Journal of Cellular Biochemistry 51:175–180 (1993)

Expression of Histone and Alkaline Phosphatase Genes in UMR 106-01 Rat Osteoblast-Like Cells Exposed to the Hoechst Dye H33342

M.H. Zheng, S. Wysocki, J.M. Papadimitriou, and D. Wood

Departments of Orthopaedic Surgery (M.H.Z., S.W., D.W.) and Pathology (J.M.P.), University of Western Australia, Nedlands, 6009, Australia

Abstract The fluorescent Dye H33342 (H342) is a *bis*-benzimidazole used for intravital fluorescent staining. In this report, we found that H342 completely abolished histone 2a mRNA but had no effect on alkaline phosphatase gene expression and protein synthesis in UMR 106-01 rat osteoblast-like cells. The complete loss of histone 2a mRNA occurred after only 20 min of treatment with H342. This effect is unlikely to be a result of inhibition of DNA synthesis, which was only partly suppressed. The mechanism of the action of H342 on histone 2a mRNA is presently unknown.

Key words: fluorescent dye H33342, histone, gene expression, cell cycle, osteoblast

The fluorescent Dye H33342 (H342) is a *bis*-benzimidazole derivative which has been employed for intravital fluorescent staining [Gregoire et al., 1984; Brenan et al., 1984]. Recently, H342 has also been used in some instances to encourage cell differentiation. For example, H342 induces a nullipotent cell line to differentiate towards endodermal lineage [Steuer et al., 1990]. Cell differentiation induced by H342, however, only occurs in stem cells and not in non-stem cells such as 3T3 fibroblasts [Steuer et al., 1990]. It is known that H342 binds selectively at AT-rich regions of DNA and arrests cells at the S/G_2 phase of the cell cycle [Murray and Martin, 1988]. The precise mechanism of the effect of H342 on cell differentiation, however, is unknown.

Research in our laboratory has focused on osteoblast and osteoclast differentiation [Zheng et al., 1991; Zheng et al., in press]. We have attempted to use H342 both as an intracellular fluorochrome in vivo and as a differentiation agent in investigations of osteoclast and osteoblast precursor cells. In the course of our investigations, however, we have found that the action of H342 had unexpected effects. In this study, we show that H342 inhibited cell cycle-regulated histone gene expression but did not have any effect on alkaline phosphatase gene expression and protein synthesis in UMR106-01 rat osteoblast-like cells.

Received July 30, 1992; accepted September 18, 1992.

Address reprint requests to Dr. M.H. Zheng, Department of Orthopaedic Surgery, QEII Medical Centre, University of Western Australia, Nedlands 6009, W.A., Australia.

MATERIALS AND METHODS

Materials

H342 was purchased from Calbiochem-Behring (Kingsgrove, Australia). ^{32}P-dCTP and a nick translation kit were supplied by Amersham (Australia). All reagents were of the highest grade available commercially. The histone 2a (H2a) cDNA probe (H2a-614) was kindly provided by Dr. W.F. Marzluff, Department of Chemistry, Florida State University [Myra et al., 1989]. The human alkaline phosphatase probe (pLBK8B/E5') and the glyceraldehyde 3' phosphate dehydrogenase (GADPH) probe (pH-cGAP) were purchased from American Type Culture Collection. A UMR 106-01 rat osteoblast-like cell line was kindly provided by Professor T.J. Martin, St Vincent's Institute of Medical Research, Melbourne, Australia.

Cell Culture

The UMR106-01 rat osteosarcoma cell line was grown in EMEM medium containing 10% fetal bovine serum, 10% non-essential amino

acids, 14 mM $NaHCO_3$, 1.2 mM L-glutamine, 0.1 mg/ml of streptomycin, and 100 units/ml of penicillin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were used between passages 6 and 12 and seeded at a density of 0.5–1.0 × 10^4 cells per cm^2 in 75 cm^2 flasks. Fresh culture medium with or without H342 was added to confluent cultures in 75 cm^2 flasks for various times before assay.

