Direct translocation of histone molecules across cell membranes

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Summary

The present work shows that histones are able to directly cross cell plasma membranes and mediate penetration of macromolecules covalently attached to them. Adding a mixture containing the five nucleosomal histones, H1, H2A, H2B, H3 and H4, as well as each of the last four individual histones to intact HeLa and Colo-205 cultured cells resulted in cell penetration and nuclear import of these externally added histones. This was observed by fluorescent and confocal microscopy using fixed and unfixed cells, showing that penetration was not due to the fixation process. Accumulation was also estimated by a quantitative assay that did not require cell fixation and allowed neutralization of surface-bound histones. Translocation into the HeLa and Colo-205 cells occurred at 4°C, in ATP-depleted cells and in cells incubated with sucrose (0.5 M) – conditions that block the endocytic pathway. Furthermore, various endocytosis inhibitors such as colchicine, nocodazole, cytochalasin D, brefeldin A, chloroquine and nystatin did not have any effect on the penetration process. Thus, cellular uptake was mostly due to direct translocation of the histones through the cell plasma membrane and not to endocytosis. The histones were also able to mediate penetration of covalently attached bovine serum albumin (BSA) molecules, indicating their potential as carriers for the delivery of macromolecules into living mammalian cells.

Key words: Histone, Direct penetration, CPP, Delivery, Endocytosis

Introduction

In the eukaryotic chromatin, the histone octamer – which is composed of the four histone classes H2A, H2B, H3 and H4 – is surrounded by DNA molecules (Van-Holde, 1989), which are electrostatically bound to the histone (Luger et al., 1997). Being positively charged, histones share several properties with other basic macromolecules such as the polylysine (PLL) (Wagner, 1998), polyornithine (PLO) and polyethanolimine (PEI) (Boussif et al., 1995). Indeed, octamer-DNA complexes or complexes formed between DNA molecules and isolated histones (such as H2A) have been used as DNA carriers to transfect animal cells (Fritz et al., 1996; Balicki et al., 2000) in a similar way to the polyplexes (complexes between DNA and basic macromolecules) (Felgner et al., 1997). A peptide derived from H2A was shown recently to mediate the transfer of a plasmid encoding the β-galactosidase gene into COS-7 cells (Balicki et al., 2002). It was generally assumed that – similar to the polyplexes – histones are being taken into cells by clatherin-coated pits and by the endocytic pathway (Ryser and Hancock, 1965; Murphy et al., 1982).

During the past few years, it became apparent that certain small molecular weight proteins are able to directly cross the cell plasma membrane without the involvement of the endocytic pathaway and thus are not susceptible to the intra-endosomal enzymes. The claim about direct penetration via a putative ‘inverted micelle pathway’ (Gariepy and Kawamura, 2001) was based on initial observations that cellular import occurred at 37°C and at 4°C, ruling out endocytosis as a possible transport mechanism. Several natural proteins or peptides such as the HIV-1 Tat or the ARM (arginine-rich motif) peptide derived from it (Vives et al., 1997) as well as mastoparan, the third alpha helix from the Antennapedia homeodomain of *Drosophila* (Higashijima et al., 1990), have been defined as cell penetrating proteins or peptides (CPPs) because of their ability to translocate cell plasma membrane independently of transporters or specific receptors. Very recently, however, this concept was re-evaluated, leading to the suggestion that the cellular accumulation of the HIV-1 Tat-ARM peptide – thus, of any positively charged peptides – is due to internalization via endocytosis and not to direct penetration (Richard et al., 2003). It was claimed that strong interaction of the CPPs with the cell membrane led to an overestimation of the observed cell-associated fluorescent signal (Vives et al., 2003). Furthermore, previous reports (Vives et al., 1997; Futaki, 2002) on Tat-ARM penetration were suggested to be an artefact due to the use of fixed cells – conditions that may cause a redistribution of the surface-bound Tat peptide, resulting in what appears by microscopic observations as cell penetration and nuclear import.

In the present work we show that histones are able to directly transverse the cell plasma membrane. This was observed using different and independent experimental systems: fluorescent microscopy observations with fixed and unfixed HeLa cells and a quantitative assay system using unfixed Colo-205 cells from which surface-bound histones were removed by neutralization. Penetration of histones was also observed at 4°C, in the

absence of ATP and in the presence of various endocytosis inhibitors, suggesting that it proceeds in an endocytosis-independent pathway. Of the different histones, H2A and H4 had the strongest ability to penetrate cells. We conclude that histones can be considered to be cell-penetrating proteins.

Materials and Methods

Cultured cells

HeLa cell monolayers were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 0.3 g/l L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Beit Haemek, Israel). Colo-205 [human Colo-205 adenocarcinoma cells (ATcc CCL 222)] were maintained in RPMI 1640 medium, supplemented with 10% FCS, 0.3 g/l L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were incubated at 37°C in 5% CO_2 atmosphere.

Human lymphocytes were obtained from fresh human blood by its fractionation on a Ficol gradient (Jazwinski, 1990).

Buffers

The following buffers were used: transport buffer (TB): 20 mM Hepes pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 0.5 mM EGTA, 2 mM DTT, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml aprotinin, 0.1 mM PMSF. Phosphate buffered saline (PBS): 140 mM sodium chloride buffered with 20 mM sodium phosphate, pH 7.3.

Expression and purification of importin alpha and beta

The vector pET28-hIMPα/β was kindly obtained from V. Citovsky (State University of New-York Stony Brook, NY) and was expressed in *Escherichia coli* strain BL21 (DE3). The histidine (His-Tag; Qiagen) tagged-importin alpha/beta fusion protein was expressed and purified by standard protocols following growth at 37°C and induction of the *E. coli* strains at 25°C.

Preparation and purification of recombinant histone proteins

Expression plasmids for the individual histones (H2A, H2B, H3 and H4) were kindly obtained from K. Luger (Department of Molecular Biology, Scripps Research Institute, La Jolla, CA) and T. J. Richmond (Institut fur Molekularbiologie und Biophysik, Zurich, Switzerland) and were expressed in *E. coli* strain BL21 (pLysS) and purified exactly as described before (Luger et al., 1999). The purified recombinant histones were solubilized in PBS to give 1 mg/ml.

Incubation of histone mixture and of pure recombinant histones with cultured HeLa cells: microscopic observations

An histone mixture containing all the five histones (Sigma H5505) as well as the individual recombinant histones (H2A, H2B, H3 and H4) were labelled with lissamine rhodamine (Molecular Probes) or covalently attached to fluorescently labelled bovine serum albumin (BSA) exactly as described previously for the labelling of other protein molecules (Hariton-Gazal et al., 2002) and solubilized in PBS.

