cytoplasts, and thus selectively concentrated in the reactivating nuclei.

(3) Of the remaining 16 h polypeptides that were not in the 'no nuclei' control, three (nos 10, 14, 17) had counterparts in the total cytoplast gel (Fig. 7). This does not necessarily mean that they are cytoplasmic contaminants, since all the labelled species in Fig. 4 must have originated in the cytoplasm. Further work will be required to determine the intracellular site(s) of these and the other 16 h labelled proteins (nos 1, 2, 3, 8, 9, 11, 20, 21–24) that did not match with polypeptides in any of the cell compartments examined.

Fig. 6. Stained two-dimensional gel of total cytoplast protein. The spot marking system is the same as in Fig. 4.
(4) There were no clear matches between total erythrocyte nuclear proteins and any of the labelled 16 h polypeptides (not shown).

(5) As expected, all the major cytoplasmic contaminants (nos 5, 6, 12, 13, 16) identified in Fig. 5 had counterparts in the gel of total cytoplast proteins. The two most abundant cytoplasmic polypeptides (nos 5, 13) of $M_r$ 43 000 and 55 000, respectively, are probably actin and vimentin. Both of these cytoskeletal proteins tend to copurify with nuclei, and often persist even in nuclear matrix preparations (Staufenbiel & Deppart, 1982, 1983). In an attempt to verify that no. 5 was actin, Western blots of one-dimensional gels were prepared and reacted with an antibody to chicken gizzard actin (Miles-Yeda Corp.). Only a small portion of the 43 000$M_r$ band showed antibody binding activity (data not shown). Positive identification of both
Table 3. List of proteins associated with the 16 h reactivated nuclei, and matches with other cellular compartments

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* For numbering system see Fig. 4.
† Since these are non-equilibrium gels, this is not necessarily the isoelectric point.
‡ Matched with one-dimensional gels only.
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proteins will require immunological evidence obtained in conjunction with two-dimensional separations.

Relationship to the nuclear matrix

Since reactivation results in the generation of an internal nuclear matrix (LaFond et al. 1983), it was of interest to determine which of the newly imported proteins contributed to the matrix structure. We first made a nuclear preparation from isolated unlabelled 16 h reactivated nuclei, and fixed and embedded the residual structures for electron microscopy. Unlike the unreactivated nuclei, which have no internal nuclear matrix, the 16 h nuclei had a typical interchromatinic matrix as previously reported (LaFond et al. 1983) for preparations made in situ (not shown). Thus, the nuclear isolation procedures were not affecting the nuclear matrix structure. Owing to the large amount of protein required for two-dimensional gel electrophoresis, the examination of matrix proteins was limited to one-dimensional gel analysis in the present study. Reactivated nuclei were isolated after 16 h fusion with prelabelled cytoplasts as in the previous experiments, mixed with unlabelled L-cell nuclei, and then treated with DNAase I followed by 2:0 M-NaCl, using standard nuclear matrix preparation methods (see Materials and Methods). The residual structures were solubilized, run on SDS/polyacrylamide gels for an extended time to provide better separation of the higher $M_r$ species, and prepared for fluorography (Fig. 8). For comparison, total L-cell nuclear proteins and L-cell nuclear matrix were run on the same gel.

After nuclear matrix preparation, the one-dimensional pattern of labelled protein from the reactivated erythrocyte nuclei was very similar to the starting material (compare Fig. 8, lane b, with Fig. 3, lane i), indicating that most of the labelled material associated with isolated nuclei was resistant to salt extraction. Once again, the major cytoplasmic contaminants dominated the gel. As seen from Table 3, many of the 16 h proteins that were not positively identified as cytoplasmic contaminants have $M_r$ values similar to contaminating material so they would be obscured in any one-dimensional separation. However, on the basis of two-dimensional analysis (Table 3) a number of the 16 h labelled proteins had $M_r$ values sufficiently different from the contaminants that they could be distinguished on one-dimensional gels. Five bands on the 16 h nuclear matrix gel appeared to fall into this category, having apparent $M_r$ values of 28 000 (1), 48 000 (7), 52 000 (8–10), 59 000 (15) and 65 000 (17). Most of these bands had counterparts in the L-cell nuclear matrix (lane d, denoted by horizontal marks). These results suggest that much of the protein that becomes associated with the reactivating nucleus consists of ‘insoluble’ salt-resistant species, perhaps homologous to those found in the host nucleus.