DNA Synthesis

DNA synthesis was studied by examining [^{3}H]thymidine incorporation into DNA. The UMR106-01 rat osteosarcoma cells were grown in 96 well culture plates (Nunc, Denmark) in EMEM medium at 37°C in a humidified atmosphere of 95% air and 5% CO_2. [Methyl-^{3}H] thymidine (specific activity 40–60 Ci/mmol; Amersham, Australia) was added to culture media to yield a final activity of 1 μCi/ml during the last 30 min of the culture period. At the end of the incubation, 100 μl of 1 M NaOH was added to the media. Thymidine incorporation was examined by liquid scintillation counting. The DNA synthesis inhibitor, hydroxyurea, was used at a concentration of 0.5 μM to compare its inhibitory effect with H342. Results were expressed as counts per minute (cpm) of [^{3}H]thymidine incorporated into DNA per well.

Total Protein Synthesis

Total protein synthesis was determined by studying the incorporation of [2,3-^{3}H] proline (specific activity 20–40 Ci/mmol, Amersham, Australia). The UMR106-01 rat osteosarcoma cells were grown in 96 well culture plates (Nunc, Denmark) in EMEM medium at 37°C in a humidified atmosphere of 95% air and 5% CO_2. [2,3-^{3}H] proline was added to the media to yield a final activity of 5 μCi/ml for the last 30 min of the culture period. Proline incorporation was terminated by adding 1 M NaOH. The cell fractions were harvested and examined by liquid scintillation counting. Results were expressed as cpm of [^{3}H]proline incorporated into total protein.

RNA Extraction and Northern Blot Analysis

Total cellular RNA from two 75 cm^2 culture flasks was obtained by cell lysis in 5 M guanidinium thiocyanate and then precipitated with 8 M LiCl overnight, and washed with 4 M LiCl/8M urea [Cathala et al., 1983]. The RNA was extracted with phenol/chloroform and precipitated in isopropyl alcohol for 2 h at −20°C. The RNA pellets were washed with 70% ethanol and then stored at −70°C. From each sample 15 to 25 μg of total cellular RNA was electrophoresed under denatured conditions in a 1% agarose gel. The RNA was then vacuum-blotted onto nitrocellulose filters (Hybond N+ Amersham). The bound RNA was hybridized with cDNA probes which had been labelled with ^{32}P-dCTP by nick translation. The bands were visualized by autoradiography on Kodak X-ray film and photographs are presented in Figures 4, 5, and 6. Because the amount of RNA in each sample loaded into agarose gels was not necessarily the same, both intensity of ethidium bromide staining of the 28S and 18S ribosomal RNAs and the expression of housekeeping gene mRNA, GADPH mRNA, were used as internal controls to allow for variation.

RESULTS

Morphology

Figure 1 shows the effect of H342 on the morphology of UMR 106-01 cells. The cells treated with H342 at a concentration of 0.5 μM for 20 min adopted a polygonal form. They were no longer able to grow as aggregates and the cells separated from one another. The morphology of nuclei in treated cells was however unchanged.

DNA and Total Protein Synthesis

H342 caused a dose-related inhibition of [^{3}H]thymidine incorporation into DNA in the UMR106-01 rat osteosarcoma cells treated for up to 6 h. At a concentration of 1 μM inhibition was almost complete and was similar to the effects of hydroxyurea, a powerful inhibitor of DNA synthesis. However, when the concentration of H342 was reduced to 0.5 μM, DNA synthesis in these cells was only partly inhibited (Fig. 2). In addition, H342 treatment at a concentration of 0.5 μM for 6 h did not have any effect on total protein synthesis in the UMR106-01 rat osteosarcoma cells (Fig. 3).

