Comparative Studies on Microinjected High-mobility-group Chromosomal Proteins, HMG1 and HMG2

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ABSTRACT The nonhistone chromosomal proteins, HMG1 and HMG2, were iodinated and introduced into HeLa cells, bovine fibroblasts, or mouse 3T3 cells by erythrocyte-mediated microinjection. Autoradiographic analysis of injected cells fixed with glutaraldehyde consistently showed both molecules concentrated within nuclei. Fixation with methanol, on the other hand, resulted in some leakage of the microinjected proteins from the nuclei so that more autoradiographic grains appeared over the cytoplasm or outside the cells. Both injected and endogenous HMG1 and HMG2 partitioned unexpectedly upon fractionation of bovine fibroblasts, HeLa, or 3T3 cells, appearing in the cytoplasmic fractions. However, in calf thymus, HMG1 and HMG2 molecules appeared in the 0.35 M NaCl extract of isolated nuclei, as expected. These observations show that the binding of HMG1 and HMG2 to chromatin differs among cell types or that other tissue-specific components can influence their binding. Coinjection of [^{125}I]HMG1 and [^{131}I]HMG2 into HeLa cells revealed that the two molecules display virtually equivalent distributions upon cell fractionation, identical stability, identical intracellular distributions, and equal rates of equilibration between nuclei. In addition, HMG1 and HMG2 did not differ in their partitioning upon fractionation nor in their stability in growing vs. nongrowing 3T3 cells. Thus, we have not detected any significant differences in the intracellular behavior of HMG1 and HMG2 after microinjection into human, bovine, or murine cells.

Chromatin contains a group of nonhistone proteins characterized by relatively low molecular weights (<30,000) and high contents of lysine and acidic amino acids. These proteins, designated high-mobility-group (HMG) proteins, can be extracted from chromatin with 0.35 M NaCl or 0.75 M $HClO_4$, and are soluble in 2% trichloroacetic acid (19, 27). The four major members of this class, HMG1, HMG2, HMG14, and HMG17, have been purified to apparent homogeneity and well characterized (17, 18, 19, 27). Partial amino acid sequences for HMG1 and HMG2 and complete primary structures for HMG14 and HMG17 have been reported (19, 51, 54, 56). HMG proteins display a number of similarities to histones, but unlike histones, their synthesis is not coupled to that of DNA (29). Their high concentration, ~10^6 molecules per nucleus, suggests that they play a role in chromatin structure. It has recently been shown that HMG14 and HMG17 bind and restore DNase I sensitivity to nucleosome core particles depleted of HMGs (58, 59). Thus, HMG14 and HMG17 appear to confer a special, nuclease-sensitive structure on active genes. The role(s) of HMG1 and HMG2 are less clear.

HMG1 and HMG2 display remarkable structural similarities and presumably share a common ancestral gene. Their N-terminal sequences differ by only one amino acid in the first 21 residues (54). The C-terminal portion of HMG1 has a continuous run of 41, and HMG2 of 35, aspartic and glutamic acid residues (52, 55). About two-thirds of their tryptic peptides appear identical (53). Between pH 4 and pH 10, both HMG1 and HMG2 exist in a highly structured conformation with 40–50% α-helix (1, 8), and both proteins interact with double-stranded DNA in a similar manner (25, 26, 47). Both have been reported to bind to specific subfractions of histone H1 (48, 61), although this has been disputed (9). Like HMG14 and HMG17, HMG1 and HMG2, or the homologous trout protein, HMGT, have been reported to be concentrated in transcriptionally competent regions of the genome, as demonstrated by their release from chromatin by nucleases under conditions which preferentially digest transcriptionally active sequences (31, 33, 34, 39, 50). However, this is also disputed (16).

Despite their many similarities, HMG1 and HMG2 are distinct proteins with different sequences and different physical properties. Recently, Seyedin and Kistler (46) measured the levels of HMG1 and HMG2 in a number of rat organs and observed a striking correlation between the HMG2 levels and the proliferative activity of the tissues. HMG1 levels, on the

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other hand, did not show marked organ variability. In an extension of this observation, it was shown that, in cryptorchid rat testis and in developing chick skeletal muscle, loss of proliferative activity was associated with a dramatic depletion of HMG2, and the authors suggested that HMG2 may play a role in cell replication.

We recently used erythrocyte-mediated microinjection to examine the fate of [^{125}I]HMG1 molecules introduced into HeLa cells and bovine fibroblasts (41). Injected HMG1 molecules rapidly concentrated within nuclei, and the chromatin-bound HMG1, which was shown to be in equilibrium with a small cytoplasmic pool of HMG1, displayed considerable stability. The observations of Seyedin and Kistler (46) suggest that HMG1 and HMG2 differ in their metabolism. Our study was undertaken, in part, to compare the intracellular behavior of microinjected HMG1 and HMG2, particularly their turnover rates. The dramatic changes in the level of HMG2 which accompany changes in proliferative activity suggested that HMG2 might have a shorter half-life than HMG1, since proteins whose intracellular concentrations are subject to wide fluctuations are frequently rapidly degraded (14, 15). We were also interested in comparing the binding of HMG1 and HMG2 to chromatin. If HMG2, but not HMG1, is involved in cell replication, the two proteins might be expected to bind to chromatin at different sites and with different affinities.

Our studies were also undertaken to resolve an apparent discrepancy in the reported intracellular localization of HMG1. Bustin and Neihart stained several types of cultured cells with fluorescent antibodies directed against HMG1 and observed considerable fluorescence over the cytoplasm (6). In fact, in some of their experiments, the cytoplasmic fluorescence was greater than that over the nucleus. Smith et al., using similar methods, failed to detect HMG1 in the cytoplasm (49). Previous microinjection studies from our laboratory also revealed a predominantly nuclear localization for HMG1 (41). Because antibodies to HMG1 cross-react with HMG2 (3, 49), it seemed possible that the cytoplasmic fluorescence observed by Bustin and Neihart might be due to high concentration of HMG2 in the cytoplasm. Therefore, we thought it desirable to determine the intracellular distribution of injected HMG2.

