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Changes in Histone H1 Phosphorylation during Differentiation of Mouse Myeloid Leukemia Cells

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The possibility that phosphorylation of protein(s) is related to the differentiation of mouse myeloid leukemia cells (M1 cells) was examined by comparing phosphoproteins in M1 cells and in M1 cells treated with dexamethasone for various lengths of time. For this purpose, M1 cells or M1 cells treated with dexamethasone were incubated with $[^{32}P]H_3PO_4$ *in vivo* and fractionated into subcellular fractions, then phosphoproteins were analyzed by gel electrophoresis followed by autoradiography. The analyses revealed no changes either in proteins detectable with Coomassie blue staining or in phosphoproteins except H1 histone at the early stage of differentiation of M1 cells. Phosphorylation of H1 histone in M1 cells occurred almost exclusively on H1B. The amount of H1B decreased remarkably after dexamethasone treatment, whereas the H1B phosphorylation level increased significantly between 16 and 48 h after dexamethasone treatment and thereafter decreased.

Keywords——histone H1; phosphorylation; differentiation; myeloid leukemia cell

Phosphorylation of proteins plays an important role in the regulation of a variety of biological processes including transformation or tumorization of normal cells. In the transformation of chicken cells by Rous sarcoma viruses, the first event leading to the transformation of the normal chicken cells is the production of the transforming protein, $pp60^{src}$, with protein kinase activity.[1] Similar protein kinase activities were found to be associated with the transforming gene products of polyoma virus,[2] SV40,[3] adenoviruses,[4] Abelson murine leukemia virus,[5] and Fujinami sarcoma virus.[6] We have previously compared the endogenous phosphorylation of the cytosol and plasma membrane fractions of cells of AH-66 hepatoma, a rat hepatoma originally induced by dimethylaminoazobenzene, with that of normal rat liver cells and found that there are several qualitative and quantitative differences in phosphoproteins between them.[7]

Mouse myeloid leukemia cells (M1 cells), which are induced naturally, are suitable for studying the control mechanism involved in the biochemical events occurring in tumor cells because they are easily induced to differentiate to macrophages or granulocytes upon treatment with a low concentration of dexamethasone.[8] If phosphorylation of proteins is related to the tumorigenicity of M1 cells, it would be expected that some phosphoproteins may disappear or appear at the early stage of differentiation of M1 cells induced by dexamethasone treatment. In order to examine this possibility, M1 cells or M1 cells treated with dexamethosone for various lengths of time were phosphorylated *in vivo* and the phosphorylated proteins in these cells were analyzed by two-dimensional gel electrophoresis followed by autoradiography.

Materials and Methods

Materials——$[^{32}P]H_3PO_4$ was purchased form the Radiochemical Center, Amersham, England. Dexamethasone

was obtained from Sankyo, K.K. Eagle's minimal essential medium (MEM) was from GIBCO Laboratories, Grand Island, N.Y.

Cell Line and Cell Culture——M1 cells (clone T22) were cultured in MEM supplemented with twice the normal concentrations of amino acids and vitamins, and 10% heat-inactivated calf serum. M1 cells were induced to differentiate with 1 μM dexamethasone as described previously by Sakagami *et al.*[8]

Phosphorylation of Cells——M1 cells (1×10^8) or M1 cells treated with dexamethasone for various lengths of time (cell number $= 1 \times 10^8$) were incubated with 300 μCi [^{32}P]H_3PO_4 (200 mCi/mmol) in 2 ml of MEM free from phosphate at 37 °C for 2 h. After incubation, the cells were washed by centrifugation at $50 \times g$ for 5 min twice in MEM and three times in 5 mM Tris-HCl buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The washed cells were suspended in 5 volumes of 10 mM Tris-HCl (pH 7.5) containing 1 mM PMSF and homogenized in a tightly fitting Teflon-glass homogenizer. The suspension was centrifuged at $1000 \times g$ for 10 min. The pellets were resuspended in 2.2 M sucrose containing 1 mM $MgCl_2$ and centrifuged at $40000 \times g$ for 1 h. The supernatant after removal of nuclei from the homogenate by centrifugation at $1000 \times g$ for 10 min was further centrifuged at $105000 \times g$ for 60 min. The supernatant was used as cytosol fraction and the sediment as microsomal fraction.

