

# Dynamic Behavior of Histone H1 Microinjected into HeLa Cells

Lily H. Wu,\* LeRoy Kuehl,\* and Martin Rechsteiner\*‡

Departments of \*Biochemistry and ‡Biology, University of Utah, Salt Lake City, Utah 84112

**Abstract.** Histone H1 was purified from bovine thymus and radiolabeled with tritium by reductive methylation or with  $^{125}\text{I}$  using chloramine-T. Red blood cell-mediated microinjection was then used to introduce the labeled H1 molecules into HeLa cells synchronized in S phase. The injected H1 molecules rapidly entered HeLa nuclei, and a number of tests indicate that their association with chromatin was equivalent to that of endogenous histone H1. The injected molecules copurified with HeLa cell nucleosomes, exhibited a half-life of  $\sim 100$  h, and were hyperphosphorylated at mitosis. When injected HeLa cells were fused with mouse 3T3 fibroblasts  $<10\%$  of the labeled H1 molecules migrated to mouse nuclei

during the next 48 h. Thus, the intracellular behavior of histone H1 differs markedly from that of high mobility group proteins 1 and 2 (HMG1 and HMG2), which rapidly equilibrate between human and mouse nuclei after heterokaryon formation (Rechsteiner, M., and L. Kuehl, 1979, *Cell*, 16:901-908; Wu, L., M. Rechsteiner, and L. Kuehl, 1981, *J. Cell Biol.*, 91: 488-496). Despite their slow rate of migration between nuclei, the injected H1 molecules were evenly distributed on mouse and human genomes soon after mitosis of HeLa-3T3 heterokaryons. These results suggest that although most histone H1 molecules are stably associated with interphase chromatin, they undergo extensive redistribution after mitosis.

IT is generally accepted that histones are arranged as nucleosomal arrays consisting of two molecules each of histones H2A, H2B, H3, and H4, around which are wrapped  $\sim 140$  base pairs (bp) of DNA. There is considerable support for this model: SDS polyacrylamide gel analysis shows the four core histones to be present in roughly equal amounts; gel filtration and cross-linking studies indicate specific histone interactions; limited nuclease digestion generates discrete particles containing the four core histones and 145 bp of DNA, electron microscopic observations show repeating knoblike structures along histone H1-depleted chromatin fibers; and x-ray diffraction patterns confirm the basic octamer structure (13, 19, 24, 29; see reference 15 for review).

There is also wide agreement that histone H1 serves to coil the nucleosomal thread into higher order, more compact structures (28, 34). The exact arrangement of nucleosomes in the thicker fibers produced by histone H1 remains an area of active study, and evidence has been presented for solenoid (7, 23, 40), superbead (32, 44), or zig-zag ribbon arrangements (41, 42; see reference 6 for review). Several recent observations indicate that histone H1 binding to chromatin can have important consequences for transcription. *Xenopus* 5S genes are protected from histone H1-mediated repression by the presence of active transcription complexes, and prior H1 binding prevents formation of such stable transcription complexes (31). Similarly, fractionation of avian red blood cell

(RBC)<sup>1</sup> chromatin produces discrete supranucleosomal particles enriched in inactive gene sequences, and it has been suggested that histone H1 maintains the structure of these particles (37).

Although traditional microscopic and biochemical approaches have provided valuable information about chromatin, dynamic aspects of its structure are not easily studied using these methods. The stability of chromatin structure is of interest since it has been proposed that the arrangement of nucleosomes may be a source of epigenetic information during development (39). In its simplest form, the hypothesis that chromatin structure, in addition to DNA sequence, is responsible for cell determination, requires little histone exchange. Furthermore, the observations in references 31 and 37 are most easily understood if the association of histone H1 to chromatin is relatively stable.

Microinjection followed by cell-cell fusion permits assessment of dynamic aspects of chromatin structure. We previously used this approach to examine the intracellular behavior of high mobility group (HMG) proteins as well as the arginine-rich histones, H3 and H4 (22, 27). In the present studies, we have injected radiolabeled histone H1 into HeLa cells and then fused the injected HeLa cells with mouse fibroblasts to determine whether histone H1 exchanges between nuclei. We observed little intranuclear exchange, but

1. *Abbreviations used in this paper:* FCS, fetal calf serum; FUDR, 2'-deoxy-5-fluorouridine; HMG, high mobility group; RBC, red blood cell.

histone H1 was evenly distributed on mouse and human chromatin once the two sets of chromosomes were incorporated within a common nuclear envelope.

## Materials and Methods

### Purification and Labeling of Histone H1

Calf thymus was obtained from Pel-Freez Biologicals (Rogers, AR) and stored at  $-70^{\circ}\text{C}$  until used. A 50-g portion of the frozen tissue was homogenized in 250 ml of 0.14 M NaCl/0.03 M NaHSO<sub>3</sub> in a blender (Waring Products Div., Dynamics Corp. of America, New Hartford, CT), and the chromatin was sedimented by centrifuging the homogenate for 10 min at 4,000 g. The chromatin pellets were washed with four 250-ml portions of homogenization solution, then extracted with 100 ml of 0.2 M H<sub>2</sub>SO<sub>4</sub>. The resulting solution of whole histones was dialyzed against water and lyophilized. Purified H1 was obtained by chromatography of the whole histones on Biogel P-60 as described by van der Westhuyzen et al. (36). The H1 fractions were pooled, dialyzed against distilled water at 4°C, and lyophilized.

### Polyacrylamide Gel Electrophoresis

For electrophoretic analysis of histone H1 the acid-urea system of Panyim and Chalkley (25) was used. Samples were applied in 6-mm lanes to 15-cm slab gels. A urea concentration of 2.5 M was used and electrophoresis was for 5 h at 250 V. After electrophoresis, the gel was stained for 1 h in 50% methanol and 7% acetic acid containing 0.5% Coomassie Blue, and then destained in 7.5% acetic acid/22.5% methanol. Detection of tritiated H1 was accomplished by treating the stained gel with 1 M salicylic acid for 30 min, then drying the gel and exposing it to Kodak XR-5 x-ray film for various lengths of time.

### <sup>3</sup>H Labeling of H1 by Reductive Methylation

[<sup>3</sup>H]Sodium borohydride (16–20 Ci/mmol) was purchased from Research Products International Corp. (Mt. Prospect, IL); the crystalline product was stored in vacuum-sealed vials at  $-20^{\circ}\text{C}$ . Histone H1 was reductively methylated by modification of the procedure by Tack et al. (33). All reactions were carried out in a well ventilated fume hood. H1 was dissolved in 0.2 M borate buffer (pH 8.9) at a concentration of 2.5 mg/ml and kept on ice for 1 h. The vial containing crystalline [<sup>3</sup>H]NaBH<sub>4</sub> was kept on ice before opening, and 200  $\mu\text{l}$  of 0.01 N NaOH was used to dissolve the [<sup>3</sup>H]NaBH<sub>4</sub> just before use. Formaldehyde reagent was prepared by diluting 37% (wt/wt) formaldehyde stock solution to 0.1 M with water, and 20  $\mu\text{l}$  was added to 0.1 ml of 2.5 mg/ml H1 solution. After 5 min on ice, tritiation was accomplished by adding 40  $\mu\text{l}$  of <sup>3</sup>H-NaBH<sub>4</sub>, mixing the solution well, and allowing it to stand another 10 min. Unbound radioactive material was removed by passing the reaction mixture over a 10-ml Sephadex G-25 column (Pharmacia Fine Chemicals Inc., Piscataway, NJ) equilibrated with 10 mM Tris-HCl buffer (pH 7.4). Fractions of 10 drops were collected and 5  $\mu\text{l}$  from each fraction was counted in a liquid scintillation counter. Fractions with activities  $>10^9$  cpm/ml were used for loading. A typical specific activity was  $1.4 \times 10^7$  dpm/ $\mu\text{g}$  corresponding to about three methyl groups per H1 molecule. In some experiments, samples containing both <sup>14</sup>C and <sup>3</sup>H were analyzed by liquid scintillation spectroscopy; correction was made for spillover of <sup>14</sup>C into the <sup>3</sup>H channel.