Gene Expression of Histone and Alkaline Phosphatase

As shown in Figure 4, incubation of UMR106-01 osteoblast-like cells for 20 min with different doses of H342 resulted in loss of H2a mRNA. At a concentration of 1 μM the loss was

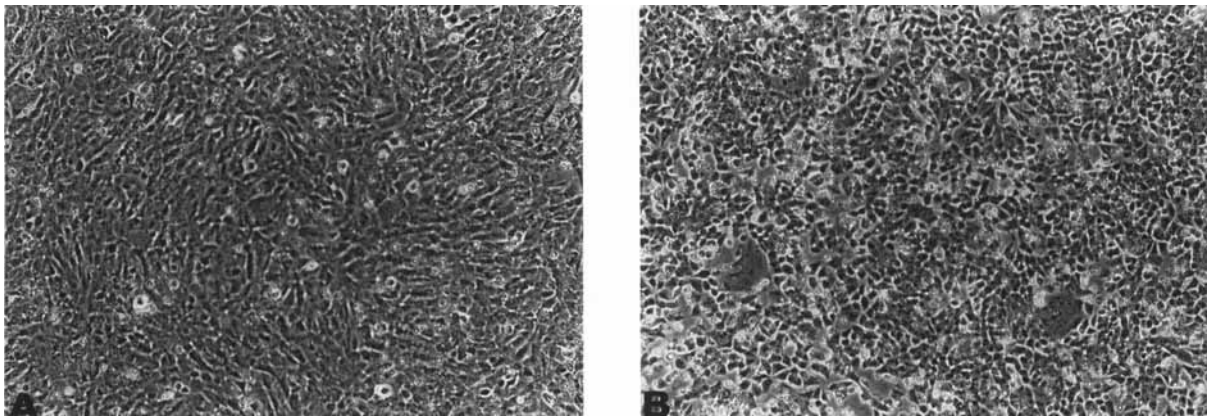

Fig. 1. Morphology of UMR 106-01 rat osteoblast-like cells incubated with vehicle (**A**) or with H342 at a concentration of 0.5 μM for 20 min (**B**). The cells adopt a polygonal form and are no longer able to grow as aggreates (×100).

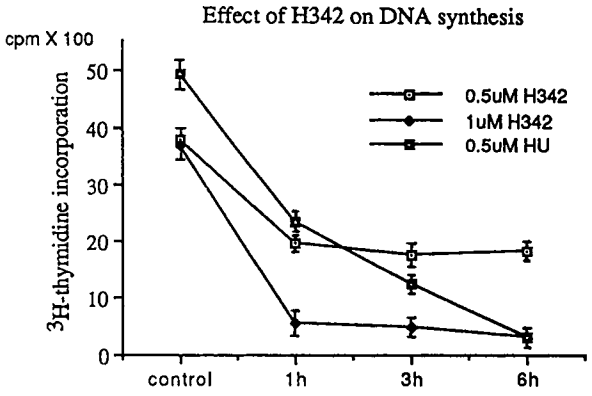

Fig. 2. Effect of H342 on [^{3}H] thymidine incorporation into DNA in UMR 106-01 osteoblast-like cells. The cells were incubated with H342 at concentration of 1 μM and 0.5 μM and hydroxyurea at a concentration of 0.5 μM, respectively, for the indicated period of time. H342 at a concentration of 0.5 μM did not totally abolish DNA synthesis. Six samples were assayed in experiments at each time point. Results were expressed as the mean ± SD of triplicate experiments.

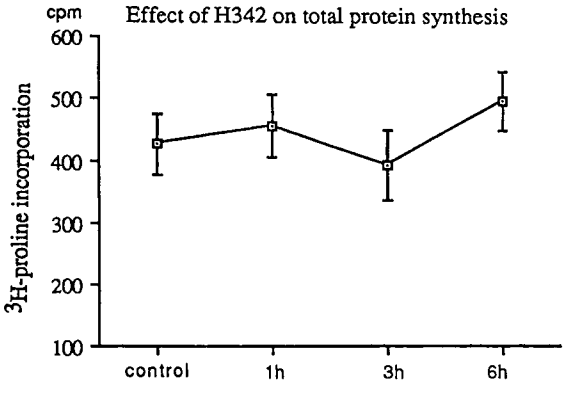

Fig. 3. Effect of H342 on [^{3}H] proline incorporation into total protein in UMR 106-01 osteoblast-like cells. The cells were incubated with H342 at a concentration of 0.5 μM for the indicated period of time. H342 at a concentration of 0.5 μM did not affect total protein synthesis. Twelve samples were assayed in each experiments at each time point. Results were expressed as the mean ± SD of duplicate experiments. ($P > 0.05$ between groups.)