For incubation with histones, HeLa cells (3×10^4 per well) were cultured on 10 mm coverslips to subconfluent density. Following the removal of the culture medium, the cells were washed three times with TB and then exposed to various concentrations of labelled-histone preparations or histones-BSA conjugates at 37°C or at 4°C in final volume of 50 μl TB. At the end of the incubation period the cells were washed three times with TB and in few experiments were observed directly by fluorescent microscopy without fixation. For most of the experiments, the cells were fixed in 4% (v/v) formaldehyde dissolved in TB. Fixed cells were examined by fluorescence microscope (Zeiss Germany, a 40× objective; Apoplan) or by confocal microscopy using an MRC 1024 confocal microscope (Bio-Rad). The microscope (Axiovert 135M; Zeiss Germany, a 63× objective; Apoplan; NA 1.4) was equipped with an argon ion laser for rhodamine excitation at 514 nm (emission 580 nm).

Estimation of the amount of histones within the cytosol and nuclei of intact Colo-205 cells

For quantitative estimation of intracellular histones, the histones were conjugated to biotin maleimide or covalently attached to biotinylated BSA (Sigma) as described previously (Hariton-Gazal et al., 2002), and for these studies a suspension of Colo-205 was used. The degree of biotinylation was estimated using a spectrophotometric assay for biotin; it was found to be in most of the experiments 4 mole biotin/mole of histone (Janolino, 1996), and the biotinylated BSA contained 9 mole biotin/1mole BSA (Sigma).

Intact Colo-205 cells ($15\text{-}20\times10^5$ cells) in TB were incubated with either biotinylated histone mixture, with the four individual biotinylated histones (H2A, H2B, H3 and H4) (0.1 mg/ml in TB) or with biotinylated BSA-histone or with biotinylated BSA (Bb) conjugates (both at 1 mg/ml in TB) in a final volume of 60 μl for 1 hour at 37°C or at 4°C. At the end of the incubation period, 200 μl TB were added and the extracellular histones were removed by centrifugation of the cell suspension for 5 minutes at 2 ***g***. Following removal of the supernatant and to neutralize any remaining cell-surface-bound biotinylated histones or conjugates, the pellets were suspended in a volume of 100 μl avidin (Sigma) (1 mg/ml) in TB. After 30 minutes incubation at 37°C, unbound avidin was neutralized by the addition of 100 μl of biocytin (2 mg/ml) in TB (Sigma). Following another 15 minutes of incubation, the samples were centrifuged and the supernatant was removed. For estimation of cytosol and nuclei biotinylated histone lysates of both compartments were obtained in two steps. First, the cells' plasma membranes were permeabilized – as followed by a phase microscope – with 30 μl of digitonin solution (0.08 mg/ml). Permeabilization was terminated by the addition of 200 μl TB, usually within 30 seconds at 37°C. The samples were centrifuged and the supernatant, containing the cell cytosols, was removed and stored in the cold. Biotinylated molecules present in these samples were considered to be those accumulated within the cell cytosol. Any exposed biotinylated histones remained in the pellet (considered as extranuclear) were neutralized by the addition 100 μl avidin (0.1 mg/ml) in TB. Following gentle suspension, the pellet was incubated for 30 minutes at 4°C, and then 100 μl of biocytin (0.2 mg/ml) in TB were added to neutralize any remaining avidin and the samples were incubated for another 15 minutes at 4°C. After centrifugation, as above, the supernatant was removed and the nuclei in the pellet were then lysed by the addition of 200 μl of lysis buffer (1% Triton X-100 in PBS). After vigorous mixing, lysis was completed by incubation overnight at 4°C. Biotinylated molecules present in these fractions were considered to be those accumulated within the cell nuclei. For estimation of total cellular accumulation of histones (cytosol + nuclei), complete cells lysates were obtained, after neutralization of surface-bound biotin molecules as described above, by the addition of 200 μl lysis buffer.

For collecting the biotinylated histones, the various lysate fractions were incubated with importin beta-coated plates (Fineberg et al., 2003). For coating, a solution of importin beta (4.3 mg/ml in $NaHCO_3/Na_2CO_3$ buffer, pH 9.6) was added to 96 maxisorp plates (Nunc Inc.) and incubated overnight at 4°C. Importin beta was used to attract and bind soluble biotinylated histones as it was shown to serve as a nuclear import receptor of histones (Johnson-Saliba et al., 2000).

When biotinylated BSA-histone conjugates were used, they were attached to anti-BSA antibody-coated plates as described previously for the attachment of nuclear localization signal (NLS)-BSA conjugates (Hariton-Gazal et al., 2002). Plates attached biotin