### Iodination of Histone H1

H1 was iodinated by a modified lactoperoxidase method as described earlier (27). Free <sup>125</sup>I was removed by filtration on a column (Sephadex G-25; Pharmacia Fine Chemicals, Inc.) equilibrated with 10 mM Tris (pH 7.4) containing 1% unlabeled BSA. <sup>125</sup>I was measured using a gamma spectrometer (Beckman Instruments Inc., Fullerton, CA).

### Cell Growth and Cell Synchronization

The heteroploid human cell line, D98/AH2, which lacks hypoxanthine-guanine phosphoribosyl transferase, was obtained from the American Type Culture Collection, Rockville, MD. The thymidine kinase-negative mouse line 3T3-4E was obtained from Dr. H. Coon (National Institutes of Health). For cell synchronization D98/AH2 cells at approximately one-third con-

fluence were rinsed with F12 medium lacking thymidine (F12-T) and grown in F12-T medium containing 1  $\mu\text{g}/\text{ml}$  2'-deoxy-5-fluorouridine (FUDR) and uridine for 36 h. The FUDR block was released by two rinses with warm ( $37^{\circ}\text{C}$ ) F12 medium containing 10% fetal calf serum (FCS) (22). 3 h after releasing the cells from the FUDR block, they were harvested by trypsinization and fused with loaded RBCs. At this stage  $\sim 90\%$  of HeLa cells were synthesizing DNA as measured by autoradiography after exposing the cells to [<sup>3</sup>H]thymidine. For experiments involving injection of <sup>3</sup>H-histone H1 into 3T3, the mouse cells were synchronized by growth to confluence and then replacement of the media with F12 containing 0.1% FCS. After 48 h, the cells were harvested by trypsinization and replated at 50% confluence in F12 containing 10% FCS. More than 80% of 3T3 cells were synthesizing DNA 12 h later. Cells at this stage were harvested by trypsinization and fused with human RBCs loaded with <sup>3</sup>H-histone H1.

### Loading, Microinjection, and Cell Fusions

The source of human RBCs and the general procedures for loading H1 into RBCs have been described in detail (26). For microinjections, the fusion mixture consists of  $1\text{--}1.5 \times 10^7$  synchronized HeLa or 3T3 cells,  $7.5 \times 10^8$  loaded RBCs, and 360 hemagglutinating units of ultraviolet-inactivated Sendai virus in a final volume of 0.5 ml of 0.15 M NaCl, 20 mM Tris (pH 7.4) containing 2 mM MnCl<sub>2</sub>, and 2 mM glucose. The components were added in the order listed and kept on ice for 10 min to ensure agglutination, then incubated at  $37^{\circ}\text{C}$  for 20 min with shaking. The fused cells were washed three times with F12/10% FCS and plated in 75-cm<sup>2</sup> flasks. About 4 h after plating, the attached cells were rinsed well with F12 medium to remove unfused RBCs. Cells were incubated overnight in fresh medium, rinsed again to remove residual RBCs, then trypsinized to remove any H1 bound to the cell surface. The injected HeLa were replated in 25-cm<sup>2</sup> flasks at  $0.5 \times 10^6$  cells per flask for stability studies, or at  $1.5 \times 10^6$  cells per flask for subsequent fusion with 3T3 mouse cells to form heterokaryons.

### Polyethylene Glycol Fusion of D98 and 3T3 Cells

3T3 cells ( $1.5 \times 10^6$ ) were plated onto 25-cm<sup>2</sup> flasks containing  $1.5 \times 10^6$  H1-injected HeLa cells. When the 3T3 cells were well attached ( $\sim 6$  h), the flasks were rinsed three times with warm serum-free F12 medium and drained. The cells were then treated with 3 ml of warm 50% polyethylene glycol (6,000) in F12 medium for 1.5 min, followed by three washes with 5 ml of serum-free F12 medium. After incubation in 5 ml of warm F12 containing 10% FCS for 3 h, the cells were trypsinized and replated into large petri dishes containing glass slides. Medium selective for hybrid cells, hypoxanthine/aminopterin/thymidine (HAT) medium, was prepared from F12 medium supplemented with additional thymidine and hypoxanthine (final concentration of 10  $\mu\text{g}/\text{ml}$ ) and with 0.2  $\mu\text{g}/\text{ml}$  aminopterin.

### Autoradiography

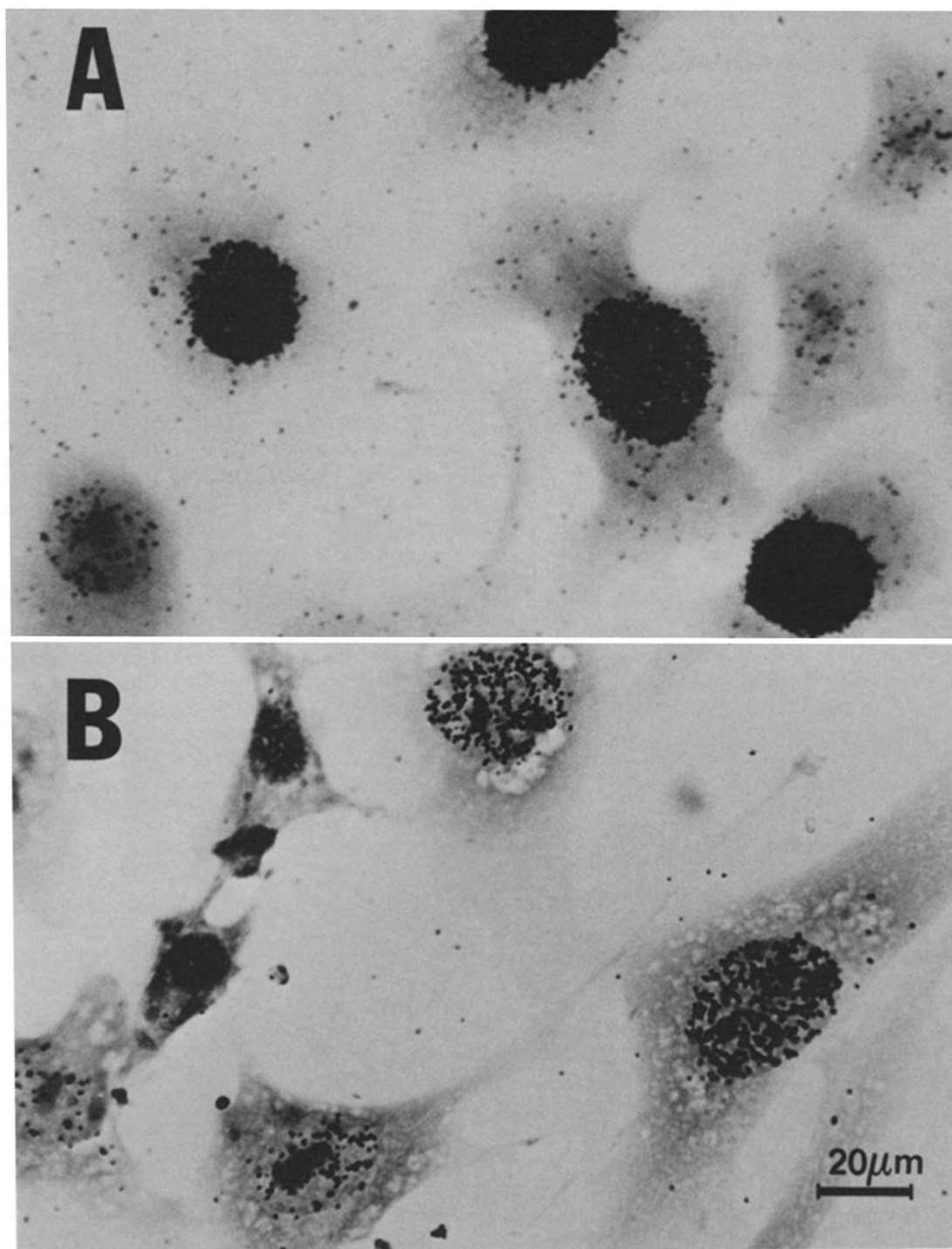
Glass slides containing hybrid cells were rinsed three times in PBS and fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h, rinsed in distilled water, and air-dried. Autoradiographic and microscopic procedures have been described (22).