almost complete. There was even evidence of some loss when the concentration was reduced to 0.1 μM. H342, however, did not change the expression of GADPH, indicating that the transcription of the housekeeping gene was unaffected by the treatment. Figure 5 demonstrates the time course (up to 6 h) of the effect of H342 on H2a mRNA at a concentration of 0.5 μM. Even though there was variability in the amounts of RNA at each time point, detected by both the ethidium bromide staining of ribosomal RNAs (not illustrated) and the expression of GADPH mRNA (Fig. 5, bottom), it was still obvious that loss of H2a mRNA was noticeable after 30 min and was complete after 1 h of H342 treatment. Over the same time period the expression of the alkaline phosphatase gene was unchanged. To determine whether the effect on H2a mRNA was reversible, cells were treated with H342 for 2 h at a concentration of 0.5 μM and then cultured in fresh medium. After 3 to 6 h, evidence of recovery of H2a gene expression was obtained (Fig. 6).

DISCUSSION

Two classes of histone genes are found in most eukaryotic cells [Osley 1991; Weintraub 1984; Smith et al., 1984]. The most abundant

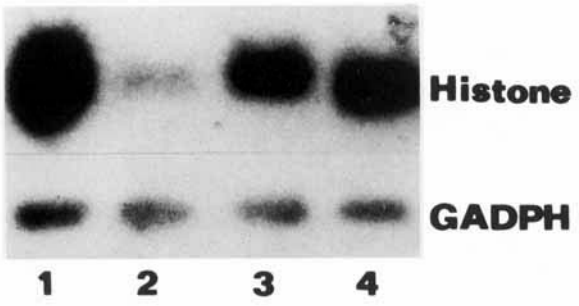

Fig. 4. Dose response of H342 inhibitory effect on H2a gene expression in UMR 106-01 cells. RNA was separated on an agarose gel, transferred to nitrocellulose, and hybridized with H2a (top) and GADPH (bottom). Lane 1, cells without H342; lanes 2 to 4, cells treated with H342 at doses of 1 μM, 0.5 μM, and 0.1 μM, respectively, for 20 min. The levels of H2a gene expression decrease with increasing doses of H342. This figure is representative of duplicate experiments.

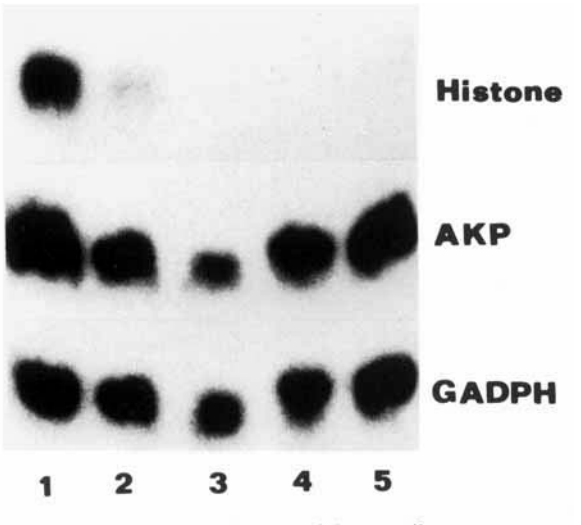

Fig. 5. Time course of H342 inhibitory effect on H2a gene expression in UMR 106-01 cells. The concentration of H342 was 0.5 μM. RNA was separated on an agarose gel, transferred to nitrocellulose, and hybridized with H2a (top), alkaline phosphatase (AKP, middle), and GADPH (bottom). Lane 1, cells without H342; lanes 2 to 5, cells exposed to H342 for 30 min, 1, 3, and 6 h, respectively. The inhibition occurs after 30 min and it is complete after 1 h. The expression of AKP is unchanged, compared to the housekeeping gene, GADPH. This figure is representative of triplicate experiments.

class contains the replication-dependent histone genes (H2a, H2b, H3, and H4) and the genes coding for this class of histones share regions of homologous sequences [Graves et al., 1985; Jacob, 1976]. It has been shown that expression of the replication-dependent histone genes is restricted to the S phase of the cell cycle and is dependent on the presence of replicating DNA [Morris et al., 1991; Plumb et al., 1984]. The