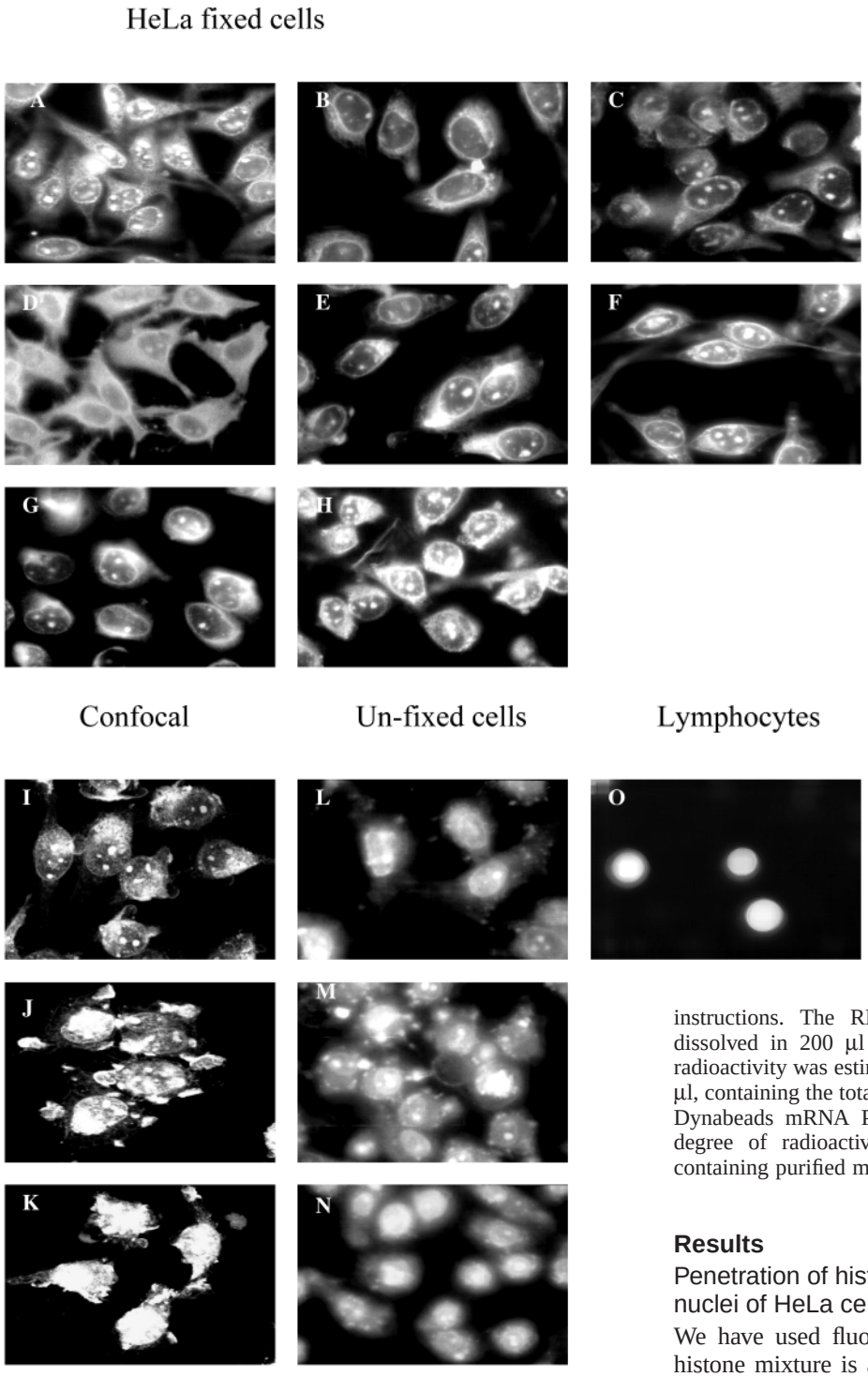


Fig. 1. Intracellular accumulation of rhodamine-labelled histone proteins in intact HeLa cells and human lymphocytes: fluorescent microscopy observations. HeLa cells were incubated for 1 hour in the presence of a mixture of rhodamine-labelled histones (2 μM) at 37°C. (A) After fixation by formaldehyde, cells were observed by fluorescent microscopy; (B-H) as in (A) but with the following conditions: (B) cells were incubated at 4°C; (C) incubation was performed in the presence of excess unlabelled histone mixture (×50 mole/mole); cells were pre-incubated for 30 minutes at 37°C before the addition of histones with the following: (D) incubated with NaF (2 mM) (ATP depletion); (E) cytochalasin D (5 μM); (F) BFA (10 μM); (G) nocodazole (20 μM); (H) nystatin (50 μg/ml) and sucrose (0.5 M); (I) as (A), (J) as (G) and (K) as (H) but using confocal microscopy; (L) as (A), (M) as (G) and (N) as (H) but with unfixed cells. (O) Human lymphocytes incubated for 1 hour in the presence of a mixture of rhodamine-labelled histones. All other experimental conditions were as described in Materials and Methods.

molecules of the various samples were estimated following the addition of avidin-HRP (horse radish peroxidase) exactly as described previously (Melchior et al., 1993). The enzymatic activity of the HRP was estimated by monitoring the optical density (O.D.) of product obtained at 490 nm. The results given are an average of triplicate determinations whose standard error is ±15%.

Estimation of the effect of the histone mixture on the overall transcription process

Colo-205 cells, 10 ml (20×10^6 cells/ml RPMI) were incubated for 1 hour at 37°C in the absence (three samples of 10 ml each) or in the presence of a 0.5 μM histone mixture (three samples of 10 ml each) and then 100 μCi of 5-[^{3}H]-uridine (28 Ci/mmole; Amersham Biosciences), and the cells were cultured at 37°C for another 30 minutes. At the end of incubation the cells were centrifuged and after three wash cycles with PBS, total RNA was extracted by TRIzol (Invitrogen) according to manufacturer instructions. The RNA pellet obtained from each sample was dissolved in 200 μl of double distilled water and the degree of radioactivity was estimated on aliquots of 100 μl. The remaining 100 μl, containing the total RNA, were used for isolation of mRNA using Dynabeads mRNA Purification (Dynal Biotech No. 610.06). The degree of radioactivity was estimated using aliquots of 20 μl containing purified mRNA.

Results

Penetration of histones (mixture) into cytoplasm and nuclei of HeLa cells: microscopic observations

We have used fluorescent microscopy to determine whether histone mixture is able to penetrate the plasma membrane of cultured HeLa cells (Fig. 1A and Table 1). Incubation of HeLa cells with a fluorescently labelled histone mixture for 1 hour at 37°C resulted in extensive fluorescent staining of both the cell cytoplasm and the intranuclear space, with a high degree of fluorescence within the nucleoli. A very similar observation was recently reported for the import of core histones into nuclei of permeabilized cells (Baake et al., 2001). Assuming that very few unoccupied binding sites for histones are available in the interphase chromatin, the added histones, being positively charged, interact with the negatively charged ribosomal RNA of the nucleoli. Accumulation within the cell cytoplasm and

Table 1. Effect of endocytic pathway inhibitors on the uptake of histone mixture, LDL and LY into intact HeLa cells: a summary of fluorescent microscopy observations

	Histone mixture†		Histone mixture‡		LDL†	LY†
Experimental conditions	Nuclei	Cytosol	Nuclei	Cytosol	Cytosol	Cytosol
Control	+	+	+	+	+	+
ATP-depleted cells*	–	+	ND	ND	–	–
Colchicine (20 μM)	+	+	+	+	–	+
Brefeldin A (10 μM)	+	+	+	+	–	–
Nystatin (50 μg/ml)	+	+	+	+	–	–
Cytochalasin D (5 μM)	+	+	+	+	–	+
Chloroquine (50 μM)	+	+	+	+	–	–
Nocodazole (20 μM)	+	+	+	+	–	+
Sucrose (0.5 M) ± Nystatin (50 μg/ml)	+	+	+	+	–	–

Following 30 minutes incubation at 37°C with all the above inhibitors HeLa cells were incubated with histone mixture for 1 hour and with LDL and LY for 4 hours.

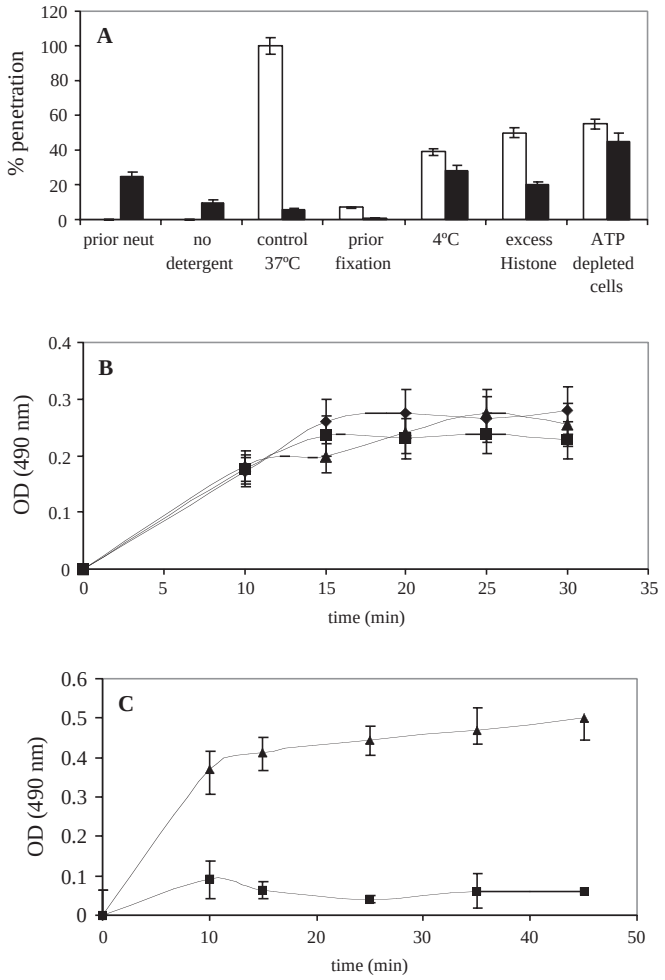
*For ATP depletion, cells were incubated, before the addition of the histones with DNP (1 mM), NaF (2 mM) and iodoaceticacid (1 mM). At the end of the incubation period the cells were washed with TB and all subsequent steps of addition of fluorescently labelled molecules as well as fluorescent microscopy were carried out as described in Materials and Methods.

