The pool of histones in the nucleosol and cytosol of proliferating Friend cells is small, uneven and chasable

Stephan TSVETKOV, Emilia IVANOVA and Lelio DJONDJUROV*

Institute of Molecular Biology, Bulgarian Academy of Sciences, 113 Sofia, Bulgaria

This study examines the histones pools in the nucleosol and cytosol of proliferating Friend cells. By using the conventional approach, detectable amounts of these molecules were found in both compartments; however, only H3 and H2B were identified in nucleosol, and H3, H2B and H4 in cytosol. The authenticity of each of these histones was verified by two independent methods, migration in SDS/polyacrylamide gels and peptide mapping. When the sensitivity of the approach was increased by radiolabelling with ^{125}I , two additional proteins, migrating as H2A and H4, were observed in nucleosol. Even by this approach, however, H1 was not detected. Direct quantitative measurements of the histones in both compartments indicated that these pools are uneven and small. This was found also in experiments involving inhibition of protein synthesis by cycloheximide. Considered together, our data do not support the idea of the existence of preformed histone heterocomplexes or octamers. Instead the assembly of nucleosomes during replication occurs by a successive deposition of individual core histones.

INTRODUCTION

In eukaryotic cells, chromatin consists of a repeating nucleosome unit containing about 200 bp of DNA, two molecules each of histones H2A, H2B, H3 and H4 and one molecule of histone H1 (Felsenfeld, 1978). Although our knowledge on the nucleosome substructure has progressed during the last 10 years (Burlingame *et al.*, 1985), there is a lack of information on the mechanisms of nucleosome assembly. Even the fundamental problems such as how the core histones are segregated and duplicated remain controversial. For example, histones that are newly synthesized have been reported to mix with pre-existing parental histones to form heterogeneous cores (Jackson & Chalkley, 1981*a,b*; Senshu *et al.*, 1985*a*). Other authors have reported that the pre-existing histone cores do not mix with newly synthesized histones and are therefore conservatively segregated (Leffak *et al.*, 1977; Worcel *et al.*, 1978; Leffak, 1983, 1984).

It is well known that at the end of a cell cycle, one half of the histones are newly synthesized. This indicates that during replication there operates a mechanism ensuring a continuous supply of histones, synthesized in the cytoplasm. It is an open question, however, as to how the deposition of these histones occurs; by a single step as preformed octamers, or by a successive deposition of preformed complexes or individual molecules. The deposition of the histones in the form of complexes or octamers is indirectly suggested from experiments *in vitro* by D'Anna & Isenberg (1973, 1974), Thomas & Kornberg (1975) and Stein *et al.* (1977, 1979). Moreover, in the cell-free systems developed recently for investigations on the assembly of chromatin, the added histones probably form artificial complexes (Glikin *et al.*, 1984; Ruperti & Worcel, 1986; Sealy *et al.*, 1986*a,b*). In contrast, Worcel *et al.* (1978) and Cremisi & Yaniv (1980) found that the formation of new nucleosomes occurs *in vivo* by the successive deposition of individual histones. Such a mode

of deposition of the histones argues against the utilization of preformed heterocomplexes or octamers and questions their actual existence in the nucleosol and cytosol.

In this study we present data on the pools of soluble histones in the nucleosol and cytosol of proliferating Friend cells. Previous studies have only partially considered this matter (Weintraub, 1973; Senshu *et al.*, 1985*b*), or concerned cell systems not committed to active proliferation (Kleinschmidt *et al.*, 1985).

MATERIALS AND METHODS

Cells, radioactive labelling and treatment with cycloheximide

Friend cells (Ostertag *et al.*, 1972) were used in all experiments. They were grown in minimal essential medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10 % (v/v) calf serum.