MATERIALS AND METHODS

Isolation and HMG Proteins

HMG1 and HMG2 were prepared from calf thymus as described by Goodwin et al. (17). Upon electrophoresis on acid-urea gels or SDS gels, each purified protein was contained in one major band.

Iodination

HMG proteins were iodinated by an adaptation of the method of Miyachi et al. (36) as described earlier (41). All steps of the labeling procedure were performed in plastic ware because HMG1 and HMG2 readily adsorb to glass. Usually ~75% of the ^{125}I or ^{131}I was incorporated into the protein. Because the reaction mixtures contained 1,000 times as much HMG as lactoperoxidase, it was assumed that little of the label was associated with the latter. This assumption was supported by electrophoresis of iodinated HMG molecules on acid-urea or SDS gels. Virtually all of the radioactivity coincided with the HMG bands.

Cell and Culture Techniques

The heteroploid human cell line D98/AH2 (CCL 18.3), the heteroploid bovine fibroblast line, EBTr (CCL 44), and Swiss 3T3 cells (CCL 92) were obtained from the American Type Culture Collection (Rockville, Md.). Cells were cultured as previously described (45).

Loading, Microinjection, and Cell Fusions

The source of human erythrocytes and the general procedures for introducing proteins into them have been described in detail (40). For microinjection, the fusion mixture consisted of 5–10 × 10^6 cultured cells, 7.5 × 10^8 loaded erythrocytes, and 500–1,000 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 0.2–1 mM $MnCl_2$. The components were added in the order listed and kept on ice for 10 min before incubation with shaking at 37°C for 20 min. The cells were washed several times in F12 medium before plating.

Fixation, Sectioning, Autoradiography, and Fluorescence Microscopy

After plating onto glass cover slips, cells were washed twice with 0.15 M NaCl–20 mM Tris, pH 7.4, fixed for 1 h with 3% glutaraldehyde in 0.1 M sodium phosphate, pH 7.4, and washed thoroughly with distilled water. For comparative purposes, cells were fixed with methanol at −20°C followed by acetone, as described by Bustin and Neihardt (6). Cells were embedded and sectioned as previously described (41). Autoradiographic procedures have been described in detail (42). After autoradiography, the slides were stained with Giemsa blood stain. Grain position and density were determined at × 1,000 magnification under oil using eyepiece reticles containing 10 × 10 or 40 × 40 grids. Cells containing fluorescent beads were examined with a Zeiss Photomicroscope II equipped with epifluorescence optics and filters matched for the detection of fluorescein.

Cell Fractionation

Nuclei were isolated from the washed cells by the double-detergent method of Penman (38) or, alternatively, by a procedure employing Triton X-100. In the former method, the cells were suspended in 10 mM NaCl–1.5 mM $MgCl_2$–10 mM Tris, pH 7.4 (RSB), at a concentration of 0.8–2.5 × 10^6 cells/ml and were ruptured by 10–12 strokes in a Dounce homogenizer with a tightly fitting pestle. The nuclei were sedimented and washed once with RSB and once with RSB containing 0.43% sodium deoxycholate and 0.86% Tween 40. Bovine fibroblast (BF) cells were more resistant to breakage than HeLa and were, therefore, homogenized after addition of detergent as well as before. In the Triton method, the cells were washed once with 25 mM KCl, 5 mM $MgCl_2$, 50 mM Tris, pH 7.6, suspended in RSB containing 0.5% Triton X-100 at a concentration of 0.5–10 × 10^6 cells/ml, transferred to a Dounce homogenizer and subjected to 10–20 strokes (HeLa cells) or 50–100 strokes (3T3 and BF cells). Nuclei were sedimented and washed once with homogenization buffer. HeLa nuclei prepared by either method were clean as judged by phase-contrast microscopy, but BF and 3T3 nuclei were contaminated with adhering fragments of cytoplasm.

For autoradiographic studies, HeLa or BF nuclei, isolated by the Triton X-100 procedure, were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate, pH 7.4, for 1 h at 4°C. The nuclei were then washed three times with absolute methanol and spread onto microscope slides. Although nuclei were often present in large aggregates, sufficient numbers of individual nuclei were present to allow autoradiographic localization of [^{125}I]HMG1.

Electrophoresis

Two methods of electrophoresis on polyacrylamide gels were employed: the acid-urea system of Panyim and Chalkley (37) and the SDS system of Laemmli (32). Samples were applied in 6-mm-wide lanes to 1.5-mm-thick slabs of gel. For the Panyim-Chalkley system, a urea concentration of 6.25 M was employed and electrophoresis was for 4 h at 250 V. For the Laemmli system, the separating gels contained 15% acrylamide and 0.4% bisacrylamide. Electrophoresis was for 4 h at 50 V before the proteins entered the separating gel and 100 V thereafter. After electrophoresis, the gels were stained for 1 h in 0.25% amido black 10B–7% acetic acid–31.5% methanol and destained electrically in 7.5% acetic acid–22.5% methanol.

The concentration of HMG proteins in cell fractions was determined by scanning the electrophoretic pattern produced by the mixture with an Ortec Model 4310 densitometer and comparing the area under the HMG peaks with the areas generated by known amounts of the pure HMG molecules. For both HMG1 and HMG2 the relationship between area and amount of protein was linear over the range investigated (0–15 μg of protein per lane).