†Cells were fixed with 4% (v/v) formaldehyde.

‡Cells were observed without fixation.

+, most of the nuclei/cytoplasm in the microscopic fields are highly fluorescent; –, no fluorescence in the nuclei/cytoplasm; ND, not determined.

nucleus was further confirmed by confocal microscopy (Fig. 1I). Recent reports suggested that the fixation process can cause artifacts that can be misinterpreted as cellular penetration (Lundberg and Johansson, 2002). However, we also observed the accumulation of externally added histones within the cell cytosol and nuclei in unfixed cells (Fig. 1L), ruling out the possibility of an artefact caused by the fixation process.



Cellular uptake of histones occurs under conditions that inhibit endocytosis

To obtain more insight into the machinery of histone penetration into cells, we have tested it under various experimental

Fig. 2. (A) Intracellular accumulation of biotinylated histone proteins (externally added) in cultured Colo-205 cells: quantitative estimation. Prior-neut (prior neutralization): biotinylated histones neutralized with avidin and biocytin before being added to the Colo-205 cells. All subsequent steps including cell permeabilization and estimation of biotinylated histones are as described in Materials and Methods. No detergent: estimation of cell-surface-bound biotin molecules. Biotinylated histones were incubated with Colo-205 cells and following neutralization of surface-bound biotin the remaining biotin was estimated on unlysed cells. Control 37°C and 4°C: the biotinylated histone mixture was incubated with Colo-205 cells at 37°C or at 4°C and after neutralizion with avidin and incubation with biocytin, the cells were treated with detergents. Prior fixation: cells were fixed by formaldehyde prior to the incubation period. Excess histone: as control 37°C but in the presence of excess unlabelled histone (×100 mole/mole). ATP-depleted cells: as control 37°C and ATP depletion was performed as described in Table 1. □, nuclei; ■, cytosol. The amount of biotinylated histone present in the nuclei of cells incubated at 37°C was considered as 100% and was calculated to be 6.2 nmol histone/mg protein, which was estimated to be about 60% of the total added histones. (B) Kinetic studies of histone penetration. Biotinylated histones were incubated with Colo-205 cells at 37°C in the absence (◆) or in the presence (■) of 0.5 M sucrose and at 4°C (▲). Pre-incubation with sucrose (0.5 M) was performed as described in Table 1. An optical density (OD) of 0.25 represents 4.7 nmol histone/mg protein. (C) Kinetic studies of histone and importin alpha penetration. Biotinylated histone (▲) and importin alpha (■) were incubated with Colo-205 cells at 37°C. Penetration of histone and importin was estimated as described in Materials and Methods, except that the incubation was performed in PBS and not in TB as in B.

conditions that inhibit the endocytic pathway. Incubation of the HeLa cells with histone mixture at 4°C resulted mainly in the appearance of the fluorescent molecules within the cell cytoplasm with very little staining of the cell nuclei (Fig. 1B), indicating inhibition only of nuclear import but not of cellular uptake. Addition of an excess (×50 mole/mole) of unlabelled histones resulted in some inhibition of nuclear import of the histones but not of their penetration, indicating a non-receptor-mediated process (compare Fig. 1A with 1C).

In a similar manner, the histone mixture was able to penetrate into ATP-depleted cells (Fig. 1D), showing that histones uptake did not require ATP, whereas the nuclear import was ATP-dependent, as expected. In these cells only the cytoplasm was fluorescent, whereas most of the nuclei remained dark. It was well established that nuclear import is an ATP- and GTP-dependent process (Gorlich et al., 2003). We next tested the effect of endocytosis inhibitors on the cellular uptake of histones. A battery of inhibitors, such as colchicine (Table 1) (Skrzypek et al., 1998), chloroquine (Table 1), as well as cytochalasin D (Table 1 and Fig. 1E) (Elliott and O'Hare, 1997), Brefeldin A (BFA) (Table 1 and Fig. 1F) and nocodazole (Bayer et al., 1998) (Table 1 and Fig. 1: G, fixed cells; J, confocal microscopy; M, unfixed cells), which are all known to affect, directly or indirectly, internalization via endocytosis or intracellular trafficking, did not cause any inhibition of cellular penetration of histones. Even extreme conditions such as incubation of cells with a combination of sucrose (0.5 M) and nystatin neither blocked cell penetration nor nuclear import (Table 1 and Fig. 1: H-fixed cells, K-confocal microscopy, N-unfixed cells). Accumulation of the fluorescent molecules within the cell cytoplasm and nuclei appeared to be the same in the presence or absence of these inhibitors. In addition to HeLa cells, the labelled histones were able to accumulate also within human lymphocytes, as evident from the nuclear staining of these cells (Fig. 1O). To confirm that the endocytosis inhibitors were active, we tested their ability to inhibit the cellular uptake of both low density lipoprotein (LDL) and lucifer yellow (LY), which are known to be taken into cells via endocytosis and pinocytosis (Skrzypek et al., 1998; Catizone et al., 1996). As shown in Table 1, nystatin ± sucrose (0.5 M) and ATP depletion of cells (Schmid and Carter, 1990) by a combination of dinitrophenol (DNP), NaF and iodoacetic acid blocked the uptake of both LDL and LY, whereas colchicine, cytochalasin D and nocodazole completely blocked the uptake of LDL, showing that the inhibitors used were functional and active.