For a continuous labelling with U- ^{14}C -labelled protein hydrolysate (Amersham; 59 mCi/mg of carbon), the cells were maintained for 22 h in the above medium containing 0.02 μCi of the labelled precursor/ml. In some experiments the cells were treated for 2 h with 5 μg of cycloheximide/ml (Sigma). Under these conditions, the rate of DNA synthesis was measured by periodical incubation of a portion of treated cells with 0.1 μCi of [6- ^3H]thymidine/ml (Amersham; 30 Ci/mmol) for 15 min. The rate of protein synthesis was estimated during the same time intervals by labelling treated duplicate cultures with 0.1 μCi of [4,5- ^3H]leucine/ml (Amersham; 120 Ci/mmol) for 15 min.

Isolation of cytosol and nucleosol

For isolation of cytosol, the collected cells were washed in buffer A containing 146 mM-sucrose, 100 mM-KCl, 10 mM-Tris/HCl, pH 7.0, and 1.5 mM-MgCl₂. The cells were then resuspended for 10 min in the same buffer containing 0.25 % (v/v) Nonidet P40. The crude nuclei

* To whom correspondence and reprint requests should be addressed.

were collected by low-speed centrifugation (550 *g*) and the supernatant was recentrifuged for 10 min at 8000 *g*. The fraction referred as cytosol was obtained after ultracentrifugation of the resulting supernatant at 220000 *g* for 4 h in a Beckman Ti50 rotor. It was then enriched in acid-soluble proteins by extraction with 0.4 M-H₂SO₄ (final concentration) for 15 min. The extract was finally precipitated with 3 vol. of cold acetone.

The nucleosol was obtained by the following approach. The crude nuclei from the previous step were washed once in buffer B containing 146 mM-sucrose, 100 mM-KCl, 10 mM-Tris/HCl, pH 7.7, and 5 mM-MgCl₂, ensuring iso-osmotic conditions, and collected by centrifugation for 10 min at 550 *g*. They were then lysed for 15 min in 0.05 M-NaCl/0.083 M-EDTA (pH 7.7) and centrifuged for 10 min at 8000 *g*. The supernatant was recentrifuged in a Beckman Ti50 rotor as described for the cytosol and extracted with 0.4 M-H₂SO₄.

All procedures were carried out at 4 °C. In all buffers 0.1 mM-phenylmethane sulphonyl fluoride was present.

Gel electrophoresis and identification of histones

The protein compositions of the acid-soluble extracts of cytosol and nucleosol were analysed by a two-dimensional gel electrophoresis [first dimension, acetic acid/urea gel according to Panyim & Chalkley (1969); second dimension, 15% (w/v) SDS/polyacrylamide gel according to Laemmli (1970)], permitting identification of the histones by staining with Coomassie Blue. In order to see whether the visually identified protein bands or spots are histones, they were excised from the gel and recovered by electroelution as described elsewhere (Tsvetkov *et al.*, 1989). The recovered samples were subjected to peptide mapping as described below.

The electroeluted samples were washed in 75% (v/v) ethanol and dissolved in 70 μ l of 0.125 M-Tris/HCl (pH 6.8)/0.5% (w/v) SDS/10% (v/v) glycerol/0.0001% Bromophenol Blue and treated at 37 °C for different times with 2 μ l of a 250 μ g/ml solution of trypsin (Merck) in 0.15 M-Tris/HCl (pH 7.5)/10% (v/v) glycerol. The hydrolysis was stopped by addition of 2% (w/v) SDS and 10% (v/v) β -mercaptoethanol and heating for 2 min at 100 °C. A portion (30 μ l) of each sample was loaded on to a 15% (w/v) SDS/polyacrylamide gel and run for 6 h at 120 V. The histone fragments were revealed by staining with Coomassie Brilliant Blue.

Radioiodination of nucleosolic samples

The dried nucleosolic samples were dissolved in 50 mM-phosphate buffer and to 50 μ l of each were added successively 100–150 μ Ci of Na¹²⁵I (Amersham; 100 mCi/ml) and 10 μ l of fresh Chloramine-T (5 mg/ml in water). After 90 s, 10 μ l of Na₂S₂O₅ (2.4 mg/ml in water) was added and the [¹²⁵I] iodine formed was diluted with 0.2 ml of KI (10 mg/ml in water). The samples were precipitated with 3 vol. of ethanol at –70 °C. The excess radiolabel was removed by a repeat precipitation with 80% (v/v) ethanol. The dried samples were finally solubilized in acid/urea sample buffer and separated in two-dimensional gel electrophoresis. After electrophoresis the gels were dried and autoradiographed.