### Collection of Mitotic HeLa Cells

H1-injected HeLa cells were plated, trypsinized 20 h later, replated into 75-cm<sup>2</sup> flasks, and then treated for 13 h with 0.5  $\mu\text{g}/\text{ml}$  of colcemid in F12 medium. Mitotic cells were collected by shake-off, and both mitotic cells and interphase cells, which remained attached to the flasks, were washed with PBS. Cell pellets were extracted with a minimum amount of 0.2 M H<sub>2</sub>SO<sub>4</sub>/50 mM NaHSO<sub>3</sub>, and the acid-soluble proteins were analyzed on 20% acid-urea polyacrylamide gel to determine the proportion of <sup>3</sup>H-H1 and phosphorylated <sup>3</sup>H-H1.

### Sedimentation of Chromatin Fragments

1 d after being injected with H1,  $2 \times 10^7$  HeLa cells were harvested by trypsinization, washed twice with 5 ml ice-cold culture medium, twice with PBS, and once with cold 10 mM KCl, 3 mM MgCl<sub>2</sub>, and 10 mM Tris (pH 7.5). The cells were then suspended in 3 ml of the same solution and after 5 min disrupted by 50 strokes with the tight-fitting pestle in a Dounce homogenizer. The nuclei were sedimented, washed twice with 5 ml of 0.25 M sucrose, 0.1 mM CaCl<sub>2</sub>, 5 mM Tris-HCl (pH 7.5) containing 0.5 mM phenylmethylsulfonyl fluoride, and suspended in 0.5 ml of the same



**Figure 1.** Intracellular localization of  $^3\text{H}$ -histone H1 microinjected into cultured mammalian cells. (A) Autoradiogram of human HeLa cells fused to  $^3\text{H}$ -histone H1-loaded human RBCs. HeLa cells were fused to  $^3\text{H}$ -histone H1 loaded RBCs, plated overnight, trypsinized, and replated before fixation in 3% glutaraldehyde. Autoradiography was performed as described in Materials and Methods and the slides were developed after 14 d. (B) Autoradiogram of mouse 3T3 cells fused to  $^3\text{H}$ -histone H1 loaded human RBCs. 3T3 cells were fused to  $^3\text{H}$ -histone-loaded RBCs and treated as described for HeLa cells except that autoradiographic exposure was 10 d.

solution containing 1 mM  $\text{CaCl}_2$  and 320 U of micrococcal nuclease (Worthington Biomedical Corp., Freehold, NJ). After a 10-min incubation at  $4^\circ\text{C}$  the nuclei were sedimented at 2,000 g for 3 min, the supernatant was removed, and the nuclei were resuspended by vortex mixing in 0.5 ml of 1 mM EDTA (pH 7.0). After 20 min, the nuclear residue was sedimented

at 5,000 g for 10 min and the supernatant fraction was analyzed on 10–30% sucrose gradients in 1 mM sodium phosphate (pH 6.8), 0.2 mM sodium EDTA, and 5 mM NaCl. Samples were spun for 10 h at 24,000 rpm in a rotor (model SW39; Beckman Instruments Inc.), and the gradients collected drop-wise. Gradients containing chromatin fragments from  $10^8$  uninjected

nuclei were sedimented under identical conditions, and individual fractions were analyzed on agarose gels to determine the approximate number of nucleosomes in the fragments at various positions on the gradients.

## Results

### *Comparison of Injected and Endogenous H1 Molecules*

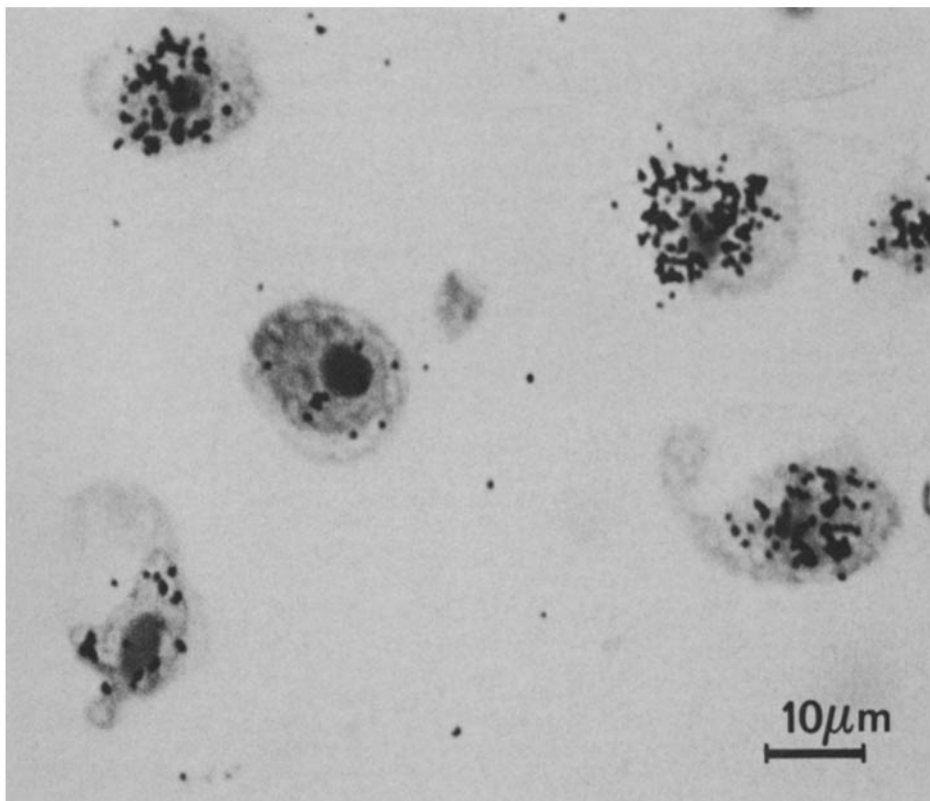
Despite their high positive charge, histone H1 molecules can be efficiently loaded into human RBCs during osmotic lysis. At low concentrations (100  $\mu\text{g/ml}$ ), 40% of the added H1 molecules were routinely associated with loaded RBCs. Trypsin treatment of intact, loaded RBCs solubilized <20% of the RBC-associated radioactivity, whereas 70% or more was released upon freeze-thaw lysis. Thus, individual RBCs can be loaded with at least  $2 \times 10^5$  histone  $^3\text{H}$ -H1 molecules, an amount small in comparison with the endogenous pool, but sufficient to trace the intracellular location of this protein after its injection into HeLa cells.