Quantitation of histone penetration into cell cytoplasm and nuclei

To quantify the amounts of histones in the cell and specifically the amounts in the cytoplasm compared with the nucleus, we have developed a quantitative ELISA-based assay system. This assay uses biotin-labelled histones and a suspension of Colo-205 cells (see Materials and Methods). Incubation of externally added biotinylated histones with the cells resulted in penetration mostly into the nucleoplasm with only about 10% in the cell cytosol (Fig. 2A). The view that only intracellular and not surface-bound histones were estimated is evident from the results shown in Fig. 2A (compare 'prior neutralization', 'no detergent' to 'control 37°C'). Any surface-bound biotin

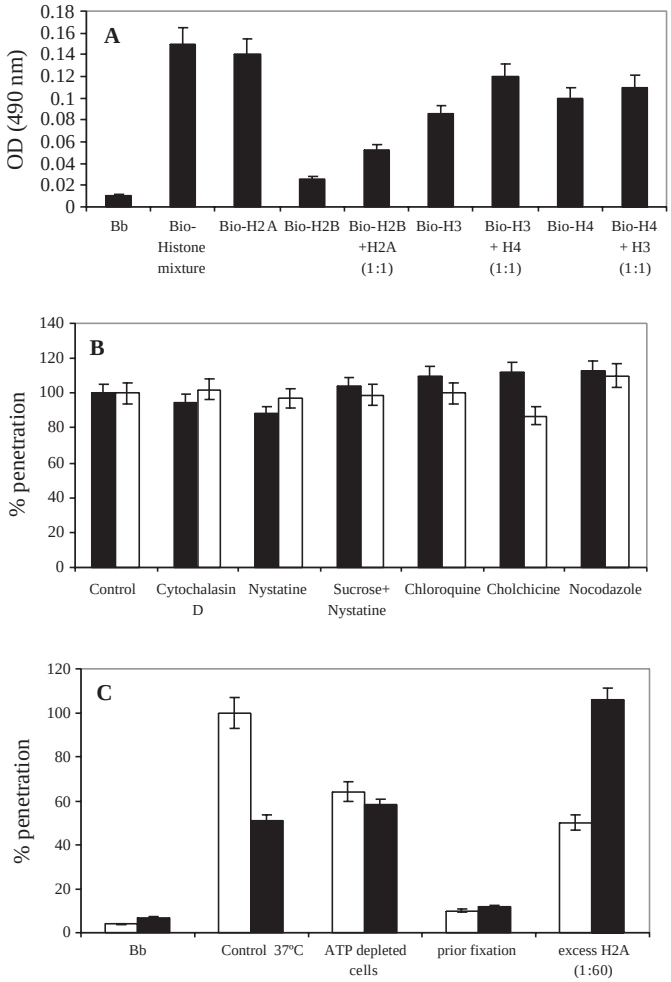


Fig. 3. (A) Accumulation of biotinylated individual histones: quantitative estimation. All experimental conditions of estimation of cellular uptake are as described in Materials and Methods. An optical density of 0.2 represents 0.47 nmol histone/mg protein. (B) The effect of various inhibitors on the intracellular accumulation of H2A and H4. (a) Accumulation of biotinylated H2A (■) and H4 (□) into Colo-205 cells. Cells were pre-incubated before the addition of the histones for 30 minutes at 37°C with the various endocytic inhibitors at the concentrations described in Table 1. The amount of biotinylated H2A and H4 present in the nuclei of cells incubated in 37°C was considered to be 100% and was found to be 5.9 and 5.5 nmol histone/mg protein, respectively. (C) Intracellular accumulation of biotinylated H2A in Colo-205 cells: effect of ATP depletion and unlabelled H2A. Accumulation of biotinylated H2A within the cell cytosol and nuclei was estimated as described in Materials and Methods. ATP depletion was performed as described in Table 1. □, nuclei; ■, cytosol. The amount of biotinylated H2A present in the nuclei of cells incubated in 37°C was considered to be 100% and represents 5.8 nmol/mg protein; it was estimated to be about 48% of the total added histones. The ratio given (in A and C) are in mole:mole.

molecules were neutralized by the addition of excess avidin, whereas accumulated intracellular biotin molecules were exposed and estimated only following treatment of the cells with detergent (see Materials and Methods). Furthermore, as can be inferred from the results in Fig. 2A, after neutralization of the surface-bound biotinylated-histones, very little – if any – biotin

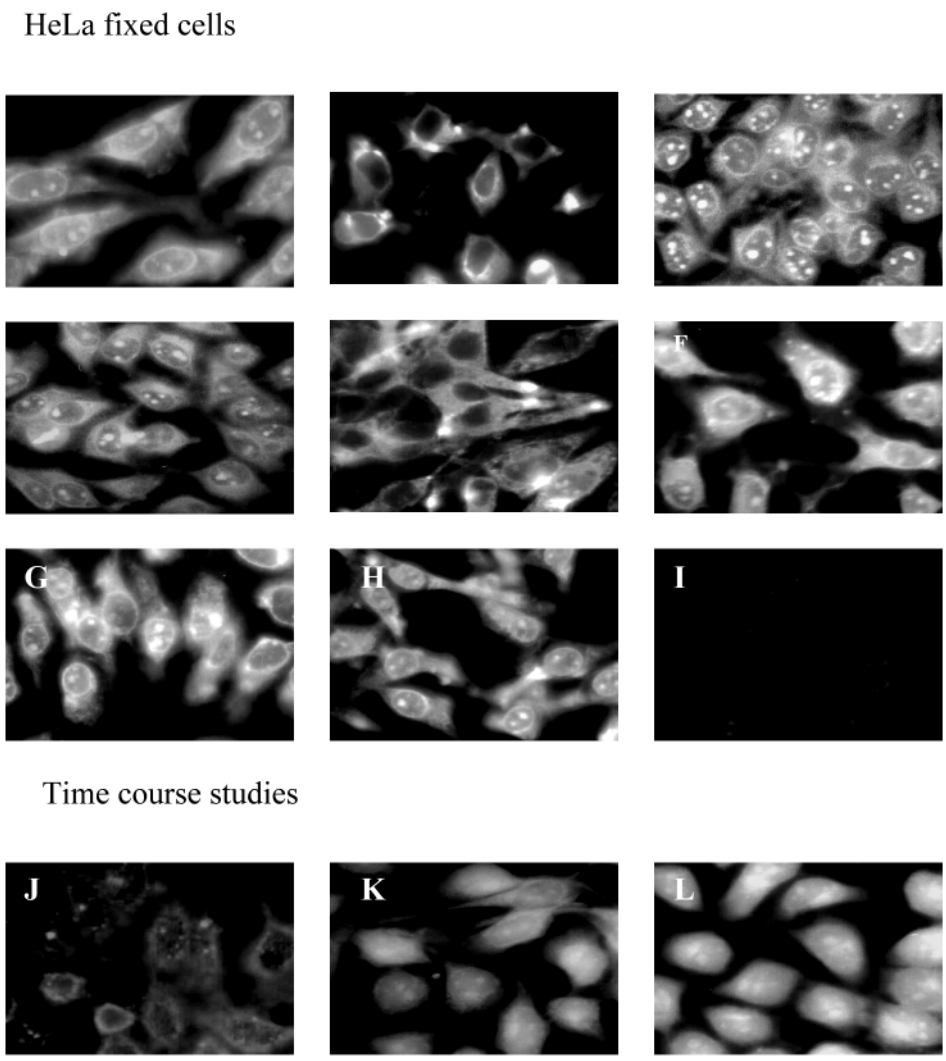


Fig. 4. Intracellular accumulation of the individual histones: fluorescent microscopy observations. HeLa cells were incubated for 1 hour at 37°C in the presence of rhodamine-labelled (A) H2A, (B) H2B, (C) labelled H2A + non-labelled H2B (1:1 mole/mole), (D) labelled H2B + non-labelled H2A (1:1 mole/mole), (E) H3, (F) H4, (G) labelled H3 + non labelled H4 (1:1 mole/mole) or (H) labelled H4 + non-labelled H3 (1:1 mole/mole), (I) lissamine rhodamine (2 mg/ml) was incubated with the Hela cells as described for incubation with the histone for 1 hour at 37°C. Timecourse studies (J-L) as in (A) but following 5 (J), 15 (K) or 30 (L) minutes of incubation. The amount of labelled-histone added as well as all other experimental conditions are as described in Materials and Methods.