Extraction of Histone H1——Histone H1 was extracted from the nuclei of M1 cells or whole cells according to the method of Lennox, Oshima, and Cohen.[9] Phosphorylated cells were homogenized in 0.4 N H_2SO_4 and 1 mM PMSF in a Teflon-glass homogenizer. The homogenate was centrifuged for 15 min at $8000 \times g$. The pellet was successively re-extracted with 0.4 N H_2SO_4 and 1 mM PMSF as described above. The combined supernatants were dialyzed against water and lyophilized. The lyophilized material was dissolved in 5% perchloric acid. After centrifugation of the solution at $18000 \times g$ for 10 min, an equal volume of 40% trichloroacetic acid was added to the supernatant. Precipitation was allowed to take place on ice for 30 min. Centrifugation at $18000 \times g$ for 10 min provided a precipitate, which was washed once with acidified acetone and twice with acetone, and dried with a stream of nitrogen.

Two-Dimensional Polyacrylamide Gel Electrophoresis of Cytosol and Microsomal Proteins——Two-dimensional gel electrophoresis of cytosol proteins was performed essentially according to O'Farrell[10] except that 1% sodium dodecyl sulfate (SDS) was added to the sample solution as described by Kaderbhei and Freedman.[11] The first dimension was run in 10-cm gels of 3.5-mm internal diameter for 15 h at 400 V. Electrode buffers were 0.01 M phosphoric acid (anode) and 0.04 M NaOH (cathode). The second dimension was run at 100 V for 8 h in a 2-mm slot in a slab gel containing 15% acrylamide and 0.1% SDS using Laemmli's discontinuous buffer system.[12] Microsomal proteins were analyzed by the same two-dimensional gel electrophoresis as used for cytosol proteins, except that 1% SDS was included in the first-dimensional gel.

Two-Dimensional Gel Electrophoresis of Nuclear Proteins——Nuclei were suspended in 10 volumes of 0.4 N H_2SO_4 containing 1 mM PMSF and homogenized in a Teflon-glass homogenizer. The supernatant, after centrifugation at $5000 \times g$ for 20 min, was dialyzed against 0.01 N HCl for 12 h, dialyzed against deionized water for 16 h, and lyophilized. Sulfuric acid-extracted proteins were analyzed by two-dimensional gel electrophoresis by the method of Yeoman, Taylor and Busch.[13] The first dimension was run in 10-cm gels of 3.5-mm internal diameter containing 10% acrylamide, 6 M urea, and 0.9 N acetic acid at 80 V for 8 h. The second dimension was run at 100 V for 24 h in a slab gel containing 12% acrylamide, 0.1% SDS, 6 M urea, and 0.1 M phosphate buffer.

Gel Electrophoresis of H1 Histone——Extracted H1 histone was analyzed by gel electrophoresis by the method of Laemmli using 15% acrylamide gel and 0.1% SDS.[12] Gels were stained with Coomassie blue R-250 and scanned at 530 nm with a Fusix type FD AIV densitometer.

Autoradiography——The gels were dried, and placed on X-ray film (Kodak RP Royal X-Omat) with intensifying screens at −80 °C for various lengths of time. For estimating relative ^{32}P incorporation into histone H1, the autoradiograph was scanned at 530 nm with the Fusix densitometer and the areas of the respective peaks of the scans were measured.