Determination of Radioactivity

Radioactive samples were solubilized in NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.) and counted on a Packard Model 3003 scintillation spectrometer (Packard Instruments Co., Inc., Downers Grove, Ill.) in toluene fluor containing PPO (4 g/l) and POPOP (0.1 g/l) or samples were

counted with a Nuclear-Chicago Gamma Spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). In those experiments which employed both ^{125}I and ^{131}I samples were counted in a Beckman Gamma 4000 spectrometer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The fraction of ^{131}I counts in the ^{125}I channel was 0.287, and corrections were made for this as well as for ^{131}I decay.

Detection of radioactivity in stained polyacrylamide gels was accomplished by the fluorographic method of Bonner and Laskey (2). Quantification of the radioactivity in each band was accomplished by scanning the fluorograms with an Ortec Model 4310 densitometer (Ortec Inc., EG&G, Inc., Oak Ridge, Tenn.) and comparing the area under each peak with the area under the peak given by a known amount of [^{125}I]HMG1 or [^{125}I]HMG2. Standard curves, prepared by scanning a fluorogram containing a series of concentrations of [^{125}I]HMG1 or [^{125}I]HMG2, were linear.

Materials

[^{125}I]Na and [^{131}I]Na were purchased from Amersham Corp. $^{32}PO_4$ was from Mallinkrodt. Lactoperoxidase was obtained from Sigma Chemical Co. (St. Louis, Mo.). Tissue culture supplies were from Flow Laboratories, Inc. (Rockville, Md.). Fluorescent polystyrene beads were obtained from The Dow Chemical Company (Indianapolis, Ind.).

RESULTS

Intracellular Distribution of Microinjected [^{125}I]-HMG2

When [^{125}I]HMG2 molecules were injected into HeLa cells or bovine fibroblasts, they concentrated in the nuclei as demonstrated by autoradiography after glutaraldehyde fixation (Fig. 1 *A*, see Fig. 2 for quantification). In parallel experiments, cells were fixed with cold methanol instead of glutaraldehyde. Although results obtained with methanol fixed cells were quite variable, it always appeared as though labeled molecules were being lost from the nuclei during fixation. In some experiments, the injected [^{125}I]HMG2 appeared to have leaked from the cells and adsorbed to the glass substratum as shown by two- to threefold higher backgrounds and a reduction in the number of injected cells when compared with parallel cover slips fixed with glutaraldehyde (Fig. 1 *B*). In other experiments, the leakage was not as extensive and was manifested primarily by lowered nuclear/cytoplasmic grain density ratios. For the data shown in Fig. 2, cover slips fixed and coded by one investigator were then evaluated by a second. The mean nuclear/cytoplasmic grain density ratio, determined after background subtraction, was 7.9 for 101 methanol-fixed cells and 16.4 for 108 cells that had been fixed in glutaraldehyde. This difference is significant at $P < 0.001$ by the Wilcoxan rank sum test. Similar results were obtained for injected [^{125}I]HMG1, so it would appear that methanol is not an entirely suitable fixative for the localization of HMG1 and HMG2 molecules within cells.

Microinjected [^{125}I]HMG1 was previously shown to be localized within nuclei and not simply adsorbed to the nuclear envelope by autoradiographic analysis on thin sections of injected HeLa cells (41). Using the same procedure we showed that injected [^{125}I]HMG1 molecules remained associated with metaphase chromosomes. In this study we have obtained the same results for injected [^{125}I]HMG2. For BF cells fixed 48 h after injection, the mean density over 50 nuclei in sectioned cells was 5.7-fold greater than the corresponding cytoplasmic grain density. Similarly, qualitative analysis of autoradiograms of thin sections of injected BF cells fixed during metaphase revealed that [^{125}I]HMG2 was concentrated in the spindle region presumably on metaphase chromosomes. Thus, the intracellular distribution of injected [^{125}I]HMG2 is equivalent to that previously measured for [^{125}I]HMG1.

Distribution of Microinjected HMG1 and HMG2 during Cell Fractionation

A major goal of this study was to compare the interaction of HMG1 and HMG2 with chromatin. If the two proteins serve different roles, as proposed by Seyadin and Kistler (46), their chromatin binding sites or binding affinities might vary. One way to detect any differences in binding would be to measure release of the two proteins from chromatin by enzymes or as a function of salt concentration. For this reason, HeLa cells were injected with a mixture of [^{125}I]HMG1 and [^{131}I]HMG2 and subsequently fractionated. As shown in Table I, the two molecules exhibited virtually identical distributions during fractionation. A large portion of each protein appeared in the cytoplasmic fraction. Because autoradiographic analysis indicated that >80% of injected HMG1 and HMG2 molecules were intranuclear, these results show that microinjected HMG1 and HMG2 are loosely associated with chromatin and leak from nuclei during the isolation procedure. The extent of this leakage precluded detailed enzyme digestion studies.

HMG1 and HMG2 molecules are tightly associated with calf thymus chromatin at low-salt concentrations. The failure of most injected HMG1 and HMG2 molecules to remain within HeLa nuclei during fractionation suggested either that the injected proteins did not act like endogenous HMG1 and HMG2 or that HMG1 and HMG2 bind to HeLa chromatin differently than to calf thymus chromatin. To distinguish between these alternatives, HeLa cells and calf thymus were fractionated as described above, and the concentration of endogenous HMG1 in each fraction was determined. In these experiments, the detergent wash was omitted because the presence of detergents would have interfered with the isolation of the HMG-containing fraction from the various extracts. A comparison of Tables I and II demonstrates that endogenous HMG1 in HeLa cells behaved similarly to microinjected HMG1 upon cell fractionation. In both cases, nearly 80% of the recovered HMG1 was found in the RSB extracts.