molecules were detected unless cells were lysed with Triton. Indeed, in all subsequent experiments the amount of biotin detected in neutralized unlysed cells never exceeded 10% of the amount of biotinylated molecules found intracellularly. Only biotin (–histones) molecules estimated after neutralization of surface-bound biotin and treatment of cells with Triton were considered to be molecules present within the intracellular space. Very little accumulation of histones was observed in formaldehyde-fixed cells (Fig. 2A), indicating that the penetration process required a functional intact plasma membrane. Our quantitative studies confirmed the microscopic observations (Fig. 1B), showing accumulation within intact cells following incubation at 4°C (Fig. 2A). However, under these conditions, translocation into the cell nuclei was inhibited, as expected, and relatively higher amounts of histones were found in the cell cytoplasm. The total amount of histones (nuclei + cytoplasm) accumulated at 4°C was 25-30% less than at 37°C (Fig. 2A). In the presence of ×100 molar excess of unlabelled histones the relative amount of the histones in the cytosol was increased but a decrease of about 50% in the intranuclear histone content was observed, showing that the unlabelled molecules did not compete with the penetration of the labelled ones. Similar to the labelled histones, the externally added unlabelled histones accumulated within the Colo-205 cells and thus inhibited translocation of labelled histones into the cells' nuclei. It appears, however, that the externally added unlabelled histones caused very little inhibition of the penetration process itself, which is consistent with a non-saturable membrane penetration process, which is not an active transport. Intracellular accumulation of histones was also obtained following the incubation of the histones with ATP-depleted cells (Fig. 2A), providing further support to the notion that penetration was energy independent. In this case the relative amount of the intracellular histones was reduced in the nuclei and increased in the cytosol, indicating inhibition of nuclear import. However, no reduction in the total intracellular histone was detected.

Kinetics of histone accumulation in the cell

We have studied the kinetics of histone accumulation in the cell using our quantitative ELISA-based system (Fig. 2B). The same amount of histones accumulated in the cells both at 37°C and at 4°C, reaching saturation after 15 minutes of incubation at both temperatures. In the presence of sucrose (0.5 M), which is known to completely inhibit the endocytic pathway, only a reduction of 20% in the total amount of the intracellular histone was observed, and the kinetics remained almost identical. The control protein importin alpha (Gorlich et al., 1995) did not penetrate into Colo-205 cells (Fig. 2C), indicating that the penetration is a specific property of the histones.

Penetration of the individual histones into cell cytoplasm and nuclei

Following the above observations, we studied whether each of the four core histones was also able to penetrate intact cells,

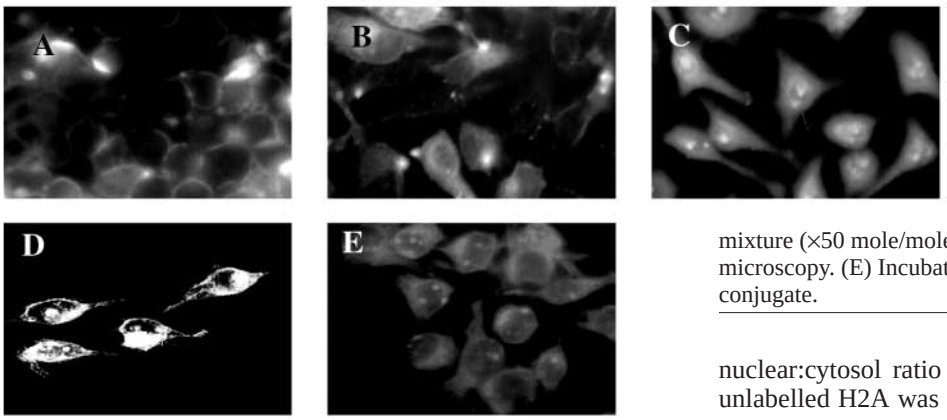


Fig. 5. Accumulation of externally added BSA-histone conjugates into HeLa cells. (A) HeLa cells were incubated for 1 hour in the presence of only rhodamine-labelled BSA. (B) Incubation of HeLa cells with Rho-BSA-histone mixture conjugate in the absence or in the presence (C) of excess unlabelled histone mixture (×50 mole/mole); (D) as in (B) but using confocal microscopy. (E) Incubation of HeLa cells with Rho-BSA-H2A conjugate.

and to what extent. This was tested using the quantitative assay system. All histones were able to penetrate into cells, in the order: histone mixture > H2A > H4 > H3 > H2B. The amount of the intracellular histone H2A was very close to that of the histone mixture, whereas the accumulation of histone H2B was significantly lower (Fig. 3A). This was confirmed by fluorescence microscopy: histone H2A readily accumulated within the cell cytosol and nuclei (Fig. 4A), whereas penetration of the H2B was low and mainly into the cytosol (Fig. 4B). The addition of labelled H2A to unlabelled H2B or of unlabelled H2A to labelled H2B increased the accumulation of the labelled histone (Fig. 3A, and see also Fig. 4C,D). The results were obtained when either unfixed cells were studied or by using the confocal microscope with fixed cells (not shown).

The extent of H3 accumulation was close to that of H4 but lower than that of the H2A or of the histone mixture (Fig. 3A and Fig. 4E,F). Most of the intracellular H3 accumulated within the cytoplasm, with very little – if any – in the intranuclear space (Fig. 4E). When a combination of the two proteins (H3 and H4) was used, the extent of their penetration was always higher than of each individual histone (Fig. 3 and compare Fig. 4E and 4F to 4G and 4H, respectively). Timecourse studies (Fig. 4J-L) confirmed our quantitative data (Fig. 2B), revealing that maximum accumulation of H2A was reached within 15-30 minutes of incubation. But no intracellular fluorescent staining was observed, even after 60 minutes incubation with lissamine rhodamine alone (Fig. 4I). Western blot analysis of the intracellular histones using HRP-strepavidin showed that after penetration, the histone molecules remained intact (not shown). Also, similar to the observations with the histone mixture, none of the endocytic pathway inhibitors had any effect on the penetration ability of the histones H2A and H4 (Fig. 3B).