In all experiments except that in which modification of histone H1 at mitosis was studied, we injected histone H1 molecules labeled with  $^3\text{H}$  by reductive methylation or  $^{125}\text{I}$  by chloramine-T oxidation. Equivalent results were obtained with either labeled molecule, but only those experiments using  $^3\text{H}$ -histone H1 are reported. When histone H1-loaded RBCs were fused with human HeLa or mouse 3T3 cells synchronized in S phase, the injected H1 molecules rapidly associated with the nuclei of the recipient cells (Fig. 1). Analysis of thin sections through injected HeLa cells confirmed the intranuclear localization of injected H1 molecules (Fig. 2), and quantitation of grain density over nucleus and cytoplasm indicated that >95% of the injected H1 molecules were intranuclear.

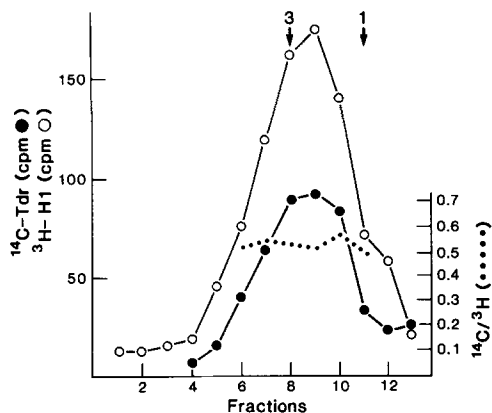
A number of experiments were undertaken to determine whether the injected H1 molecules were properly associated with the newly assembled HeLa cell chromatin. HeLa cells synchronized in S phase were injected with  $^3\text{H}$ -histone H1 and plated. After overnight incubation the cells were collected by trypsinization, disrupted by Dounce homogenization, and a nuclear fraction was prepared. Under these conditions, 94% of the labeled H1 molecules were found in the nuclear pellet, compared with 93% of the radioactivity from  $^3\text{H}$ -thymidine-labeled HeLa cells similarly fractionated. When isolated nuclei containing  $^3\text{H}$ -histone H1 were extracted with increasing concentrations of NaCl, trace amounts of the injected molecule were extracted with 0.2 M NaCl; 76% was extracted at 0.4 M NaCl and another 12% at 0.6 M salt. The two higher NaCl concentrations have been found to extract endogenous histone H1 from a variety of cells, so we conclude that the injected H1 molecules display binding properties expected for endogenous H1.

Evidence that the injected H1 molecules were associated with nucleosomes was obtained by sucrose gradient analysis of nuclease-treated chromatin. Synchronized HeLa cells were labeled with [ $^{14}\text{C}$ ]thymidine for 2 d, grown an additional 2 d, then treated with FUdR for 35 h and injected with  $^3\text{H}$ -histone H1. The next day, nuclei were isolated and treated with micrococcal nuclease on ice for 10 min at  $0^\circ\text{C}$ . Chromatin fragments released by nuclease treatment were then analyzed on 10–30% sucrose density gradients, and injected H1 molecules were found to be associated with nucleosomes or larger chromatin fragments (Fig. 3).

Intracellular stability was also used to compare injected H1 molecules with their endogenous counterparts. In these experiments  $^3\text{H}$ - or  $^{125}\text{I}$ -histone H1 was injected into synchronized

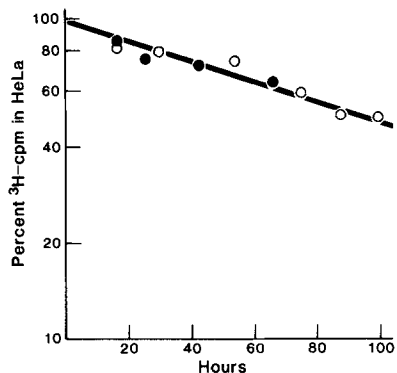


*Figure 2.* Autoradiogram of thin sections through HeLa cells injected with  $^3\text{H}$ -histone H1. The cells shown were fused to  $^3\text{H}$ -histone H1 loaded RBCs, plated overnight, collected by trypsinization, fixed, embedded, and sectioned. The sections were coated with emulsion and exposed for 6 d.



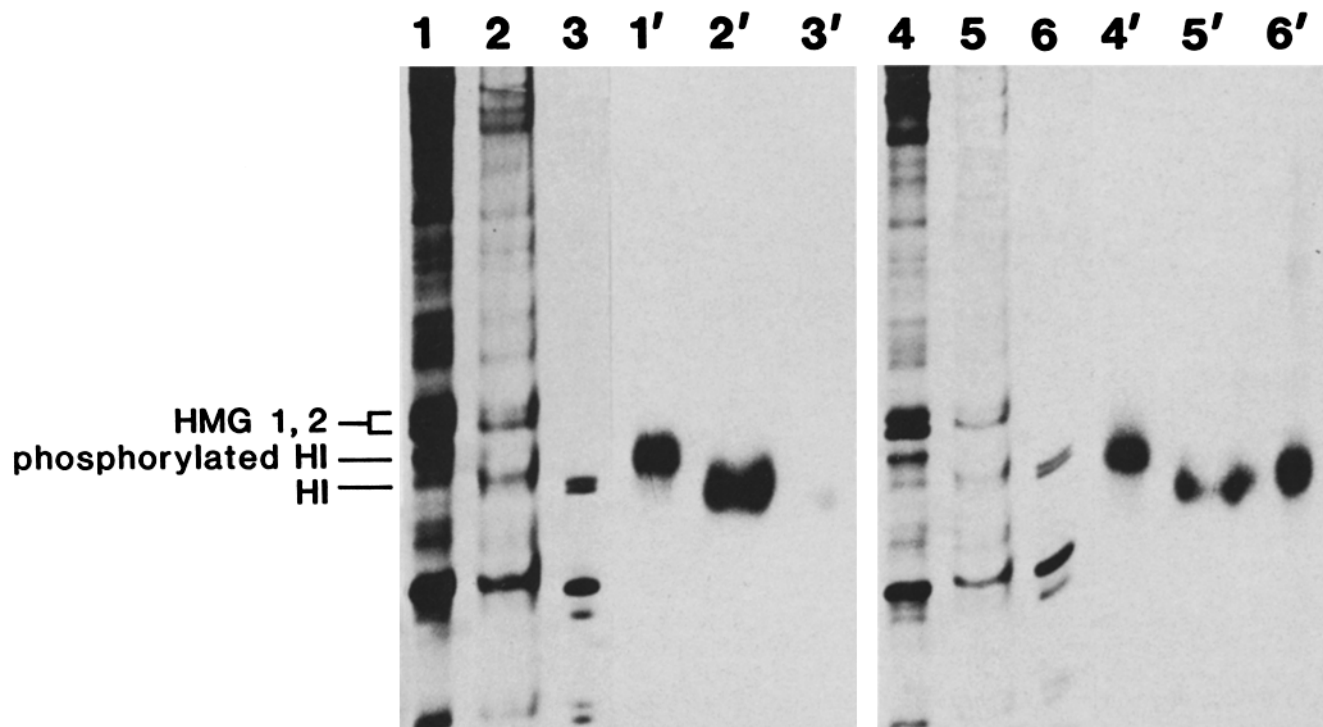
**Figure 3.** Sedimentation of chromatin fragments from injected HeLa cells. Nuclei prepared from [<sup>14</sup>C]thymidine-labeled HeLa cells that had been injected with <sup>3</sup>H-H1, were digested with micrococcal nuclease. The EDTA supernatant fraction was sedimented on a 10–30% sucrose gradient as described in Materials and Methods. The distributions of <sup>3</sup>H and <sup>14</sup>C in the gradients were measured by liquid scintillation spectroscopy. Arrows 1 and 3, positions in the gradients where mononucleosomes and trinucleosomes were predominate species, as determined by agarose gel electrophoresis of samples from equivalent gradients.