The endogenous HMG1 of calf thymus behaved differently from that of HeLa cells, because most of it appeared in the 0.35 M NaCl fraction as previously reported by Johns et al. (27). A trace amount of ^{125}I-labeled HMG1 added to the homogenates, fractionated similarly to the endogenous HMG1 of both HeLa cells and calf thymus. If it is assumed that HMG1 is quantitatively extracted from cells with 0.35 M NaCl, then it can be calculated from the data of Table II that HeLa cells contain 2.3×10^6, and calf thymus 2.9×10^6 molecules of HMG1 per cell. Comparison of the distribution of endogenous and injected HMG2 molecules during cell fractionation gave results virtually identical to those obtained for HMG1, except that there appear to be 1.2×10^6 HMG2 molecules per HeLa cell.

The experiments presented above indicate that injected HMG1 and HMG2 fractionate like their endogenous counterparts in HeLa cells. The distribution of injected [^{125}I]HMG1 was also examined upon fractionation of injected BF cells, but the distribution of endogenous HMG1 in BF cells was not measured because of the expense in growing sufficient numbers of these cells. As with HeLa cells, a large portion (45%) of microinjected [^{125}I]HMG1 entered the low ionic strength buffer during homogenization of BF cells. However, after extraction with 0.35 M NaCl significantly more label (50%) remained associated with the residual nuclear fraction from BF cells.

Autoradiography of BF nuclei extracted with 0.35 M NaCl

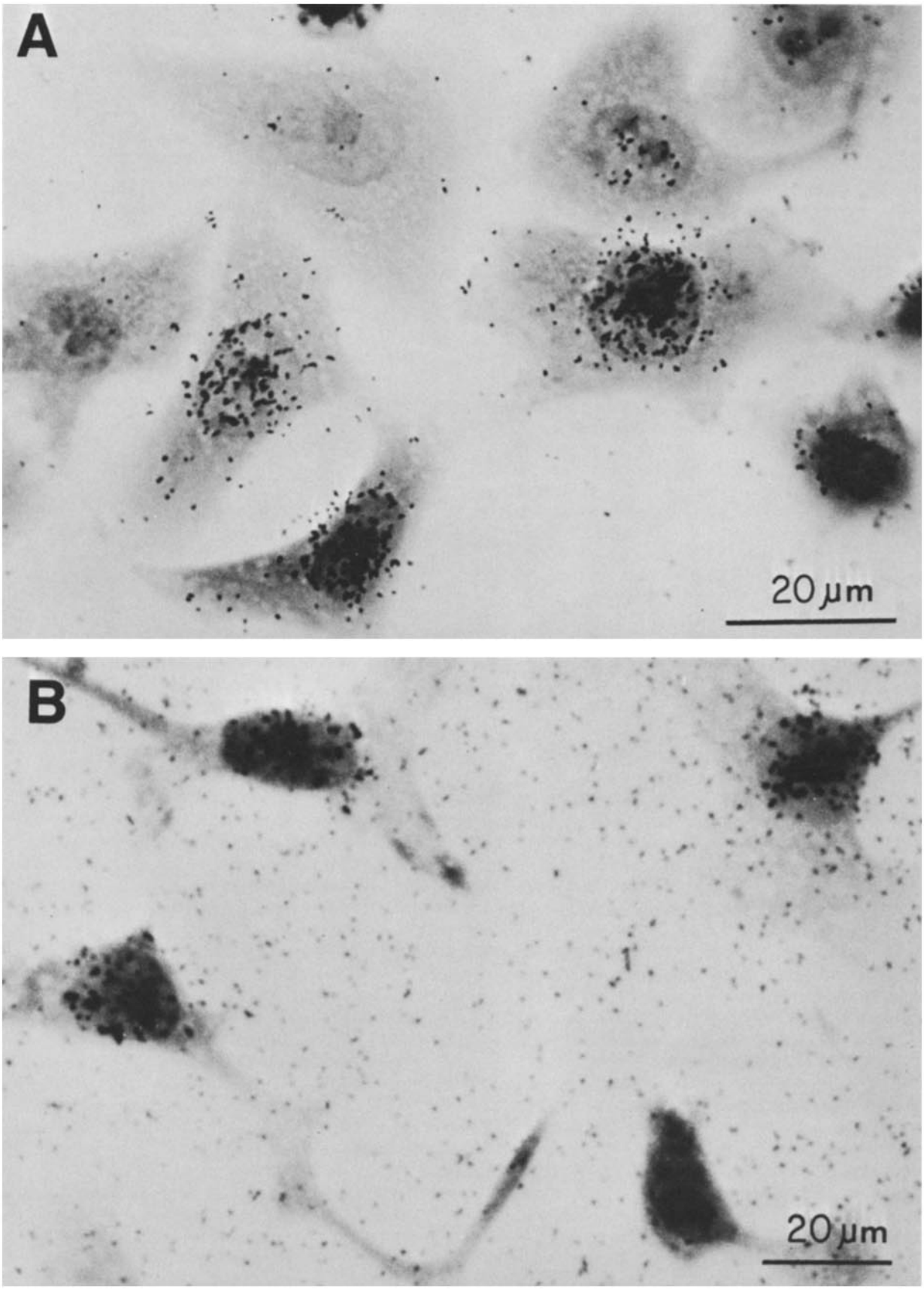

FIGURE 1 Autoradiograms of [^{125}I]HMG2-injected HeLa cells. HeLa cells were fused with [^{125}I]HMG2 loaded human erythrocytes, plated onto glass cover slips and fixed 16 h later with either 3% glutaraldehyde (*A*) or methanol-acetone (*B*). Autoradiographic exposure was 7 d.

showed that the residual ^{125}I was not present within the nuclei. Rather, the label was associated with material adhering to the nuclei. Nuclease digestion studies provided further evidence that the residual label in the nuclear fraction was not associated with chromatin. HeLa and BF cells were grown in medium containing 1 μCi/ml $^{32}PO_4$ for 48 h and then injected with [^{125}I]HMG1. 24 h later, nuclei were isolated from each cell type and incubated in a mixture containing 400 U/ml DNAse I and 2,700 U/ml micrococcal nuclease for 20 min at 37°C as described by Weintraub and Groudine (57). Whereas ^{32}P loss from the nuclei indicated that >90% of the DNA was digested, <15% of the ^{125}I in the nuclear fraction was solubilized. Attempts to solubilize the residual label with 5 M urea–2 M NaCl were also unsuccessful.