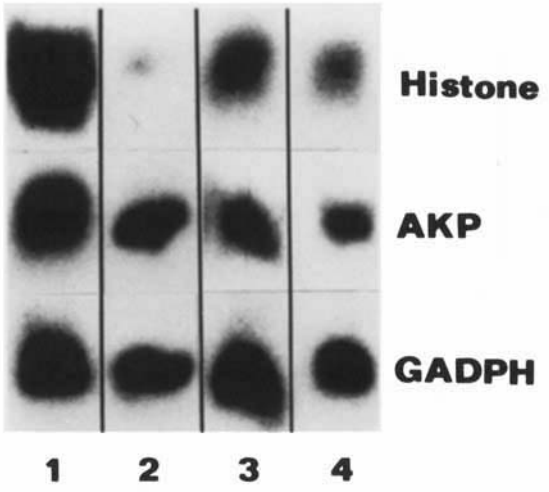

Fig. 6. Reversibility of H342 inhibition of H2a gene expression in UMR 106-01 cells. RNA was separated on an agarose gel, transferred to nitrocellulose, and hybridized with H2a (top), AKP (middle), and GADPH (bottom). Lane 1, cells without H342; lane 2, cells exposed to H342 at a dose of 0.5 μM for 2 h; lanes 3 and 4, cells incubated in fresh medium for 3 and 6 h after 2 h exposure to H342 at a dose of 0.5 μM. Recovery of H2a gene expression was found 3 h after removal of H342. The expression of AKP is unchanged. This figure is representative of duplicate experiments.

mRNAs for this class of histones therefore accumulate to maximal levels during the S phase of the cell cycle [Morris et al., 1991], and H2a expression has been often used as being representative of the synthesis of replication-dependent histone proteins [Majesky et al., 1990].

In this report, we have demonstrated that H342 effectively reduces the level of H2a mRNA. The concentration used approximates that for optimal intravital staining [Gregoire et al., 1984; Brenan et al., 1984]. Previous investigation indicated that the inhibition of histone gene expression is linked to the inhibition of DNA synthesis [Osley, 1991]. However, the concentration of H342 (0.5 μM) which induced the complete loss of H2a mRNA in our study did not abolish DNA synthesis. It therefore appears that the effect of H342 on H2a mRNA may not be secondary to the inhibition of DNA synthesis; in fact the diminished DNA synthesis may be the result of the loss of H2a mRNA.

The effect on H2a gene message was readily detectable after 20 min of treatment with H342. If the reduced levels of H2a mRNA are caused by inhibition of gene expression then this indicates that the half-life of histone 2a mRNA in osteoblast-like cells is between 10–15 min. This

result is consistent with reports that the half-life of most of the histone gene mRNA is approximately 15 min [Morris et al., 1991; Heintz et al., 1983].

At this stage, however, the mechanism by which H342 reduces histone gene mRNA is unclear. It has been suggested that three major processes contribute to the regulation of histone mRNA: transcription, pre-mRNA processing, and mRNA stability. In HeLa cells, however, it has been shown that although histone mRNA levels can rise or fall some 10- to 20-fold during the cell cycle, histone gene transcription only changes 3- to 5-fold [Majesky et al., 1990]. This difference between vastly increased levels of the histone mRNA and only a moderate increase in the histone gene transcription suggests that a post-transcriptional mechanism is involved in the regulation of the level of this message; the presence of a stem-loop structure at the 3′ end of all replication-dependent histone mRNAs may target them for degradation via an unknown mechanism [Pandey and Marzluff, 1987]. It is possible, therefore, that an alteration in a degradative mechanism may partly account for the results obtained in our experiments.