HeLa cells, and the cells were plated into numerous T-flasks. The stability of the injected molecules was determined both by TCA precipitation of the culture medium and by solubilization of the cellular monolayer in 1% SDS. Both methods of analysis revealed the half-lives of injected histone H1 molecules to be 90–110 h (see Fig. 4).

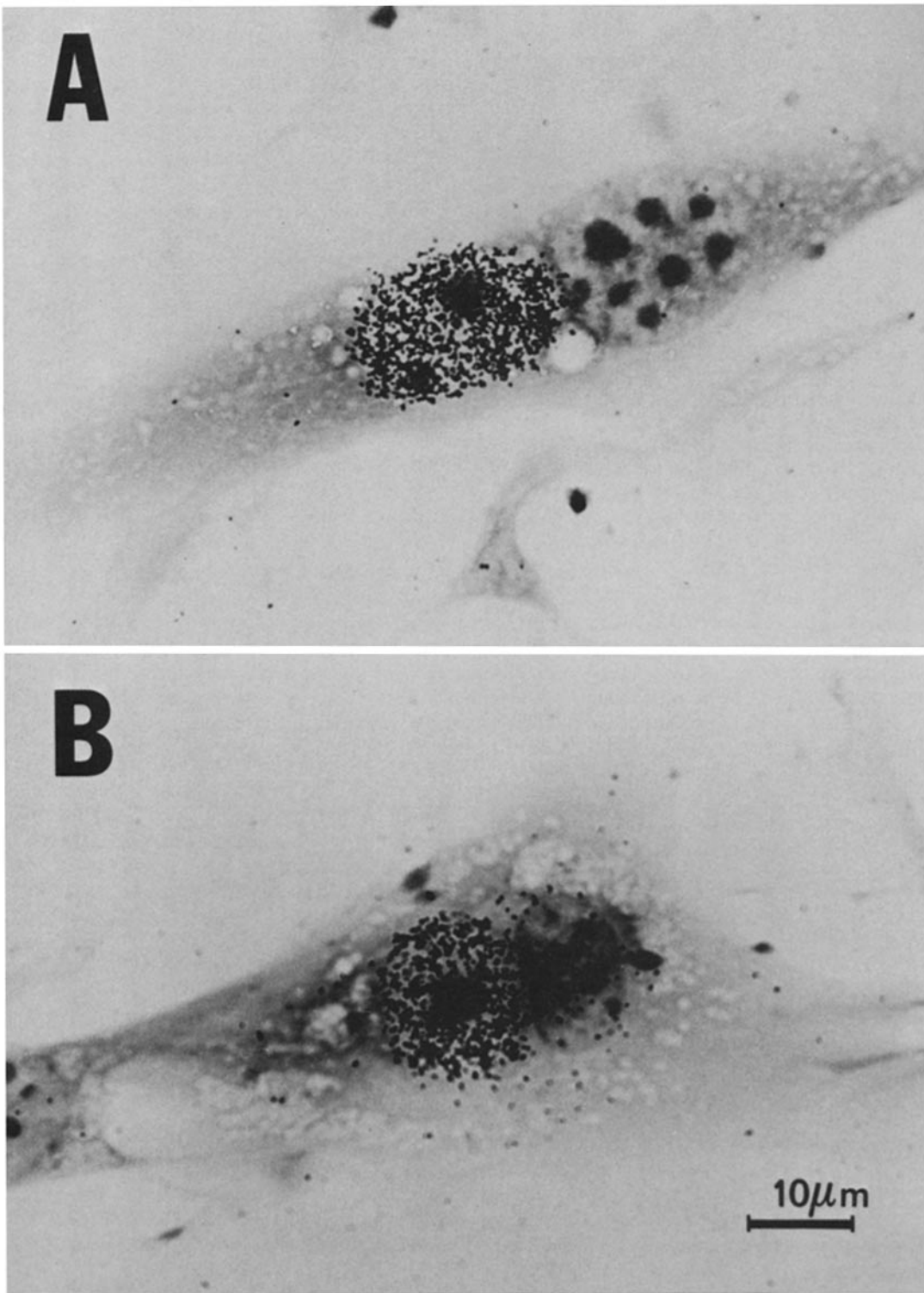


**Figure 4.** Intracellular stability of <sup>3</sup>H-histone H1 after injection into HeLa cells. RBCs loaded with <sup>3</sup>H-histone H1 were fused to HeLa cells, and the HeLa cells were then plated in 25-cm<sup>2</sup> T-flasks. At various times after fusion, cells from two flasks were dissolved in 1% SDS. The amount of <sup>3</sup>H-histone remaining within the cells was then determined (●). Alternatively, injected HeLa cells were plated into 75-cm<sup>2</sup> flasks and the degradation rate of <sup>3</sup>H-H1 was determined from the rate of release of TCA-soluble <sup>3</sup>H to the medium (○).

Histone H1 is hyperphosphorylated at mitosis (1, 8), and this provided another test for determining whether the injected histone H1 molecules behaved like their endogenous counterparts. Synchronized HeLa cells were injected with <sup>3</sup>H-histone H1 and plated. The next day the injected cell populations were placed in 0.05 μg/ml colcemid for 13 h, and metaphase cells were collected by mitotic shake-off. The mobility of injected H1 molecules was then determined for in-



**Figure 5.** Acid-urea polyacrylamide gel electrophoresis of <sup>3</sup>H-histone H1 recovered from HeLa cells. Lanes 1–3 are from a Coomassie Blue-stained gel. Acid-soluble proteins from mitotic HeLa cells (lane 1); acid-soluble proteins from interphase cells (lane 2); histone standards (lane 3). Lanes 1'–3', fluorograms of the same gel region after 2 mo exposure at –70°C. Note that injected <sup>3</sup>H-histone H1 migrates with the hyperphosphorylated mitotic H1 in lane 1' and interphase H1 in lane 2'. Lanes 4–6 are equivalent to lanes 1–3' except that samples from a separate experiment were analyzed.



*Figure 6.* Autoradiograms of HeLa-3T3 heterokaryons. (A) HeLa cells were grown in medium containing 0.1  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]thymidine for 24 h and fused to 3T3 cells using polyethylene glycol. Cells were fixed 24 h after fusion and autoradiograms were prepared. (B) HeLa cells were injected with  $^3\text{H}$ -histone H1 and fused to 3T3 cells as described in Materials and Methods. Cells were fixed 24 h after fusion and autoradiograms were prepared. Note the absence of autoradiographic grains over 3T3 nuclei in A, indicating that DNA or DNA digestion products did not migrate between nuclei; B shows that very little histone H1 has migrated from human to mouse nuclei in the 24-h period after fusion.

terphase and metaphase cells by electrophoresis on acid-urea gels. It is evident from the fluorogram in Fig. 5 that injected  $^3\text{H}$ -histone H1 extracted from mitotic cells migrates with the hyperphosphorylated histone H1 characteristic of metaphase cells, whereas that extracted from interphase cells comigrates with interphase histone H1.