Identification of the histones was achieved by running, in parallel, iodinated histones extracted from total chromatin.

Analytical procedures

Protein was measured according to Lowry *et al.* (1951). For quantification of H3, H2B and H2A in nucleosol and cytosol, standard curves were prepared for each of these histones. Radioactivity was counted in a Beckman spectrometer after dissolving the samples in toluene/Triton X-100 scintillation mixture (Anderson & McClure, 1973). Autoradiography of radioiodinated samples, separated in polyacrylamide gels, was performed on Kodak XR-5 film. When necessary, the stained gels were recorded on an ISCO densitometer.

RESULTS

Some considerations on the origin of histones in nucleosol and cytosol

Although the terms 'nucleosol' and 'cytosol' are used here to describe the fractions obtained (see the Materials and methods section), it is accepted that both leakage of nuclear proteins and contamination of the nuclear fraction with cytosolic material are likely to have occurred during cell fractionation. However, the individuality of the two fractions is supported by the results obtained.

It is generally accepted that the nucleosol represents the non-structural compartment of the nucleus. We use 'nucleosol' to refer to the nuclear fraction obtained after lysis of nuclei, isolated under iso-osmotic conditions, and successive low- and high-speed centrifugations of the

Table 1. Quantification of the histones of chromatin, nucleosol and cytosol in a proliferating Friend cell

In these experiments, quantification of each histone was carried out after electroelution from a preparative gel and a Lowry reaction. The data presented are mean values of five determinations; in each determination 1×10^{10} cells were used. For convenience, the total histones in chromatin (6.05 pg) was referred to as 100% and each histone found in nucleosol or cytosol was recalculated as a percentage of this; these values are given in parentheses. n.d., not done.

Fraction	Nucleosol (pg)	Cytosol (pg)
Total protein	1.61 $\pm$ 0.1530 (26.61)	n.d.
Histone H3	0.0083 $\pm$ 0.0009 (0.14)	0.0259 $\pm$ 0.0021 (0.43)
Histone H2B	0.0054 $\pm$ 0.0006 (0.09)	0.0147 $\pm$ 0.0020 (0.24)
Histone H2A	–	0.0090 $\pm$ 0.0010 (0.15)

Fig. 1. Two-dimensional gel electrophoresis of acid-soluble proteins (a) released from isolated chromatin by treatment with lysis buffer and (b) in nucleosol after incomplete (30 min) ultracentrifugation of the low-speed supernatant

In (a) note the absence of similarity to the protein pattern of nucleosol shown in Fig. 3. (b) The histone pattern includes H1, H3, H2B, H2A and traces of H4. In order to illustrate the presence of H4, the gel was overloaded.

lysate (see the Materials and methods section). Since among the proteins of this fraction some histones are also present, their origin should be carefully investigated. Two lines of evidence indicate that these histones do not originate from the structurally organized compartment of the nucleus, i.e. the chromatin. First, if isolated chromatin is treated with lysis buffer in the same way as whole nuclei, the proteins released into the high-speed supernatant comprise only 0.2% of the histones of chromatin (referred to arbitrarily in Table 1 as 100%).

According to the data given in the Table, this value is significantly less than the amount of these proteins in nucleosol. Moreover, the protein pattern of this fraction does not show similarity to that of nucleosol and, most importantly, does not contain detectable amounts of histones (Fig. 1). Secondly, upon treatment of the cells with cycloheximide, the histones of nucleosol are sensitive to this compound whereas the histones of chromatin are not (Fig. 2).