The intracellular distribution of H2A was very similar to that observed with the histone mixture (compare Fig. 3C to Fig. 2A). In control, untreated cells the large majority of the intracellular H2A accumulated within the cell nuclei (Fig. 3C), whereas in ATP-depleted cells it was equally distributed between the nucleus and the cytosol. However, the total intracellular amount of the H2A in ATP-depleted cells was very close to that found in control untreated cells (Fig. 3C), indicating again that the penetration process is energy independent. Similarly, very little change was observed in the total amount of the intracellular H2A, although its nuclear:cytosol ratio was altered when ×50 molar excess of unlabelled H2A was added (Fig. 3C).

Histones are able to mediate cellular accumulation and nuclear import of covalently attached BSA

Molecules that have cell-penetration ability are of potential use as carriers of drugs into cells. To test whether histones could carry covalently attached molecules into cells we used histone-BSA constructs in which only the BSA was labelled. Therefore, the appearance of intracellular fluorescence or biotin-labelled molecules indicated the presence of intracellular BSA. The results in Figs 5-7 show that the histone mixture, as well as the histone H2A, were able to mediate the penetration of covalently attached BSA into intact cells. Penetration into the cells rather than absorption by the cell surface, as well as nuclear uptake of histone-BSA conjugate, was evident from the confocal microscopy studies (Fig. 5D) and from the quantitative assay system (Fig. 6). The addition of unlabelled histone mixture (×50 molar excess) to the labelled histone-BSA conjugates stimulated the penetration of the labelled conjugate as detected by the microscopic observations (Fig. 5C), as well as by the quantitative assay (Fig. 6). Labelled BSA molecules that were not conjugated to histones did not accumulate within cells (Fig. 5A and Fig. 6), proving the intactness of the cell plasma membrane as well as the penetration properties of the histone moiety. BSA bearing the NLS of the large SV40 T antigen (Adam and Gerace, 1991) failed to accumulate within the Colo-

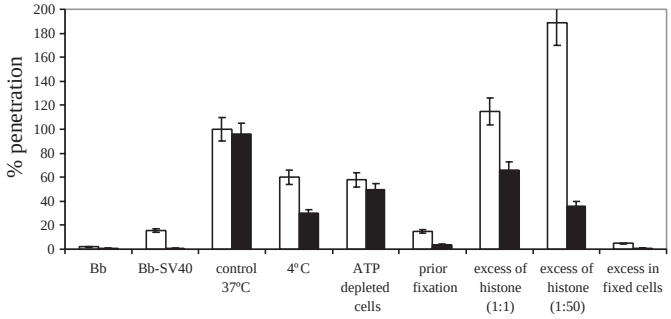


Fig. 6. Quantitative accumulation of externally added BSA-histone conjugates into Colo-205 cells. Bb-histone conjugates were prepared and their accumulation within the cell cytosol and nuclei was estimated as described in Materials and Methods. ATP depletion was performed as described in Table 1. □, nuclei; ■, cytosol. The amount of Bb-histone present in the nuclei of cells incubated in 37°C was considered as 100% and represents 6.3 nmol/mg protein; it was estimated to be about 28% of the total added Bb-histone. The ratio given are in mole:mole.

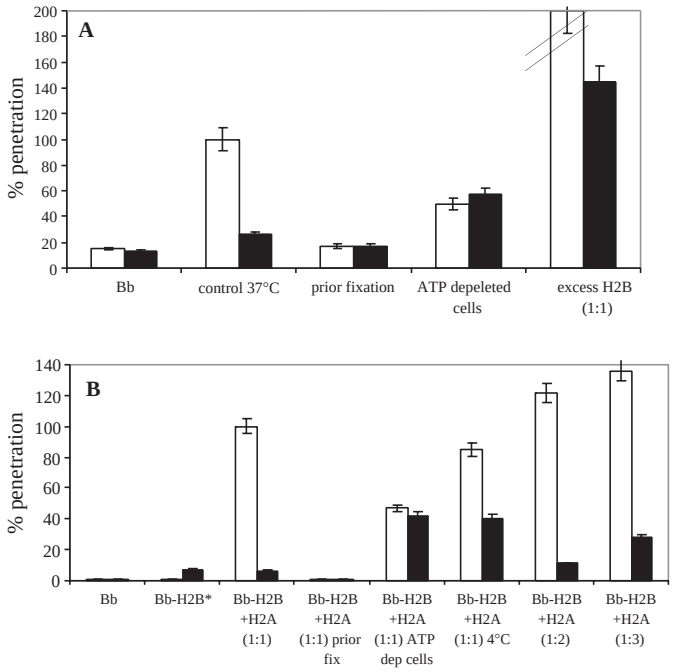


Fig. 7. Cellular and nuclear accumulation of Bb-histone conjugate within the Colo-205 cells – cytoplasm and nuclei. (A) The amount of Bb-H2A present in the nuclei of cells incubated in 37°C was considered to be 100% and represents 6.4 nmol/mg protein; it was estimated to be 58% of the total added Bb-H2A. (B) Bb-H2B conjugates. ATP depletion was performed as described in Table 1 and cells were fixed (prior fixation) as described in Fig. 2. 100% represents 6.2 nmol histone/mg protein and was estimated to be 62% of the total added Bb-H2B. *Bb-H2B incubated in 37°C, 4°C, ATP-depleted cells or after prior fixation gave the same results as biotinylated Bb-H2B alone. Biotinylated conjugates were prepared and their accumulation within the cell cytosol and nuclei was estimated as described in Materials and Methods. □, nuclei; ■, cytosol. The ratio given (in A and B) are in mole:mole.

205 cells (Fig. 6). Estimation of the intracellular amounts of the biotinylated BSA (Bb)-histone within cells incubated at 4°C or in ATP-depleted cells revealed about 40-50% reduction compared with control, untreated cells (Fig. 6). Probably, some of the added histones – the uptake of which was found to be ATP dependent – was taken into the cells by endocytosis. The same results were obtained when Bb-H2A conjugate was used (Fig. 7A). However, it appears that similar to what has been observed with the H2A itself, most of the Bb-H2A conjugates accumulated within the intranuclear space (Fig. 7A). Also, the addition of excess unlabelled H2B greatly stimulated the penetration of the labelled H2A (Fig. 7A). By contrast, Bb-H2B hardly penetrated into the recipient cells (Fig. 7B), but the addition of non-biotinylated H2A greatly stimulated its penetration, resulting in almost complete translocation of the intracellular Bb-H2B into the cell nuclei (Fig. 7B). Very little inhibition of penetration was observed following incubation of a mixture containing Bb-H2B and unlabelled H2A with cells incubated in the cold or with ATP-depleted cells (Fig. 7B).