### *Exchangeability of Histone H1*

The experiments presented above demonstrate that injected  $^3\text{H}$ -histone H1 exhibits characteristics that match those of the endogenous H1 molecules. Injected  $^3\text{H}$ -histone H1 appears, therefore, to be a valid probe for the behavior of endogenous histone H1. The intent of this study was to assess the exchangeability of histone H1, and this was accomplished by fusing HeLa cells injected with  $^3\text{H}$ -histone H1 to 3T3-4E mouse fibroblasts and determining the intracellular location of the injected molecules.

HeLa cells in S phase were injected with  $^3\text{H}$ -histone H1 and fused with 3T3-4E cells on the next day. The fused cell population was dispersed onto glass slides, and samples were fixed at intervals over the next 72 h. Autoradiographic analysis of the intracellular distribution of  $^3\text{H}$ -H1 in the resulting heterokaryons (Fig. 6) revealed extremely slow exchange of  $^3\text{H}$ -H1 from HeLa to 3T3-4E nuclei. Grain counts over the nuclei in heterokaryons fixed 1 d after fusion showed that 5.6% of the histone H1 had migrated to 3T3-4E nuclei. This value increased to 7.5% by 48 h. The low levels of exchange cannot result from rapid proteolysis of histone H1 molecules that leave the HeLa nucleus since there was little degradation of  $^3\text{H}$ -histone H1 after fusion. Nor can the apparent low level of exchange be explained by rapid internuclear equilibration with a marked preference of tritiated histone H1 for HeLa chromatin. Two results ruled out this possibility:  $^3\text{H}$ -histone H1 did not rapidly migrate between nuclei in HeLa-HeLa homokaryons, and  $^3\text{H}$ -histone H1 did not migrate from 3T3-4E nuclei to HeLa nuclei when the injection protocol was reversed (data not shown). Thus, unlike the nonhistone proteins, HMG1, HMG2, and HMG17, injected  $^3\text{H}$ -histone H1 did not rapidly equilibrate between nuclei within heterokaryons.

HeLa-3T3 heterokaryons give rise to hybrid cell pairs in which human and mouse chromosome sets occupy distinct sectors within the nucleus (22). This spatial segregation permitted us to determine whether  $^3\text{H}$ -H1 molecules would exchange from HeLa to mouse chromosomes when both were contained within a common nuclear envelope. About 40 hybrid pairs were examined in three separate experiments, and in contrast to the slow internuclear exchange observed for heterokaryons,  $^3\text{H}$ -histone H1 was always uniformly distributed among human and mouse chromosomes in newly formed hybrid cells (Fig. 7).

### *Discussion*

We have introduced radiolabeled histone H1 into HeLa cells in an attempt to determine dynamic aspects of chromatin structure. Central to our approach is the assumption that injected histone H1 molecules mimic the behavior of endogenous H1, and a number of results support this supposition.  $^3\text{H}$ -labeled histone H1 rapidly accumulated in nuclei after

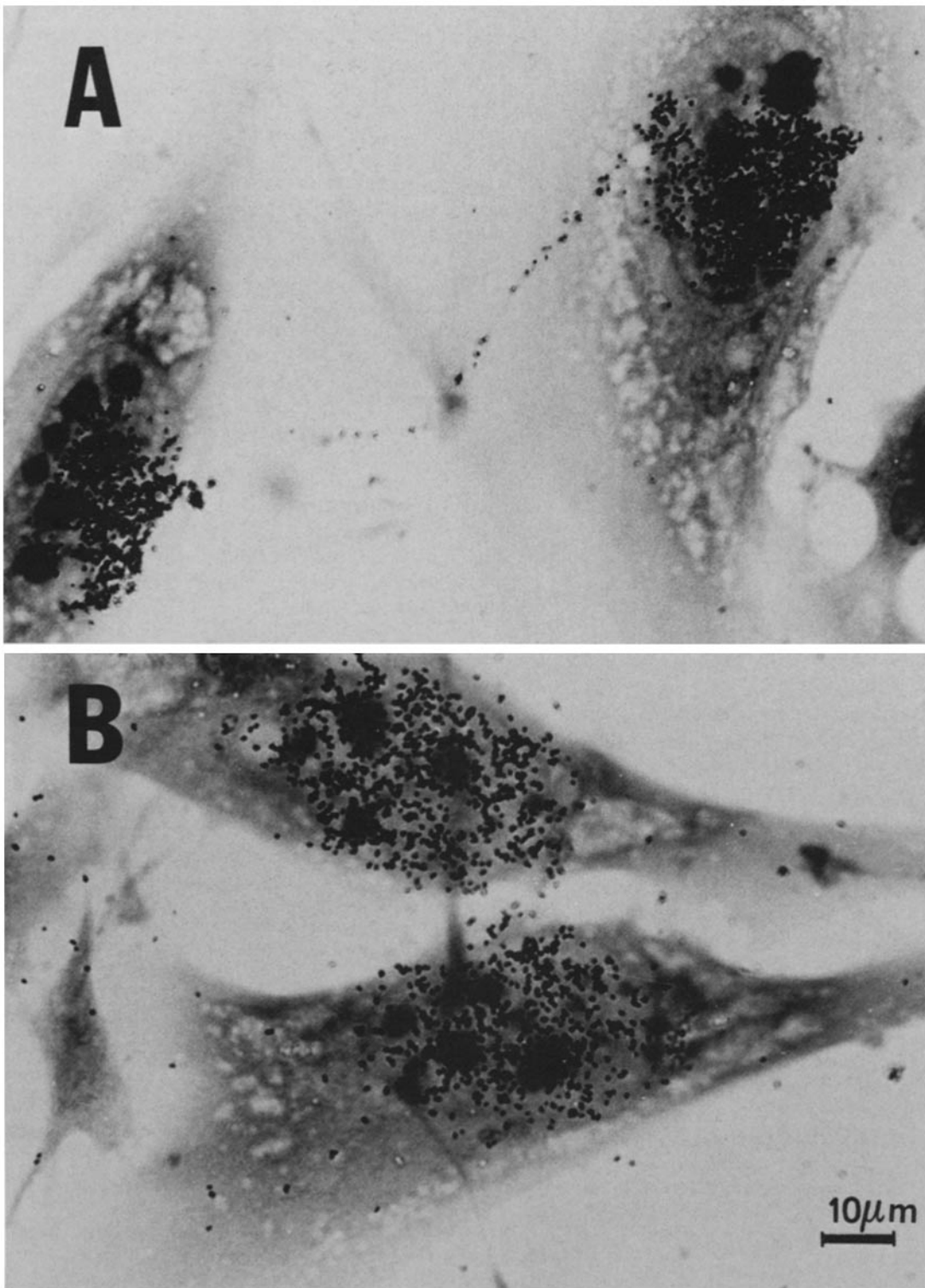
its injection into S phase HeLa or 3T3 cells (Figs. 1 and 2). The injected  $^3\text{H}$ -histone H1 copurified with HeLa nuclei and remained associated with them at NaCl concentrations  $<0.4$  M. Moreover, sucrose density gradient centrifugation revealed a constant ratio of  $^3\text{H}$ -histone H1 to [ $^{14}\text{C}$ ]thymidine-labeled DNA in chromatin fragments released by nuclease digestion, indicating that injected H1 molecules had become associated with nucleosomes (Fig. 3). Also,  $^3\text{H}$ -histone H1 exhibited a half-life of 110 h (Fig. 4), in good agreement with several *in vivo* labeling studies (5, 11, 30). Finally, injected histone H1 molecules were hyperphosphorylated at mitosis like endogenous H1 molecules (Fig. 5). These specific tests for the fidelity of injected H1, similar demonstrations of expected behavior for HMG proteins in previous microinjection studies (43), and the observation that various derivatized cytoskeletal proteins can assemble into actin filaments or microtubules (9, 18) indicate that proteins labeled at a few residues can retain a high degree of native structure. Consequently, we believe that injected  $^3\text{H}$ -histone H1 is a valid probe for the behavior of its endogenous counterpart.