Results

Approximately 190 phosphorylated spots in the cytosol fraction (Fig. 1), 30 spots in the microsomal fraction (Fig. 2), and 20 spots in the nuclear fraction (Fig. 3) of M1 cells were detectable on the autoradiographs. In Fig. 1, the cytosolic proteins that were clearly detectable by both Coomassie blue staining and autoradiography are indicated by arrows. As is evident from this figure, most of the phosphorylated proteins are not the major proteins that are strongly stained with Coomassie blue. Moreover, a significant number of spots seen on the autoradiographs could not be detected by Coomassie blue staining on the gels. Although we did not examine whether these spots are really proteins, Rickwood, Riches, and MacGillivray[14] have proved that nearly all of the radioactive spots which are phosphorylated

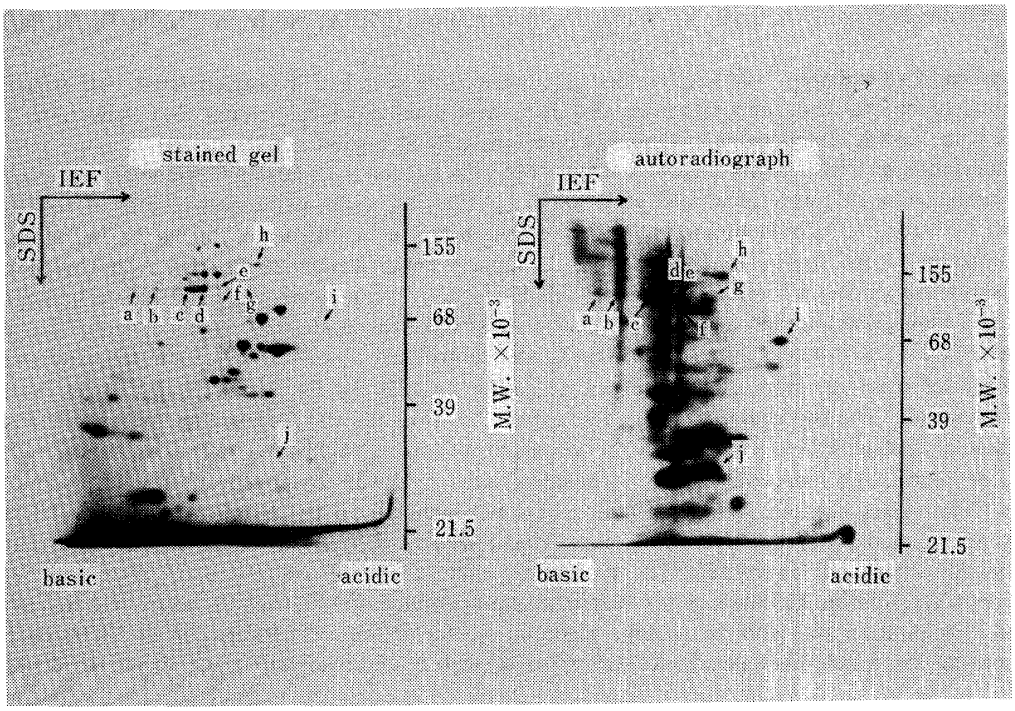

Fig. 1. Two-Dimensional Gel Electrophoretic Analysis of Cytosolic Proteins of M1 Cells

M1 cells were phosphorylated *in vivo* and fractionated as described in Materials and Methods. Proteins (250 μg) were applied to the gel and electrophoresis was carried out by a slight modification of the method of O'Farrell[10] as described in the text. The gel was stained with Coomassie blue and then autoradiographed. The arrows (a—j) indicate proteins clearly detectable by both Coomassie blue staining and autoradiography.