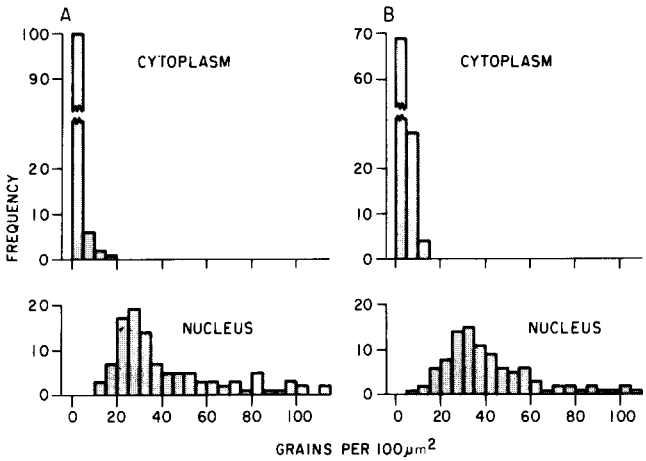

FIGURE 2 *A* shows the grain densities over nuclei and cytoplasm of 108 cells fixed with 3% glutaraldehyde. Similar data are presented in *B* for 101 cells fixed in methanol-acetone.

TABLE I

Distribution of Microinjected [^{125}I]HMG1 and [^{131}I]HMG2 during Cell Fractionation

Fraction	[^{125}I]HMG1		[^{131}I]HMG2	
	cpm × 10⁻³	*% of total*	*cpm × 10⁻³*	*% of total*
Whole cells	43.4	100	33.1	100
Cytoplasmic supernate	29.3	68	21.9	66
0.35 M NaCl extract of nuclei	9.1	19	5.9	18
Residual nuclear pellet	6.1	14	5.9	18

HeLa cells: 1.2 × 10^7 were microinjected with [^{125}I]HMG1 and [^{131}I]HMG2, and on the following day 5.5 × 10^6 cells were fractionated by the Triton X-100 procedure. Note the very similar distribution of HMG1 and HMG2 during fractionation.

Stability of Microinjected HMG1 and HMG2

To determine whether [^{125}I]HMG1 and [^{131}I]HMG2 were degraded or altered after injection, extracts prepared from injected HeLa or BF cells between 7 and 43 h after injection were analyzed by electrophoresis and fluorography, as previously described (41). In eight different experiments between 84 and 100% of the radioactivity which was extracted into RSB during nuclear isolation migrated as HMG1 or HMG2. Thus, the radioactivity which can be reextracted from HeLa or BF cells injected with [^{125}I]HMG1 or [^{125}I]HMG2 is present as undegraded HMG molecules.

Although intracellular ^{125}I was quantitatively associated with the injected HMG molecules, this does not indicate that injected HMG molecules are stable. The degradation of any individual protein molecule is rapid, and iodotyrosine is quickly lost to the medium (62). Previously measured half-lives for microinjected [^{125}I]HMG1 ranged from 60 to 120 h in HeLa and BF cells (41). Similar extractions of HeLa or BF cells at various times after injection of [^{125}I]HMG2 indicated apparent half-lives of 55–120 h in HeLa and BF cells. The relative stabilities of HMG1 and HMG2 were directly compared by coinjection of [^{125}I]HMG1 and [^{131}I]HMG2 into HeLa cells. According to the data in Fig. 3, the half-lives of HMG1 and HMG2 are identical. Identical degradation rates for [^{125}I]-HMG1 and [^{131}I]HMG2 were also observed in a second experiment with HeLa cells. Although we cannot exclude the possibility that the turnover rates of the microinjected HMG molecules differ from their endogenous counterparts, it should be noted that the half-lives determined in the above experiments are similar to those which have been reported for total HeLa cell proteins (12, 23). Evidence suggesting that the microinjected and endogenous HMGs are equivalent, will be considered in the discussion section.

Equilibrium of Microinjected [^{125}I]HMG2 Molecules between Cytoplasm and Nucleus

We previously showed that chromatin-bound HMG1 is in equilibrium with a cytoplasmic HMG1 pool (41). To determine whether this is also true of HMG2, HeLa cells microinjected

TABLE II

Extraction of Endogenous HMG1 and Added ^{125}I-Labeled HMG1 from HeLa Cells and Calf Thymus

Fraction	HeLa cells				Calf thymus			
	Endogenous HMG1		^{125}I Tracer HMG1		Endogenous HMG1		^{125}I Tracer HMG1	
	μg	*%*	*cpm × 10⁻³*	*%*	*μg*	*%*	*cpm × 10⁻³*	*%*
RSB extracts	43.2	79	3,840	64	12.1	13	1,590	33
0.35 M NaCl extract	11.6	21	1,990	33	79.6	87	3,030	63
Pellet	ND	ND	210	3	ND	ND	180	4