Cytosol is one of the seven major cellular compartments common to most eukaryotic cells (Alberts *et al.*, 1983). The presence of histones in cytosol should not be considered as contamination from nucleosol, because in a Friend cell the volume of cytosolic compartment exceeds many-fold the volume of the nucleosolic one, and if such contamination really exists it should be negligible. For the same reason, a possible contamination of nucleosol with histones from cytosol is more likely. It seems, however, that the bulk of nucleosolic histones do not originate from the cytosol, because if this were the case then the histone patterns of the both compartments should be similar, whereas Fig. 3 shows that these patterns are different. Contamination of cytosol with histones released from chromatin also seems unlikely because in this case they would also heavily contaminate the small nucleosolic compartment, which, as we have already mentioned, does not occur. Such a conclusion is in accordance with the differential sensitivity of the histones of cytosol and chromatin to treatment with cycloheximide (Fig. 2).

It should be noted that each step of the procedure for the isolation of nucleosol and cytosol is of importance for the final preparation. For example, if the ultracentrifugation of the nuclear lysate at 220000 *g* is as short as 30 min, the resulting nucleosolic fraction exhibits an exotic histone complement including H1, H3, H2B and H2A and only traces of H4, which suggests a contamination with light chromatin fractions (Fig. 1).

The pool of individual nucleosolic histones is uneven

In order to study the pattern of the nucleosolic histones, 1×10^{10} cells were collected and processed for the isolation of nucleosol. The nucleosol was enriched in acid-soluble proteins by extraction with H_2SO_4 and the resulting fraction was analysed by a two-dimensional gel electrophoresis (Fig. 3). Fig. 3(b) shows that among the huge number of proteins only three species, migrating in the zone of the core histones, are of interest. In order to check their affiliation to the histones, each spot was excised from a preparative gel and electroeluted. The following confirmed that two of the spots represent histones H3 and H2B. First the eluted proteins comigrate with the above-mentioned histones in SDS gels (Fig. 4), and secondly, when subjected to peptide mapping, they give the characteristic protein fragments (Fig. 5). It appears from the quantitative measurements given in Table 1 that the protein masses of the two recovered histones are not equal. The same conclusion is also reached by direct densitometry of stained polyacrylamide gels, which separates the proteins of nucleosol (Fig. 2).

By the conventional approach used for identification of the histones described above, we did not observe polypeptides resembling H2A, H4 or H1. The question that arises is whether the presence of these histones might be revealed by increasing the sensitivity of the approach. In order to investigate this, isolated nucleosolic samples

Fig. 2. Densitometry performed in the zone of the histones of acetic acid/urea gels, separating acid-soluble proteins of nucleosol and cytosol stained with Coomassie Blue

(a) Trace 1, nucleosolic histones; trace 2, nucleosolic histones after treatment of the cells with cycloheximide for 2 h; trace 3, cytosolic histones; trace 4, cytosolic histones after treatment with cycloheximide for 2 h. (b) Trace 1, control chromatin histones extracted from untreated cells; trace 2, histones extracted from the chromatin of cells treated with cycloheximide for 2 h.

Fig. 3. Two dimensional gel electrophoresis of acid-soluble proteins of nucleosol

(a) Lane a, separation of the proteins in the first dimension (acetic acid/urea gel). The asterisk indicates a protein migrating in the zone of the histones but not related to them. Lane b, separation of histones extracted from chromatin. (b) Separation of the nucleosolic proteins in an

were radioiodinated and then analysed by two-dimensional gel electrophoresis. Fig. 6(a) shows an autoradiograph of such a gel in which, as well as a confluent spot containing H3 and H2B, the zone of the core histones includes spots corresponding to H2A and H4. Because of the insignificant amount of the presumed H4 and the overlapping of the presumed H2A with H2B, additional evidence for their authenticity was not obtained. Even this approach did not reveal iodinated polypeptides resembling H1, which suggests that the amount of this histone in the nucleosol is very low.

The pool of the nucleosolic histones is small and is chased rapidly

It was interesting to investigate further the size of the nucleosolic histone pool. In order to measure their amount, the electroelution procedure was used; the data obtained were recalculated for a single cell and are presented in Table 1. Since H3 and H2B together comprise 0.24 % of all histones of chromatin and less than 1 % of the total protein content of nucleosol, it is obvious that their pool in this compartment is small.