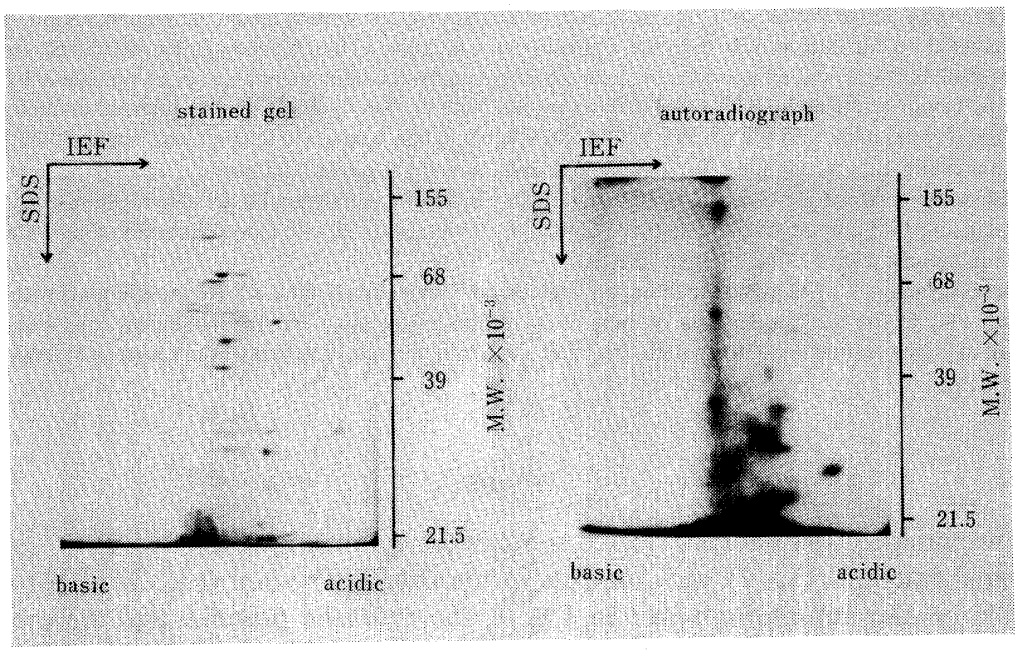

Fig. 2. Two-Dimensional Gel Electrophoretic Analysis of Microsomal Proteins of M1 Cells

Phosphorylated M1 cells were fractionated as described in Materials and Methods. Proteins (250 μg) were applied to the gel and electrophoresis was carried out by a modification of the method of O'Farrell[10] as described in the text.

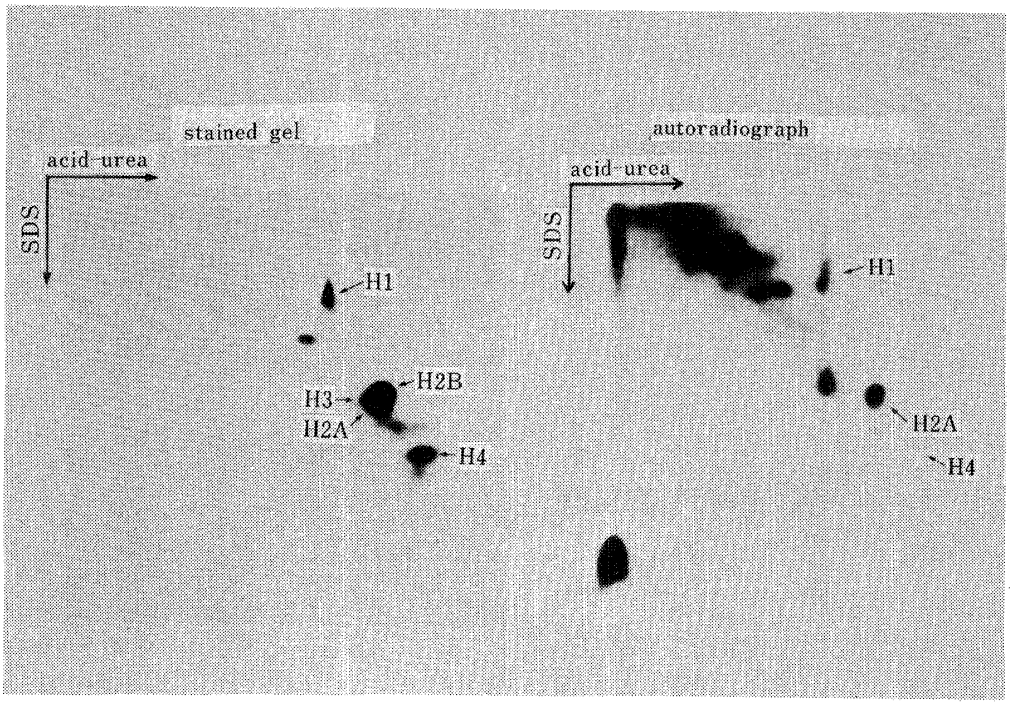

Fig. 3. Two-Dimensional Gel Electrophoretic Analysis of Nuclear Proteins of M1 Cells