A suspension of 5.5 × 10^6 HeLa cells or 0.19 g of finely minced calf thymus (7.1 × 10^8 cells) in 5 ml of RSB was homogenized by 12 strokes in a Dounce homogenizer. To serve as a tracer, 5 × 10^6 cpm of [^{125}I]HMG1 was added to the resulting suspension. In a previous study it has been shown that when a tracer amount of HMGT, the trout homologue of HMG1 and HMG2, is added to a suspension of trout testis nuclei it enters the nuclei and binds to the chromatin with an affinity similar to that of the tightly bound, endogenous HMGT (30). The resulting suspension was centrifuged 10 min at 600 *g*. The nuclear pellet was extracted with a second 5-ml portion of RSB, then with 5 ml of 0.35 M NaCl. Each extract was brought to a trichloroacetic acid (TCA) concentration of 2% by addition of 100% wt/vol TCA. The material which precipitated was sedimented and discarded. The supernates were brought to 18% TCA and the precipitates, which contained the HMG proteins, were collected, dissolved in 60-μl portions of electrophoresis sample buffer, and run on acid-urea gels. The amount of HMG1 present in each extract was determined by densitometry of the stained gels. Based on the distribution of the [^{125}I]HMG1 tracer, corrections were made for the amount of HMG1 which was lost with the 2% TCA precipitate and for that which failed to precipitate with 18% TCA. The amount of HMG1 which could be extracted with 0.2 M H_2SO_4, 0.2 M NaCl from unfractionated cells was: HeLa cells, 106 μg/5.5 × 10^6 cells; thymus, 114 μg/7.1 × 10^8 cells. ND, not determined.

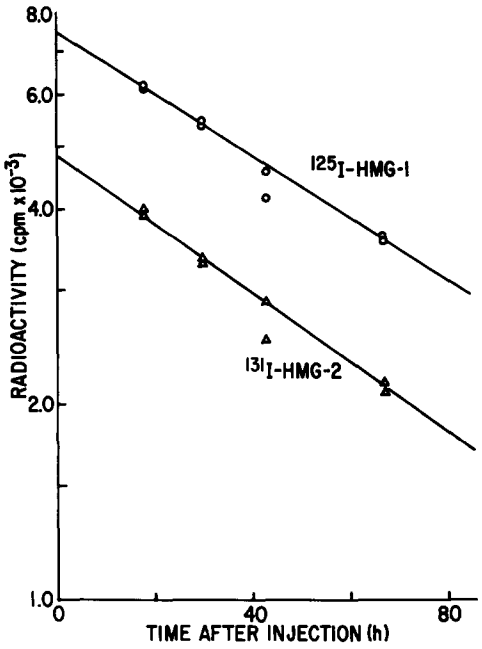

FIGURE 3 Stabilities of [^{125}I]HMG1 and [^{131}I]HMG2 after coinjection into HeLa cells. Calf thymus HMG1 and HMG2 labeled with ^{125}I and ^{131}I, respectively, were injected into HeLa cells, and the cells were dispersed into culture flasks. Cells from duplicate flasks were removed at the times indicated, and the amounts of ^{131}I and ^{125}I in the cells were determined.

with [^{125}I]HMG2 were fused with unlabeled cells which had been allowed to phagocytize fluorescent polystyrene latex beads. Binucleate cells produced by fusion of an injected and uninjected cell were identified by the presence of both radioactivity and fluorescent beads, and grain densities over the two nuclei in a number of such cells were determined at various times following cell fusion. The results of this experiment, which are presented in Fig. 4, demonstrate that [^{125}I]HMG2 molecules fully equilibrated between the two nuclei by 12 h after the formation of binucleate cells. These results are indistinguishable from those previously obtained with [^{125}I]HMG1 (41).

Stability of HMG1 and HMG2 in Growing and Nongrowing 3T3 Cells

Although expected on the basis of protein structure, the high degree of similarity between HMG1 and HMG2 in the studies just presented was surprising in view of the results of Seyedin and Kistler (46). Their observation that HMG2 rapidly disappeared in nongrowing tissues suggested that HMG1 and HMG2 would differ in the properties we have examined. However, because HeLa cells are continuously dividing, any difference between HMG1 and HMG2 strictly coupled to cell division might not be manifested. Accordingly, we re-examined the stability and partitioning of HMG1 and HMG2 in mouse 3T3 cells, since division of these cells can be controlled by serum concentration and cell density.

3T3 cells were injected with a mixture of [^{125}I]HMG1 and [^{131}I]HMG2 and then plated into regular F12 medium. After 20 h, the cells were collected by trypsinization, and some of the cells were plated into 100-mm petri dishes at high density in F12 medium containing 0.1% fetal calf serum (FCS); others were plated into 100-mm petri dishes at low density in F12 medium containing 10% FCS; and still others were fractionated to determine the partitioning of [^{125}I]HMG1 and [^{131}I]HMG2 in 3T3 cells. The stabilities of HMG1 and HMG2 in growing and nongrowing 3T3 cells were determined by dissolving the cells in 1 ml of 100 mM NaOH at various times after plating. The amounts of [^{125}I]HMG1 and [^{131}I]HMG2 in each sample were determined by gamma spectroscopy. From these data, which are presented in Table III, along with further experimental details, it can be seen that there was no difference in the stabilities of HMG1 and HMG2 in 3T3 cells grown in high serum as compared to those grown in low serum. Yet, the proportion of cells synthesizing DNA differed 20-fold between high density and low density 3T3 cells by 5 d after plating, so we conclude that for 3T3 cells the degradation rates of HMG1 and HMG2 are unaffected by growth rate. The distribution of HMG1 and HMG2 during cell fractionation also did not vary with growth rate. Table IV presents the subcellular distribution of HMG1 and HMG2 before growth in high or low serum and 120 h later. It is clear that for 3T3 cells, at least, growth rate does not affect the distribution of HMG1 and HMG2 upon fractionation.

DISCUSSION

We have used microinjection to study the behavior of HMG molecules within living cells. The validity of our approach depends upon the extent to which the microinjected molecules and endogenous molecules behave similarly. Three observations suggest that the iodinated HMG1 and HMG2 molecules, employed as probes, are equivalent to their endogenous counterparts. First, HMG1 and HMG2 concentrate in the nuclei of injected cells, and they are present on mitotic chromosomes.