In accordance with this conclusion are the data from experiments in which the cells were treated for 2 h with 5 μ g of cycloheximide/ml. Under these conditions the complete cessation of protein synthesis occurs in about 30 min and the process of replication continues, albeit at a reduced rate. As is seen from Fig. 2, whereas the histones organized in chromatin do not exhibit visible

SDS gel (second dimension). Note that of the histones, only H3 and H2B are present. The asterisk is as in (a). (c) Separation of the control histones extracted from chromatin.

Fig. 4. Comigration of the histone-like proteins eluted from nucleosol and cytosol with authentic histones H3, H2B and H2A

Lane a, total histone showing the position of the individual histones. Lane b, H3-like protein eluted from nucleosol.

changes, the two nucleosolic histone species have been almost completely chased. Since the histones are metabolically conservative (Djondjurov *et al.*, 1983; Tsvetkov *et al.*, 1989) the above suggests (i) the existence of a limited number of histone molecules in the pool, and (ii) a continuous and rapid movement of these molecules to outside the pool, probably to the regions of deposition. It might be supposed that they participate in both the replicative assembly of new nucleosomes and in a 'replacement' assembly of old nucleosomes (Louters & Chalkley, 1985).

The pool of histones in cytosol

After isolation, the fraction referred to as cytosol was enriched in acid-soluble proteins and analysed by two-dimensional gel electrophoresis. Three protein species resembling core histones were seen regularly on such electrophoretograms (Fig. 7). After electroelution, the recovered proteins were re-electrophoresed on an SDS gel and analysed by peptide mapping. Both approaches confirmed that these proteins are indeed the histones H3,

The H3-like protein eluted from cytosol exhibited the same migration characteristic and is not shown. Lane c, H2B-like protein eluted from nucleosol. The H2B-like protein from cytosol exhibited the same migration characteristic and is not shown. Lane d, H2A-like protein eluted from cytosol.

Fig. 5. Identity of the histone-like proteins eluted from nucleosol and cytosol with authentic histones extracted from chromatin as determined by one-dimensional peptide mapping

(a) Lane a, migration of a control H3 histone extracted from chromatin; lane b, migration of the same H3 histone after proteolytic degradation as described in the Materials and methods section; lane c, migration of H3-like protein eluted from nucleosol and treated as in lane b. The H3-like protein, eluted from cytosol and subjected to proteolytic degradation, gives the same pattern and is not shown. (b) Lane a, migration of a control H2B histone eluted from chromatin; lane b, migration of the same H2B histone after proteolytic degradation; lane c, migration of H2B-like protein eluted from nucleosol and treated as in lane b. The H2B-like protein eluted from cytosol and subjected to proteolytic degradation give the same pattern, and is not shown. (c) lane a, migration of control undigested H2A eluted from chromatin; lane b, migration of the same H2A histone after proteolytic degradation; lane c, migration of H2A-like histone eluted from cytosol and treated as in lane b.

Fig. 6. Radioiodination of the proteins of nucleosol reveals the existence of H2A- and H4-like molecules

The acid-soluble extract obtained from nucleosol was precipitated and dried and the proteins were radioiodinated as described in the Materials and methods section. The labelled samples were then separated by two-dimensional gel electrophoresis, and the gels were dried and autoradiographed (a). Note the absence of H1-like molecules. (b) Autoradiograph of control radiolabelled histones from chromatin.