Phosphorylated M1 cells were fractionated as described in Materials and Methods. Proteins (150 μg) were applied to the gel and electrophoresis was carried out by the method of Yeoman, Taylor and Busch.[13]

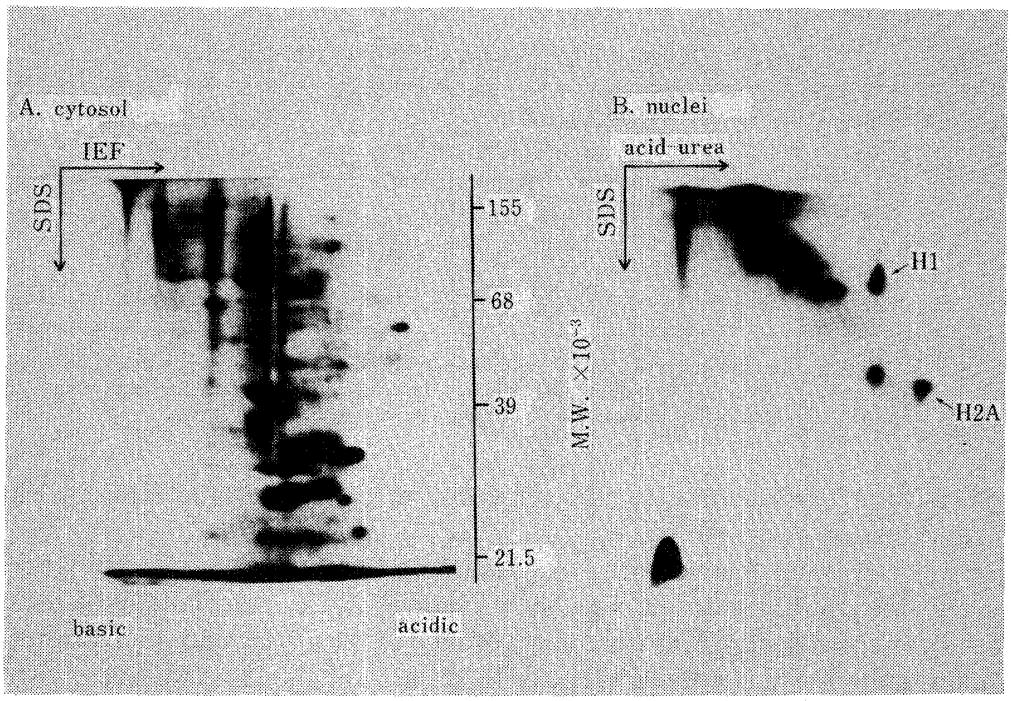

Fig. 4. Autoradiographs of Cytosolic and Nuclear Phosphoproteins of M1 Cells Treated with Dexamethasone for 16 h

Phosphorylation of M1 cells was carried out under the same conditions as in Figs. 1 and 3. Cytosolic proteins (A) were electrophoresed by a modification of the method of O'Farrell[10] and nuclear proteins (B) by the method of Yeoman, Taylor and Busch,[13] and then autoradiography was carried out.