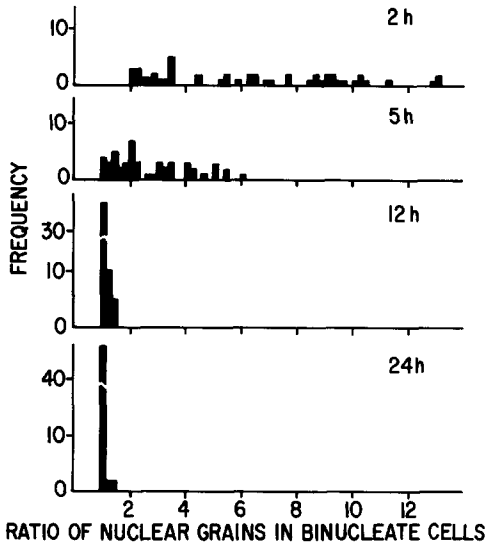

FIGURE 4 Distribution of [^{125}I]HMG2 in binucleate cells formed by fusion of injected and uninjected cells. 20 h after injection of [^{125}I]HMG2 into HeLa cells, the injected cells were collected and mixed with a twofold excess of uninjected cells containing fluorescent beads. The resulting mixture was plated onto glass cover slips and grown overnight. The cells were then fused with Sendai virus and fixed with 3.7% glutaraldehyde at various times. After autoradiographic processing, binucleate cells were examined, and the number of grains overlying the more heavily labeled nucleus was divided by the number of grains over the less heavily labeled nucleus to produce the ratio plotted on the abscissa. Only cells which contained fluorescent beads and at least 25 grains over nuclei were scored.

TABLE III

Relationship Between Cellular Growth Rate and Degradation Rates of HMG1 and HMG2

Hours after injection	Growth condition	Cells in S-phase	Radioactivity		
			[^{125}I]HMG1	[^{131}I]HMG2	HMG2/HMG1
		%		*cpm*	
24	High density	10	1,311	1,238	0.94
	Low density	65	1,299	1,206	0.93
72	High density	9	1,219	1,151	0.94
	Low density	57	1,479	1,372	0.93
120	High density	2	643	606	0.94
	Low density	48	588	566	0.96

3T3 cells were trypsinized and collected 20 h after coinjection with [^{125}I]HMG1 and [^{131}I]HMG. The injected cells were suspended in F12 medium with 0.1% FCS and were distributed into two sets of 75-cm flasks, each of which contained a glass cover slip. One set (high cell density) contained 0.9×10^6 injected cells plus 2.4×10^6 uninjected 373 cells per flask, and a FCS concentration of 0.1%. The other set of flasks (low cell density) contained 0.9×10^6 injected cells per flask and 10% serum. At 24, 72, and 120 h after plating, the cover slips were taken from the flasks and placed in 60-mm petri dishes containing regular growth medium and F12 medium containing 5% serum and 10 μCi/ml [^{3}H]thymidine. After incubation for 2 h, the cover slips were rinsed, and the cells were fixed with 3% glutaraldehyde. The fraction of cells in S-phase was determined by radioautography. At each sampling the remaining cells in the flasks were rinsed with phosphate-buffered saline, dissolved in 100 mM NaOH, and the intracellular radioactivity was measured.

TABLE IV

Fractionation of 3T3 Cells Injected with ^{125}I-HMG1 and ^{131}I-HMG2

Fraction	20 h after injection				120 h after injection							
					Low density				High density			
	[^{125}I]HMG1		[^{131}I]HMG2		[^{125}I]HMG1		[^{131}I]HMG2		[^{125}I]HMG1		[^{131}I]HMG2	
	cpm	%	*cpm*	%	*cpm*	%	*cpm*	%	*cpm*	%	*cpm*	%
Whole cell	10,400	100	14,375	100	24,600	100	22,600	100	20,228	100	18,000	100
RSB extracts	3,336	32	3,659	28	4,453	18	3,712	16	3,488	18	4,395	18
Triton extract	1,138	13	2,048	14	3,353	14	3,858	17	1,892	7	1,514	10
0.35 M NaCl extract	392	7	746	5	1,884	7	2,546	11	2,218	10	2,309	11
Nuclear pellet	5,347	51	7,331	51	13,876	58	12,361	55	11,144	62	10,852	54

3T3 cells were microinjected with [^{125}I]HMG1 and [^{131}I]HMG2 and trypsinized the next day. The recovered cells were divided into three portions; one portion was immediately fractionated; the other two portions were replated into flasks. The low density flasks contained 1.8×10^6 cells and 10% FCS. The high density flasks contained 1.8×10^6 injected cells, 8×10^6 uninjected cells, and FCS in a final concentration of 0.1%. After incubating 120 h, the cells were collected and fractionated as described in Materials and Methods.

The labeled proteins, therefore, display at least some of the binding characteristics of the native proteins. Second, injected HMG1 and HMG2 display high stability in HeLa, 3T3, and BF cells. Because altered proteins are often rapidly degraded (7, 24, 28), this observation provides further evidence that the labeled HMGs behave like native molecules. Third, microinjected HMG1 and HMG2 distribute similarly to their endogenous counterparts upon fractionation of HeLa cells. Clearly, we cannot be certain that the behavior of microinjected, labeled HMG1 and HMG2 mirrors their endogenous counterparts in every detail.