DISCUSSION

The reactivation of erythrocyte nuclei is a complex process involving the morphological events of swelling, chromatin decondensation and internal nuclear matrix generation, accompanied by an influx of proteins and the onset of nucleic acid
synthesis (Harris, 1970; Appels & Ringertz, 1975; LaFond & Woodcock, 1983). Most of the work on reactivation has been concentrated on heterokaryons where the presence of the host nucleus adds further complexity to studies of protein traffic. In cybrids, there is no host nucleus that could act as a source of proteins, and although these hybrid systems have a shorter life span, reactivation seems to be morphologically equivalent (Ege et al. 1975; Lipsich et al. 1978; LaFond et al. 1983). RNA synthesis is observed in cybrids and, in cases where the cytoplasts were made from cells in S phase, DNA synthesis also occurred (Lipsich et al. 1978). The protein that is imported by the reactivating nucleus in a cybrid could have one or more of the following origins: host (pre-existing in the cytoplasm), host (synthesized in response to the foreign nucleus on pre-existing mRNA), or chick (synthesized on message made as part of the reactivation process). However, previous results indicate that the morphological aspects of cybrid and heterokaryon reactivation occur in the absence of protein synthesis (Appels et al. 1974a; Lipsich et al. 1978; Woodcock et al. 1984), as does the importation of nucleus-specific antigens (Woodcock et al. 1984), and the generation of an internal nuclear matrix (Woodcock & Baldwin, unpublished data). This suggests that protein migration can be effectively studied by focusing exclusively on the movement of pre-existing proteins in cybrids. Thus, all the experiments reported here used cytoplasts that were prepared from methionine-labelled L-cells, and the redistribution of the label was examined after 16 h of fusion with erythrocyte nuclei. The insensitivity of this type of nuclear reactivation to protein synthesis inhibitors is in contrast to the nuclear 'activation' observed in lymphocytes in response to stimulation by concanavalin A (Setterfield et al. 1983). Although the changes in nuclear morphology are quite similar in the two systems, lymphocyte stimulation is completely blocked by cycloheximide (Setterfield et al. 1983).

The design of these experiments made the problem of cytoplasmic contamination of the isolated reactivated nuclei of particular concern. The long exposure times needed to detect the low levels of imported proteins resulted in the small amounts of contaminating material dominating the fluorograms. We were fortunate, however, to find that much of the contaminating cytoplasmic label was in the form of copurifying, but not attached, material. Thus, we were able to use mock fusions without nuclei to identify major cytoplasmic contaminants (rectangles in Figs 4–7). Each of the remaining labelled proteins associated with the 16 h reactivated nuclei (circles in Figs 4–7) could have an intranuclear location, be specifically involved in the nuclear envelope or pore-complex lamina, or be adventitiously bound to the nuclei during isolation. It is probable that some of each category are present. However, comparison with total cytoplasmic proteins (Fig. 7) makes it clear that there is not an indiscriminate uptake of cytoplasmic proteins by the erythrocyte nucleus. Instead, a discrete subset is imported, each component of which is concentrated in the nuclear preparation to a different degree. Some of the nucleus-associated polypeptides could be matched with spots on gels of total cytoplasm protein (Table 3), while others constituted such a minor component of the cytoplasm that they could not be detected
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there. These findings are consistent with other reports of nuclear reactivation in both heterokaryons and cybrids in which the accumulation of nucleus-specific protein, and exclusion of 'cytoplasmic' proteins has been demonstrated (Appels et al. 1975; Appels & Ringertz, 1975; Scheere et al. 1983).

Most of the labelled proteins associated with the reactivated nuclei (including the cytoplasmic contaminants) are not extracted by the extensive treatment with DNase I followed by 2·0·m-NaCl used to reveal the nuclear matrix (Fig. 8). Moreover, there appear (on the basis of one-dimensional gels) to be homologous proteins in the nuclear matrix preparations from 16 h reactivated nuclei and L-cell nuclei. Thus, it is possible that many of the major proteins that become associated with reactivated nuclei are resistant to extraction and contribute to the nuclear matrix. However, further work will be required to determine whether the generated nuclear matrix is composed exclusively of imported proteins. It should also be noted that there is a population of loosely bound protein that, according to the autoradiographic results, is lost during the nuclear isolation process.