Earlier injection studies using the high mobility group chromosomal proteins, HMG1, HMG2, or HMG17, showed that they rapidly localized in HeLa nuclei. When injected cells were fused to uninjected cells, all three proteins fully equilibrated between nuclei within 12 h (27, 43; for HMG17, unpublished observations). In contrast, microinjected  $^3\text{H}$ -histone H1 did not shuttle between nuclei in heterokaryons (Fig. 6). Less than 10% of the  $^3\text{H}$ -histone H1 molecules originally within HeLa nuclei had transferred to 3T3 nuclei even 2 d after heterokaryon formation. The apparent absence of shuttling cannot be attributed to degradation of H1 in the cytoplasm since the molecule was reasonably stable after injection (Fig. 4). Nor can it be attributed to a preference of the injected H1 for HeLa chromatin, since labeled H1 did not exchange between HeLa nuclei or migrate from 3T3 to HeLa nuclei. Thus, despite similarities between HMG proteins and histone H1 with regard to size, proposed chromatin-binding sites, intracellular half-lives, and salt extractability, the two classes of molecules differ in the extent to which they exit from nuclei.

In similar fusion studies on the behavior of arginine-rich core histones, we showed that H3 and H4 neither migrated between nuclei within heterokaryons nor transferred from human to mouse chromosomes within newly formed hybrid cell nuclei (22). This was evident from the sectorized distribution of autoradiographic grains over hybrid nuclei formed from [ $^3\text{H}$ ]arginine-labeled HeLa cells and unlabeled 3T3 cells. Similar analysis of newly formed hybrid cells in the present study revealed a uniform distribution of grains over hybrid nuclei (Fig. 7 *b*). We never observed sectorized nuclei in hybrid cells formed from  $^3\text{H}$ -histone H1 injected HeLa and 3T3 cells. For this reason and because human and mouse DNAs remain segregated in hybrid nuclei (Fig. 7 *a*), we conclude that histone H1 rapidly equilibrates between human and mouse chromosomes soon after mitosis.

The behavior of injected histone H1 in hybrid nuclei was somewhat unexpected given its behavior in heterokaryons. The molecule did not equilibrate between nuclei, but it was rapidly transferred to mouse chromosomes after mitosis. There are two explanations for this apparent discrepancy. Histone H1 could undergo continuous, rapid exchange with





**Figure 7.** Autoradiograms of HeLa-3T3 hybrid cells. (A) A hybrid pair formed from an [ $^3\text{H}$ ]thymidine-labeled HeLa cell and an unlabeled 3T3 cell. Note the segregation of human and mouse chromosomes within newly formed hybrid nuclei. (B) A hybrid pair formed from a HeLa cell injected with  $^3\text{H}$ -histone H1 and an unlabeled 3T3 cell. Despite the segregation of human and mouse chromosomes in most hybrid cells,  $^3\text{H}$ -histone H1 molecules originally localized on human chromosomes are now evenly distributed on mouse and human chromosomes in all hybrids.

chromatin-binding sites during interphase, but unlike HMG proteins, might be unable to exit the nucleus. Alternatively, most histone H1 molecules could permanently associate with chromatin during most of interphase and then undergo wide-

spread redistribution after mitosis. Although little compelling evidence exists in support of either hypothesis, we favor the latter.

It is clear that histone H1 and other histones can migrate



between chromatin fractions in vitro (3, 14, 20, 35), although recent studies indicate that some H1 molecules do not exchange in vitro (17). Likewise, there is little doubt that histone H1 exchange can occur in vivo. During male pronucleus formation in sea urchin zygotes, sperm H1 molecules are replaced by maternal histone H1 stored in the oocyte (10). The present study has shown that H1 molecules on human chromosomes at the start of mitosis are distributed on both human and mouse chromosomes soon after telophase. Furthermore, several studies on chromatin assembly in S phase indicate that newly synthesized H1 molecules deposit on old as well as newly synthesized DNA molecules (16, 21). Thus, the question is not whether histone H1 can exchange, but rather what fraction of the H1 pool is normally capable of exchange during interphase.

We suspect that the exchangeable fraction of histone H1 is small based on the following considerations. First, the results in Fig. 6 showing that some H1 molecules can migrate from HeLa nuclei to mouse nuclei, demonstrate that there is no inherent barrier to H1 molecules exiting the nucleus. In light of this observation, the apparent kinetics of exchange, 6% at 1 d postfusion and 7.5% at 2 d, suggest that only a fraction (~15%) of the H1 molecules are exchangeable. Second, the chromatin assembly experiments (16, 21), from which some might argue that all H1 molecules are exchangeable, demonstrate only that newly synthesized histone H1 has access to chromatin-binding sites other than those containing DNA synthesized during the same pulse. If H1 generates compact chromatin structures, it is not surprising that extensive regions of chromatin would be devoid of H1 during S phase. It might also be expected that there would be a progressive transit of H1 molecules into an exchangeable pool as different portions of the genome underwent replication. Third, certain aspects of gene regulation are most easily understood in terms of prolonged condensation of chromatin domains (2, 38). To the extent that histone H1 produces chromatin condensation, it is simpler to envision "permanent" association of H1 in repressed chromatin regions.

There is a possible test of the idea that most H1 molecules are stably associated with inactive chromatin domains. If this hypothesis is correct, then H1 molecules injected into HeLa cells just completing S phase should preferentially associate with late replicating regions and remain there until mitosis. The autoradiographic demonstration of enrichment of injected <sup>3</sup>H-histone H1 in late replicating regions of metaphase chromosomes would constitute evidence for stable association of H1 with inactive chromatin domains. Unfortunately, we have yet to find conditions that both fix H1 to the chromosomes and produce cytological preparations of sufficient quality for the high resolution analysis required to test the prediction.

In summary, we have shown that although histone H1 exchanges slowly between nuclei in heterokaryons, it rapidly equilibrates between chromosomes within hybrid nuclei. Despite the clear demonstration of wholesale H1 exchange after mitosis, we favor the view that most H1 molecules are stably associated with chromatin during interphase. We attribute the extensive exchange after cell division to the rapid chromatin decondensation and dephosphorylation of H1 that occurs in telophase and early G1.

We thank Drs. K. V. Rote and S. Rogers for helpful comments on the manuscript and Maurine Vaughan for expert word processing.

These studies were supported by grants GM-30445 and GM-13864 from the National Institutes of Health.

Received for publication 31 March 1986.