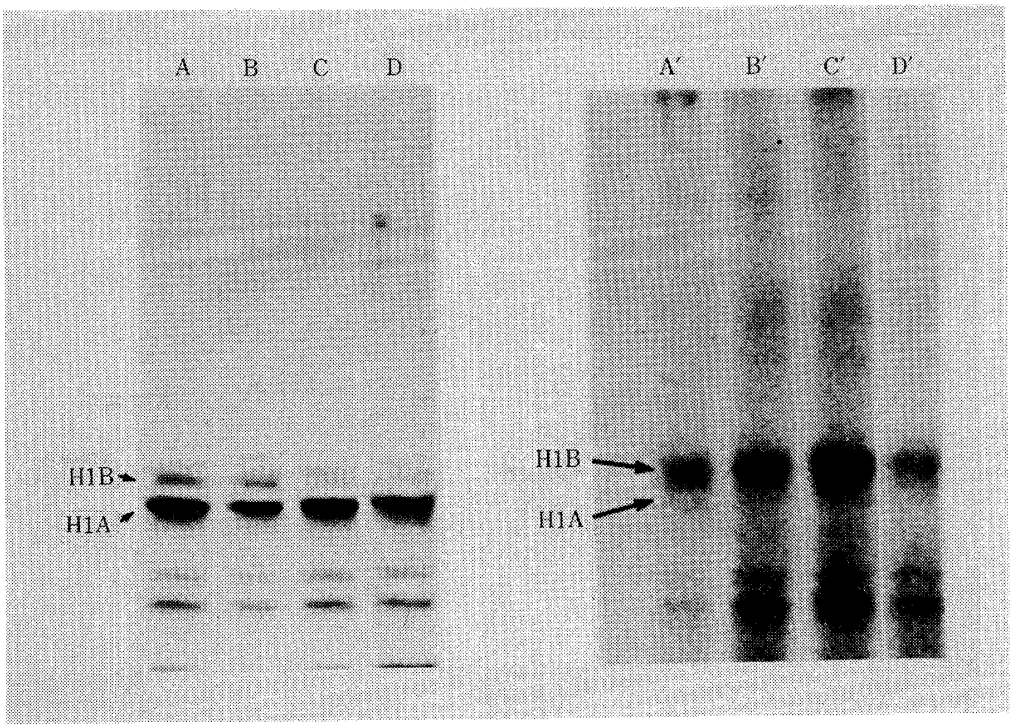

Fig. 5. SDS–Polyacrylamide Gel Electrophoretic Analysis of Perchloric Acid-Soluble Proteins Obtained from M1 Cells or M1 Cells Treated with Dexamethasone

Electrophoresis was carried out according to the method of Laemmli.[12] The four lanes for each sample each contained 20 μg of protein. The gel was stained with Coomassie blue (lanes A—D), and then autoradiographed (lanes A′—D′): A and A′, M1 cells without treatment; B and B′, 16 h after dexamethasone treatment; C and C′, 40 h after the treatment; D and D′, 64 h after the treatment.

TABLE I. Relative Incorporation of ^{32}P into Histone H1B

Time (h)	Relative ^{32}P incorporation[a]	Relative amount of H1B[a]
0	1.00	1.00
16	1.46	0.80
40	1.72	0.61
64	0.87	0.32

a) The relative amounts of H1B and ^{32}P incorporation into it were estimated by scanning the radioactive and Coomassie blue stained bands in Fig. 5 with a densitometer and measuring the areas of the peaks of the scans as described under Materials and Methods.

in isolated nuclei of various mouse tissues are due to phosphorylated proteins rather than other phosphorylated macromolecules.

When M1 cells were treated with 1 μM dexamethasone for 72 h, they differentiated into macrophages and granulocytes and significant changes in the morphology of the cells were observed under a microscope. This treatment resulted in the appearance and disappearance of many proteins and phosphoproteins in the cells (results not shown). However, when the time of dexamethasone treatment was shortened to 16 h, we could not detect either appearance or disappearance of any phosphoprotein or protein, nor was there any change in the state of phosphorylation of proteins in the cytosolic (compare Fig. 4A with Fig. 1) and microsomal fractions (result not shown). Upon close examination of the phosphoproteins in the nuclear fraction, however, we detected an apparent increase in the state of phosphorylation of histone

H1 (compare Fig. 4B with Fig. 3). By contrast, it can be seen that the phosphorylation of histone H2A was not changed.