Fig. 8. SDS/polyacrylamide gel of nuclear matrix preparations from 16 h reactivated nuclei and L-cell nuclei. All lanes are from the same gel: a,b, fluorograms; c–e, stain. Lane e, Mr markers as in Figs 4–7, with the addition of myosin heavy chain (205 000). Lane a, nuclear matrix from 16 h reactivated nuclei. The marked bands correspond to proteins that were identified in the two-dimensional gels as associated with these nuclei. All matched with bands of similar Mr in the L-cell nuclear matrix (lane d). Lanes b,c, fluorogram and stain of total L-cell nuclear proteins. Lane d, stain of L-cell nuclear matrix proteins. The common major bands at about 55 000 and 43 000 Mr in all the lanes include the major cytoplasmic contaminants identified in Fig. 5.
In no instance did we detect the presence of labelled histone in the reactivated nuclei. This was anticipated in view of the extremely small cytoplasmic histone pools, but emphasizes the contrast between cybrids and heterokaryons, where about half of the labelled protein associated with the erythrocyte nuclei consisted of core histone (Appels et al. 1974b). In their study, in which HeLa cells were used as the host, these authors found that the non-histone proteins associated with the reactivating nuclei were largely concentrated into two bands in the 40 000–60 000 M region of one-dimensional SDS/polyacrylamide gels (Appels et al. 1974b; also discussed by Appels & Ringertz, 1975). The appearance of the chick-specific histone H5 has been observed in the host nuclei of heterokaryons (Linder et al. 1982), suggesting that a direct exchange of proteins between the two nuclei may occur in these hybrid cells.

The evident selective concentration of proteins in reactivating nuclei raises questions concerning the reported dramatic increases in mass of the nuclei. Ege et al. (1975), using interferometry, found a threefold increase in the mass of the erythrocyte nuclei in L-cell cybrids, and suggested that this increase was mostly due to imported protein. Using the measurements made by Zentgraf et al. (1971), the mass of the erythrocyte nucleus is approximately $5 \times 10^{-9}$ mg, of which $1.7 \times 10^{-9}$ mg is DNA and $3.0 \times 10^{-9}$ mg is protein. In order to increase threefold in mass by the addition of protein, the nucleus would have to import about $13 \times 10^{-9}$ mg, which is more than 15% of the total protein content of the cytoplast ($8.7 \times 10^{-8}$ mg by Lowry determination). Moreover, in cases where multiple nuclei are reactivated in a cybrid, the available evidence suggests that the total protein transfer may be about twice as much as for the single nucleus. A single cytoplast has the ability to reactivate several erythrocyte nuclei to a considerable degree (e.g. see LaFond et al. 1983, fig. 1) and, in a study of this phenomenon in heterokaryons, Carlsson et al. (1973) found that approximately twice as much 'nuclear antigen' was transferred when four nuclei were reactivated as when only one was present, and the total nuclear volume was also doubled. This implies that when four nuclei are reactivated about 30% of the total cytoplasmic protein would have to be donated to the nuclei, and would seemingly be possible only if the uptake included proteins that were normally confined to the cytoplasm.

These considerations led us to make an independent estimate of the protein uptake using autoradiographic data. The results suggest that the erythrocyte nuclei undergo a much more modest gain, such that in the above example of a cytoplast containing four reactivated nuclei, only about 4.8% of the total cytoplast protein would be involved. This would be more consistent with the imported protein being confined to pools of nucleus-specific species.

It is likely that we have identified only a small fraction of the proteins taking part in the reactivation process. Not only are many soluble species lost during nuclear isolation, but also many others, such as the enzymes involved in nucleic acid metabolism, are likely to be imported in very small quantities, and are therefore not detectable in these experiments. However, we were interested in identifying the most abundant insoluble species, since these would be most likely to make a structural
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contribution to the nucleus. In continuing this work, it will be important to confirm the presence of these proteins in the nuclear matrix, and then determine their distribution in vivo. Especially favourable candidates for initial studies in this direction are the proteins numbered 7, 15 and 17, which appear to be present in L-cell nuclei also (Table 3), and are thus available in sufficient quantity for antibody production. Once these proteins are characterized, it will be of interest to compare them with others, such as nucleoplasmin, whose entry into the nucleus appears to be mediated by specific polypeptide sequences (reviewed by Dingwall, 1985).

A second feature of interest will be the time course of nuclear reactivation. We have found that at 3 h post-fusion an internal nuclear matrix can be detected in few reactivated nuclei (Woodcock & Baldwin, unpublished data), and it will be important to determine the protein content of the reactivated nuclei at intermediate time points. Preliminary experiments suggest that most of the imported proteins are already present in the reactivated nuclei at this early time, in agreement with other reports that protein transfer is extremely rapid (Appels et al. 1974b). Thus other factors, in addition to the presence of the necessary proteins, may be required for nuclear matrix formation.

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REFERENCES


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