## References

- Ajro, K., T. W. Borun, and L. H. Cohen. 1981. Phosphorylation states of different histone 1 subtypes and their relationship to chromatin functions during the HeLa S-3 cell cycle. *Biochemistry*. 20:1445-1454.
- Brown, D. D. 1984. The role of stable complexes that repress and activate eucaryotic genes. *Cell*. 37:359-365.
- Caron, F., and J. O. Thomas. 1981. Exchange of histone H1 between segments of chromatin. *J. Mol. Biol.* 146:513-537.
- Delabar, J. M. 1985. Non-random location of H1-H1<sup>o</sup> histones on chromatin of mouse liver and brain. *J. Biol. Chem.* 260:12622-12628.
- Djondjurov, L. P., N. Y. Yancheva, and E. C. Ivanova. 1983. Histones of terminally differentiated cells undergo continuous turnover. *Biochemistry*. 22:4095-4102.
- Felsenfeld, G., and J. D. McGhee. 1986. Structure of the 30 nm chromatin fiber. *Cell*. 44:375-377.
- Finch, J. T., and A. Klug. 1976. Solenoidal model for superstructure in chromatin. *Proc. Natl. Acad. Sci. USA*. 73:1897-1901.
- Fischer, S. G., and U. K. Laemmli. 1980. Cell cycle changes in *Physarum polycephalum* histone H1 phosphate: relationship to deoxyribonucleic acid binding and chromosome condensation. *Biochemistry*. 19:2240-2246.
- Glacy, S. D. 1983. Pattern and time course of rhodamine-actin incorporation in cardiac myocytes. *J. Cell Biol.* 96:1164-1167.
- Green, G. R., and D. L. Poccia. 1985. Phosphorylation of sea urchin sperm H1 and H2B histones precedes chromatin decondensation and H1 exchange during pronuclear formation. *Dev. Biol.* 108:235-245.
- Gurley, L. R., R. A. Walters, and R. A. Tobey. 1972. The metabolism of histone fractions. IV. Synthesis of histones during the G1-phase of the mammalian life cycle. *Arch. Biochem. Biophys.* 148:633-641.
- Hannon, R., E. Bateman, J. Allan, N. Harborne, and H. Gould. 1984. Control of RNA polymerase binding to chromatin by variations in linker histone composition. *J. Mol. Biol.* 180:131-149.
- Hewish, D. R., and L. A. Burgoyne. 1973. Chromatin substructure. The digestion of chromatin DNA at regularly spaced sites by a nuclear deoxyribonuclease. *Biochem. Biophys. Res. Commun.* 52:504-510.
- Huang, H.-C., and R. D. Cole. 1984. The distribution of H1 histone is nonuniform in chromatin and correlates with different degrees of condensation. *J. Biol. Chem.* 259:14237-14242.
- Igo-Kemenes, T., W. Hörz, and H. G. Zachau. 1982. Chromatin. *Annu. Rev. Biochem.* 51:89-121.
- Jackson, V., and R. Chalkley. 1985. Histone synthesis and deposition in the G1 and S phases of hepatoma tissue culture cells. *Biochemistry*. 24:6921-6930.
- Jin, Y. j., and R. D. Cole. 1986. H1 histone exchange is limited to particular regions of chromatin that differ in aggregation properties. *J. Biol. Chem.* 261:3420-3427.
- Keith, C. H., J. R. Feramisco, and M. Shelanski. 1981. Direct visualization of fluorescein-labeled microtubules in vitro and in microinjected fibroblasts. *J. Cell Biol.* 88:234-240.
- Kornberg, R. D. 1974. Chromatin structure: a repeating unit of histones and DNA. *Science (Wash. DC)*. 184:868-871.
- Louters, L., and R. Chalkley. 1984. *In vitro* exchange of nucleosomal histones H2a and H2b. *Biochemistry*. 23:547-552.
- Louters, L., and R. Chalkley. 1985. Exchange of histones H1, H2a and H2b *in vivo*. *Biochemistry*. 24:3080-3085.
- Manser, T., T. Thacher, and M. Rechsteiner. 1980. Arginine-rich histones do not exchange between human and mouse chromosomes in hybrid cells. *Cell*. 19:993-1003.
- McGhee, J. D., J. M. Nickol, G. Felsenfeld, and D. C. Rau. 1983. Higher order structure of chromatin: orientation of nucleosomes within the 30 nm chromatin solenoid is independent of species and spacer length. *Cell*. 33:831-841.
- Olins, A. L., and D. E. Olins. 1974. Spheroid chromatin units ( $\mu$  bodies). *Science (Wash. DC)*. 183:330-332.
- Panyim, S., and R. Chalkley. 1969. High resolution acrylamide gel electrophoresis of histones. *Arch. Biochem. Biophys.* 130:337-346.
- Rechsteiner, M. 1982. Transfer of macromolecules using erythrocyte ghosts. In *Techniques in Somatic Cell Genetics*. J. Shay, editor. Plenum Publishing Corp., New York. 385-398.
- Rechsteiner, M., and L. Kuehl. 1979. Microinjection of the nonhistone chromosomal protein HMGI into bovine fibroblasts and HeLa cells. *Cell*. 16:901-908.
- Renz, M., P. Nehls, and J. Hozier. 1977. Involvement of histone 3 H1 in the organization of the chromosome fiber. *Proc. Natl. Acad. Sci. USA*. 74:1879-1883.
- Richmond, T. J., J. T. Finch, B. Rushton, D. Rhodes, and A. Klug. 1984. Structure of the nucleosome core particle at 7 Å resolution. *Nature (Lond.)*. 311:532-537.
- Sakagami, H., Y. Mitsui, S.-I. Murota, and M.-A. Yamada. 1982. Effect of growth stage on histone H1 metabolism in human diploid fibroblasts. *J. Cell*.

*Physiol.* 110:213-218.

31. Schlissel, M. S., and D. D. Brown. 1984. The transcriptional regulation of *Xenopus* 5S RNA genes in chromatin: the roles of active stable transcription complexes and histone H1. *Cell*. 37:903-913.

32. Strätling, W. H., and R. Klingholz. 1981. Supranucleosomal structure of chromatin: digestion by calcium/magnesium endonuclease proceeds via a discrete size class of particles with elevated stability. *Biochemistry*. 20:1386-1392.

33. Tack, B. F., J. Dean, D. Eilat, P. E. Lorenz, and A. N. Schechter. 1980. Tritium labeling of proteins to high specific radioactivity by reductive methylation. *J. Biol. Chem.* 255:8842-8847.

34. Thoma, F., and T. Koller. 1977. Influence of histone H1 on chromatin structure. *Cell*. 12:101-107.

35. Thomas, J. O., and C. Rees. 1983. Exchange of histones H1 and H5 between chromatin fragments. *Eur. J. Biochem.* 134:109-115.

36. van der Westhuyzen, D. R., E. L. Böhm, and C. von Holt. 1974. Fractionation of chicken erythrocyte whole histone into the six main components by gel exclusion chromatography. *Biochim. Biophys. Acta.* 359:341-345.

37. Weintraub, H. 1984. Histone-H1-dependent chromatin superstructures

and the suppression of gene activity. *Cell*. 38:17-27.

38. Weintraub, H. 1985. Assembly and propagation of repressed and de-repressed chromosomal states. *Cell*. 42:705-711.

39. Weintraub, H., S. J. Flint, I. M. Leffak, M. Groudine, and R. M. Grainger. 1977. The generation and propagation of variegated chromosome structures. *Cold Spring Harbor Symp. Quant. Biol.* 42:401-407.

40. Widom, J., and A. Klug. 1985. Structure of the 300 Å chromatin filament: X-ray diffraction from oriented samples. *Cell*. 43:207-213.

41. Woodcock, C. L. F., L.-L. Y. Frado, and J. B. Rattner. 1984. The higher-order structure of chromatin: evidence for a helical ribbon arrangement. *J. Cell Biol.* 99:42-52.

42. Worcel, A., S. Strogatz, and D. Riley. 1981. Structure of chromatin and the linking number of DNA. *Proc. Natl. Acad. Sci. USA.* 78:1461-1465.

43. Wu, L., M. Rechsteiner, and L. Kuehl. 1981. Comparative studies on microinjected high-mobility-group chromosomal proteins, HMG1 and HMG2. *J. Cell Biol.* 91:488-496.

44. Zentgraf, H., V. Muller, and W. W. Franke. 1980. Supranucleosomal organization of sea urchin sperm chromatin in regularly arranged 40 to 50 nm large granular subunits. *Eur. J. Cell Biol.* 20:254-264.