In order to minimize the opportunity for phosphorylation and dephosphorylation of histone to occur during homogenization and centrifugation, histone H1 was directly extracted from the cells with 0.4 N H_2SO_4 as described in Materials and Methods. Histone H1 is known to be composed of several subtypes. According to Tan *et al.*,[15] histone H1 is resolvable into two principal subtypes, H1A and H1B, by SDS-gel electrophoresis. As is evident from Fig. 5, the radioactive phosphate was incorporated almost exclusively into the H1B band. Phosphorylation of H1A was nearly undetectable. The extent of ^{32}P incorporation into H1B increased with the passage of time up to 40 h and then decreased (lanes A′—D′ in Fig. 5 and Table I). These results were reproducibly observed. In different experiments, however, the time of maximum phosphorylation varied between 8 and 40 h after dexamethasone treatment of M1 cells. It should be noted that the intensity of the phosphorylated band on the autoradiogram in Fig. 5 is not in parallel with the amount of H1B estimated from the Coomassie blue staining on the gel in the same figure. The amount of H1B decreased progressively with the passage of time after dexamethasone treatment (lanes A—D in Fig. 5 and Table I). This indicates that the levels of phosphorylation of H1B change independently of the change in the amount of H1B during the differentiation of M1 cells induced by dexamethasone treatment.

Discussion

Although about 240 proteins are phosphorylated in M1 cells *in vivo*, neither appearance or disappearance of phosphoproteins and proteins nor change in the state of phosphorylation was observed except in histone H1 at the early stage of dexamethasone treatment. Therefore, we conclude that the appearance and disappearance of several proteins and phosphoproteins other than histone H1 after the differentiation of M1 cells is the result but not the cause of the differentiation. However, the possibility that proteins or phosphoproteins which are not detectable by Coomassie blue staining or by autoradiography may change during the differentiation of M1 cells is not excluded.

Histone H1 is known to play an important role in the control of chromatin structure and function. Phosphorylation of histone H1 has been observed in rat liver cells in response to stimulation by hormones such as glucagon and insulin.[16] Extensive phosphorylation of histone H1 has also been shown to occur during S-phase and late G2-phase just preceding M-phase.[17] Based on a study of phosphorylation of the nuclear proteins of *Physarum polycephalum*, Bradbury, Inglis and Matthews[18] have proposed that phosphorylation of histone H1 may trigger chromosome condensation. The dependence of the histone H1 phosphorylation on the cell cycle is compatible with the marked phosphorylation of histone H1 in rapidly growing and dividing cells.[19]

Since histone H1 is composed of several subtypes which are slightly different in primary structure and molecular weight, these subtypes and phosphorylation of these subtypes are believed to play different roles in regulating chromatin structure and function. Ajiro, Borun and Cohen[17a] showed that H1B was phosphorylated by over twice as much as H1A at all phases of the cell cycle of HeLa S-3 cells and proposed that H1B has more phosphorylated sites than H1A. In accordance with the result obtained by Ajiro *et al.*,[17a] the present study shows that histone H1B is almost exclusively phosphorylated. Moreover, the phosphorylation of H1B increased significantly between 16 and 40 h after dexamethasone treatment and thereafter decreased. We could not determine in the present study whether the ratio of phosphorylated H1B to unphosphorylated H1B or the number of phosphorylated sites in phosphorylated H1B is increased in M1 cells after dexamethasone treatment. The pheno-

menon observed in the present study is similar to that found by Harrison *et al.*[20] who showed that isoproterenol treatment of rat C6 glioma cells results in increased phosphorylation of histone H1. One interpretation of the increased phosphorylation of histone H1 induced by dexamethasone treatment is that M1 cells treated with dexamethasone may be ready to synthesize chromatin proteins indispensable for differentiation of the cells. Nagata and Ichikawa[21] suggested that activation of a certain genome is needed for the induction of phagocytosis by dexamethasone treatment of M1 cells. Further work is necessary to elucidate the molecular mechanism of differentiation of M1 cells induced by dexamethasone.

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