Effect of the histone mixture on the transcription process

The results in Fig. 8 show that incubation of Colo-205 cells with the histone mixture stimulated the transcription process within the recipient cells. On the basis of the degree of [^{3}H]-uridine incorporation, it appears that about 30% increase was observed in total RNA synthesis following incubation with the histone mixture. A much higher degree of stimulation was observed in the total amount of the mRNA in cells incubated in the presence of histones compared with those incubated in their absence (Fig. 8).

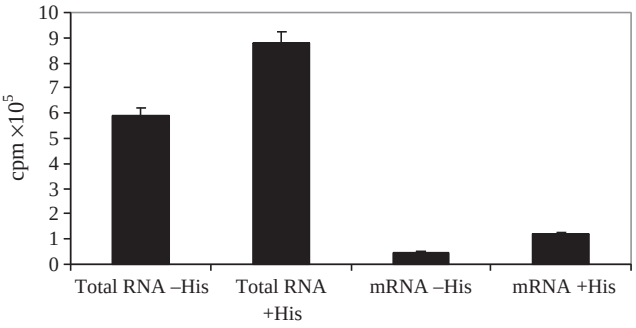


Fig. 8. Effect of the histone mixture on transcription process in Colo-205 cells. Colo-205 cells were incubated in the presence and absence of the histone mixture as well as with [^{3}H]-uridine as described in Materials and Methods. The degree of transcription was estimated by monitoring the amount of radioactivity in total RNA and in mRNA.

Discussion

Our results suggest that penetration of histones into tissue-cultured cells occurs by direct translocation through the cell plasma membrane and not by a typical endocytosis. Penetration of the histones occurred under various conditions that are known to inhibit endocytosis: it was observed at 4°C, was not competitively inhibited by endocytosis inhibitors or by the addition of molar excess of unlabelled histones and, under the experimental conditions used, was not saturable. Also, it was an energy-independent process, as it was observed in ATP-depleted cells. By contrast, internalization via endocytosis and intracellular membrane trafficking are blocked at low temperature (Kuismanen and Saraste, 1989). ATP was shown to be required for endocytosis, especially for clatherin-coated pits-mediated internalization (Schmid and Carter, 1990).

Our studies with various inhibitors of microtubules, microfilaments and the Golgi apparatus rule out the possibility that histones undergo endocytosis. The possible involvement of cell microtubules and microfilaments in the penetration of the histones, as well as in their trafficking into the nucleoplasm, was studied by the addition of colchicine, nocodazole and cytochalasin D. Colchicine is known to inhibit the function of microtubules, nocodazole was shown to block transport to late endosomes by the depolymerization of the microtubules, and cytochalasin D is a microfilament-disrupting drug. The involvement of the endoplasmic reticulum (ER)/Golgi in the intracellular trafficking of the histones, especially into the cell nuclei, was studied by the use of BFA, which disrupts the Golgi apparatus and inhibits classical vesicle-mediated secretion. All these inhibitors did not have any effect on the internalization of the histones as well as on the pattern of their intracellular accumulation. Also, the internalization of the histones was not a caveoloe-mediated endocytic or potocytotic processes

(Anderson et al., 1998; Grider and Vazquez, 1996), as nystatin, which is known to disrupt caveoloe formation, did not have any effect on the penetration process. The sucrose (0.5 M) treatment, which completely blocks internalization via the endocytic pathway, also did not have any effect. Moreover, even the combination of nystatin and sucrose did not inhibit the penetration process, completely ruling out the involvement of either clathrin- or caveoloe-mediated endocytosis. The fact that the various inhibitors were indeed active should be inferred from the experiments with LDL and LY, in which the various drugs and the metabolic energy inhibitors completely blocked LDL as well as LY internalization. LDL is taken into intact cells via receptor-mediated endocytosis, whereas LY enters the cells via pinocytosis. The view that the penetration of histone is not mediated by pinocytosis was also strengthened by our observations showing translocation into human lymphocytes, which do not support efficient pinocytosis (Wettey et al., 2002).

Further support for the claim that histones are able to directly translocate biological membranes was obtained from our recent experiments showing penetration of histones into phospholipid liposomes (J.R., E.H.-G., S. Rottem, A. Dagan, A.G. and A.L., unpublished).

The translocation of the histones observed in the present work exhibited very similar characteristics to that reported for the translocation of the HIV-1 Tat protein (Vives et al., 1994) or the peptide derived from it (Vives et al., 1997). The HIV-1 Tat protein and basic peptides bearing the Tat and the Rev ARM sequences have been reported to penetrate directly through the cell membrane (Futaki, 2002). However, during the preparation of this manuscript, a work re-evaluating the mechanism by which the CPPs, and especially the Tat ARM, are taken into cells has been published (Richard et al., 2003). It is suggested that, owing to their positive charges, the CPPs are absorbed to the cell surface, and even mild cell fixation causes their redistribution and leads to what may appear as cell penetration and translocation into the cell nucleus. In our studies, the intracellular accumulation of the biotinylated histones resulted neither from estimation of cell-membrane-absorbed molecules nor from redistribution of these molecules following cell fixation. First, our quantitative assay system necessitates the use of unfixed cells, and second, any cell-absorbed-biotinylated molecules were neutralized and washed, therefore practically removed from the assay system. Furthermore, to eliminate any artefacts due to the fixation procedure, unfixed cells were also examined by the fluorescent microscopy and the results obtained were identical to those observed with fixed cells. Similar results were obtained following the use of the confocal microscope, again strengthening the conclusion that the intracellular fluorescent staining was indeed due to molecules present within the cell cytoplasm and nucleoplasm.

Our results also show that the various histones differ in their ability to penetrate intact cells. H2A and H4 were the most effective, whereas H2B and H3 were less potent. Currently, experiments to study the penetration abilities of peptides bearing various domains derived from the individual histones are being conducted in our laboratory. Such experiments may help to identify protein domains involved the penetration process as well as further clarify its detailed mechanism. Cellular penetration, and especially nuclear import of the BSA-histone conjugates, was highly stimulated by the addition of excess histones. It is difficult to explain this phenomenon, but it is possible that the addition of excess histone promotes the formation of histone oligomers containing pairs of nonconjugated and conjugated histones, such as unconjugated H2A and conjugated H2B; similar hetero-oligomers may be formed between conjugated H3 and unconjugated H4, on the basis of the stimulation observed by H2A on the cellular penetration and nuclear import of H2B and similarly of H4 on H3.

The penetration of histone-BSA conjugates was distinguished by the same features that characterize the penetration of the histones, indicating that both the conjugates and the histones are taken into the cells by the same route. However, the extent of the conjugate penetration was slightly decreased in ATP-depleted cells and in the cold, suggesting that a small fraction of the conjugates were taken into the cells via the endocytic route.

Preliminary experiments in our laboratory also indicate that histones mediate, in addition to the BSA, the translocation of specific plasmids expressing the luciferase gene, which attach to the histone molecules. The utilization of such histone-DNA conjugates may open new possibilities for in vivo use of histones as a gene delivery system.

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