Studies by several groups of investigators have demonstrated that labeled nuclear proteins, when injected into the cytoplasms of homologous cells, become concentrated in the nuclei, whereas non-nuclear proteins do not (4, 5, 11, 21, 60). The ability of nuclear proteins and the failure of other proteins to accumulate in the nucleus is not obviously related to the isoelectric points or sizes of the protein. Our present data for HMG2 and previous data from HMG1 (41) show that in BF cells >90% of the injected HMG molecules localize in nuclei; in HeLa cells this value is slightly lower. However, because HeLa cells do not spread to a great extent, some autoradiographic grains overlying the cytoplasm result from iodinated molecules within the nucleus. Therefore, the proportion of endogenous HMG1 and HMG2 molecules bound to HeLa chromatin is higher than our data suggest. Our findings that HMG1 and HMG2 localize in nuclei are consistent with those obtained by Seyedin and Kistler using cell fractionation (46) and those of Gordon et al. (20) using an enucleation approach. Taken together, these three studies identify both HMG1 and HMG2 as chromosomal proteins. An explanation for the high levels of cytoplasmic fluorescence observed by Bustin and Neihart (6) upon staining various cultured cells with fluorescent antibody directed against HMG1 may be provided by our observation that when HeLa cells which had been microinjected with either ^{125}I-labeled HMG1 or HMG2 were fixed with cold methanol and acetone, a higher proportion of autoradiographic grains were distributed over the cytoplasm. These results indicate that the methanol-acetone fixation may permit redistribution of HMG1 and HMG2 and is, therefore, not entirely suitable for studies on these proteins.

The presence of HMG1 and HMG2 in cytoplasmic fractions is not surprising, since a number of nuclear proteins are known to leak from nuclei during aqueous cell fractionation. For example, DNA polymerase α, which is present in cytoplasmic fractions upon aqueous cell fractionation, is located within nuclei isolated by nonaqueous techniques (13, for review, reference 22). Because our autoradiographic studies show [^{125}I]HMG1 and [^{125}I]HMG2 to be intranuclear (Fig. 1), their recovery in cytoplasmic fractions clearly represents leakage during fractionation.

The affinity with which HMG1 is bound to chromatin varies from one cell type to another, and this appears to be due to differences in the chromatin rather than to differences in the

HMG1 molecules. When HeLa cells were fractionated, endogenous HMG1 and HMG2 were found mainly in the cytoplasm, whereas most of the HMG1 of calf thymus remained associated with the nuclear pellet during fractionation. That this is due to tissue, rather than species specificity, is suggested by the observation that ^{125}I-labeled HMG1 from a single species (calf), when added to homogenates of the two tissues, fractionated in each case like the endogenous HMG1.

Although we have observed apparent tight-binding forms of HMG1 and HMG2, these are probably of little physiological significance. The fact that the tight-binding fraction of injected [^{126}I]HMG1 was not within isolated BF nuclei indicates either that it is artificially produced during fractionation or that HMG1 molecules are bound to some nuclear component which is released during isolation. The possibility that [^{125}I]HMG1 was degraded and that [^{125}I]iodotyrosine was incorporated into some insoluble protein is not tenable. First, it is known that iodotyrosine is not incorporated into proteins (44); and second, both acid-urea and SDS-acrylamide electrophoresis gave no evidence for labeled proteins other than HMG1.

Grain counts obtained from cells microinjected with [^{125}I]-HMG2 suggest that 5–10% of the HMG2 molecules are localized in the cytoplasm. However, as discussed above, some cytoplasmic grains may arise from HMG2 molecules which are present in the nucleus. The HMG2 found in cytoplasmic fractions by Seyedin and Kistler could, likewise, result from redistribution of the protein during cell fractionation. However, our observation that the introduction of an unlabeled nucleus into a previously microinjected cell is followed by an equilibration of the labeled HMG2 molecules between the two nuclei provides evidence for the existence of a cytoplasmic pool of HMG2 and further demonstrates that the HMG2 molecules in this pool are in equilibrium with those bound to the chromatin. We previously found that HMG1 migrates between nuclei (41). The rapidity with which microinjected HMG1 and HMG2 enter the nucleus and the rapidity with which these proteins exit from the nucleus upon disruption of the cell are also consistent with a model in which there is an equilibrium between nuclear and cytoplasmic pools of these proteins. Thus, in contrast to the core histones which appear to be permanently associated with DNA (35, 43), microinjected HMG1 and HMG2 (and presumably endogenous HMG1 and HMG2 molecules) maintain a state of dynamic equilibrium between the cytoplasm and the chromatin. Comings and Harris have proposed that many nonhistone chromosomal proteins behave in this manner (10).

In our study we failed to detect any differences in the intracellular behavior of injected HMG1 and HMG2. This high degree of similarity between the two proteins, expected on the basis of protein structure, was somewhat surprising, given the results of Seyedin and Kistler (46). Their studies suggested that HMG1 and HMG2 would differ in the properties examined here. Proteins whose intracellular concentration fluctuates greatly in response to environmental stimuli, usually turn over rapidly (14, 15), but the half-life of HMG2 was not significantly different from that of HMG1 in either growing or nongrowing 3T3 cells. Moreover, if HMG2 plays a role in cell replication, it presumably does so by interacting with chromatin in a specific fashion. Yet, the apparent binding of HMG2 to chromatin was equivalent to that of HMG1, which has not been implicated in replication. Injected HMG1 and HMG2 displayed identical distributions upon fractionation of HeLa cells, and both molecules equilibrated between nuclei with similar kinetics. While these measurements are not extremely sensitive, they clearly suggest that HMG1 and HMG2 bind to chromatin at similar sites with association constants of the same magnitude. All of our results, then, are consistent with HMG1 and HMG2 performing closely related functions within cells. While we did not observe any growth-related differences in the degradation of HMG2 in cultured cells, it is possible that this regulatory mechanism only operates in vivo or that differential synthesis of HMG1 and HMG2 is responsible for growth-related changes in their concentration. Clearly, further work will be required to determine whether the hypothesis that HMG2 plays a role in cell replication is generally valid.

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