H2B and H2A (Figs. 4 and 5). No other histone-like proteins were found after staining with Coomassie Blue. The quantitative data for the recovered H3, H2B and H2A per Friend cell are given in Table 1: these data, as well as those obtained from direct densitometry of gels with separated cytosolic proteins (Fig. 2) illustrate a situation similar to that present in the nucleosol, i.e. an obvious molar difference between the histones. Moreover, on the basis of the data on Table 1 and the

Fig. 7. Two-dimensional gel electrophoresis of acid-soluble cytosolic proteins

(a) Separation of the cytosolic proteins in the second dimension. The asterisk indicates a protein ubiquitous for nucleosol and cytosol (see Fig. 3). Note that this compartment contains three identifiable core histones, H3, H2B and H2A. (b) Separation of the control histones extracted from chromatin in the second dimension.

experiments with cycloheximide (Fig. 2), we conclude that the pool of individual histones in cytosol is also small. Their efficient chase with cycloheximide coinciding in time with the chase in nucleosol suggests a dynamic equilibrium between the processes of histone synthesis in cytoplasm and histone utilization in nucleus.

DISCUSSION

The results from this study show that detectable amounts of histones are present in the nucleosol and cytosol of proliferating Friend cells (Figs. 3 and 7; Table 1). By the conventional approach for identification, a complete set of the known histone species was not observed: in nucleosol only H3 and H2B were identified while in cytosol H3, H2B and H2A were present. The authenticity of the above histones was confirmed by two independent methods, migration in SDS gels and peptide

mapping (Figs. 4 and 5). In both compartments the relative molar amounts of histones measured after electroelution (Table 1) or densitometry of stained gels (Fig. 2) were not equal, as is the case for chromatin. This observation indicates that the pool of individual histones is uneven and raises a number of questions concerning the mode of transcription of the histone genes, the rate of translation of the histone RNAs and the mechanisms of transport of the newly synthesized histone molecules to the sites of deposition.

It is reasonable to believe that the failure to identify the rest of the histones, i.e. H4 and H1 in cytosol and H2A, H4 and H1 in nucleosol, is not due to a complete absence of these molecules but to their insignificant amount. Such an assumption is supported by the fact that when the proteins of nucleosol were radioiodinated and then separated by two-dimensional gel electrophoresis, two additional histones were identified (Fig. 6). Moreover, we obtained direct evidence that the size of the histone pool in both compartments is small (Table 1). The latter was also confirmed in experiments involving inhibition of protein synthesis: treatment of the cells with cycloheximide for 2 h chased almost completely the histones from the two compartments (Fig. 2). Since the histones are proteins which turn over only slowly (Djondjurov *et al.*, 1983; Tsvetkov *et al.*, 1989), such treatment should not be able to chase them if they are immobilized within a given compartment. Thus we assume a continuous and rapid movement of histones, directed outside each of the two compartments. It might be supposed in such a case that under physiological conditions there is an equilibrium between the histones synthesized in cytosol, the amount of soluble histones both in cytosol and nucleosol, and the histones deposited in chromatin. It is likely that the equilibrium is realized by an intensive traffic of the histones between the compartments. Thus in our experiments with cycloheximide, the pool of the histones in cytosol and nucleosol drops rapidly because of a deficient supply of newly synthesized histones on the one hand, and their continuous chase toward the sites of utilization on the other.

As was mentioned in the Introduction, one of the reasons stimulating this study was to discover whether the soluble histones of the two compartments interact to form heterocomplexes. If such complexes really exist, our experiments would confirm the following two predictions. First, since in the complexes the histones interact in 1:1 molar ratio, we should observe heterogeneous doublets or tetrads of core histones in equimolar amounts. Secondly, since it is known that under physiological conditions the core histones give three strong complexes, i.e. H3/H4, H2B/H4 and H2A/H2B (D'Anna & Isenberg, 1974), we should find evidence for their existence. Our experiments, however, did not confirm the above predictions. This conclusion is also supported in the literature. In experiments performed *in vivo* Worcel *et al.* (1978) and Cremisi & Yaniv (1980) found that during assembly of nucleosomes the histones deposit on DNA as individual molecules but not as dimers or tetramers. Moreover, the turnover of the histones in proliferating cells, which might be considered to be a process opposite to that of chromatin assembly,

indicates that the protein moiety of nucleosomes is not replaced as one unit or as doublets but as a 'mosaic' in which each histone species follows its own rate of replacement (Tsvetkov *et al.*, 1989).

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