H342 has been reported to be relatively non-toxic to cells [Gregoire et al., 1984; Brenan et al., 1984]. It has been used as an intracellular fluorochrome to trace migration of cells in vivo [Brenan et al., 1984] and to separate cells on the basis of their relative DNA content [Arndt-Jorin and Jovin, 1977]. In addition, H342 has been shown to induce differentiation in a variety of stem cells; this effect however does not occur in terminally differentiated immortalized cells, such as 3T3 cells [Steuer et al., 1990]. In our experiments, although H342 changed the morphology of UMR 106-01 rat osteoblast-like cells, we could not find any changes in the expression of alkaline phosphatase, which is a marker of osteoblast differentiation and enhances mineralization by converting inorganic phosphate into organic phase calcium phosphate [Zheng et al., in press] . These results therefore indicated that H342 does not encourage osteoblast differentiation in UMR 106-01 rat osteoblast-like cells.

Recently, a new growth factor family termed osteogenic growth peptides (OGP) was found to be identical to the C-terminus of histone H4. OGPs are present in normal serum and induce bone formation [Bab et al., 1992]. Since H342 inhibits H2a gene expression and the genes coding for H2a and histone H4 share homologous sequences [Graves et al., 1985; Jacob, 1976], H342 may prove to be a useful tool for the analysis of the role of histones and related growth factors in osteogenesis.

ACKNOWLEDGMENTS

We are grateful to Dr. E.A. Wang at the Genetics Institute for useful discussions and comments on the manuscript. This work was supported by grants from the Medical Research Fund of Western Australia and the University of Western Australia. Dr. M.H. Zheng is a holder of the Wyn Spence Fellowship in Medical Research.

REFERENCES

Arndt-Jorin DJ, Jovin TM (1977): Analysis and sorting of living cells according to deoxyribonucleic acid content. J Histochem Cytochem 25:585–589.

Bab I, Gazit D, Chorev M, Muhlrad A, Shteyer A, Greenberg Z, Namdar M, Kahn A (1992): Histone H4-related osteogenic growth peptide (OGP): A novel circulating stimulator of osteoblastic activity. EMBO J 11:1867–1873.

Brenan M, Parish CR (1984): Intracellular fluorescent labelling of cells for analysis of lymphocyte migration. J Immun Methods 74:31–38.

Cathala G, Savouret JF, Mendez B, West BL, Karin M, Mertial JA, Baxter JP (1983): A method for isolation of intact, translationally active ribonucleic acid. DNA 2:329–335.

Graves RA, Wellman SE, Chiu IM, Marzluff WF (1985): Differential expression of two clusters of mouse histone genes. J Mol Biol 183:179–194.

Gregoire M, Hernandez-Verdun D, Bouteille M (1984): Visualization of chromatin distribution in living PTO cells by Hoechst 33342 fluorescent staining. Exp Cell Res 152:38–46.

Heintz N, Sive H, Roeder RG (1983): Regulation of human histone gene expression: kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during the Hela cell cycle. Mol Cell Biol 3:539–550.

Jacob E (1976): Histone gene reiteration in the genome of mouse. Eur J Biochem 75:275–284.

Majesky MW, Reidy MA, Bowen-Pope DF, Hart CE, Wilcox JN, Schwartz SM (1990): PDGF ligand and receptor gene expression during repair of arterial injury. J Cell Biol 111:2149–2153.

Morris TO, Weber LA, Hickey E, Stein GS, Stein JC (1991): Changes in the stability of a human H3 histone mRNA during the Hela cell cycle. Mol Cell Biol 11:544–553.

Murray V, Martin RF (1988): Sequence specificity of ^{125}I-labelled Hoechst 33258 in intact human cells. J Mol Biol 201:437–442.

Myra MH, Chodchoy N, Marzluff WF (1989): The mouse histone H2a2 gene from chromosome. Nucl Acids Res 17:8876.

Osley MA (1991): The regulation of histone synthesis in the cell cycle. Annu Rev Biochem 60:827–861.

Pandey NB, Marzluff WF (1987): The stem-loop structure at the 3′ end of histone mRNA is necessary and sufficient for regulation of histone mRNA stability. Mol Cell Biol 7:4557–4559.

Plumb M, Marashi F, Green L, Zimmerman A, Zimmerman S, Stein J, Stein G (1984): Cell cycle regulation of human histone H1 mRNA. Proc Natl Acad Sci USA 81:434–438.

Smith BJ, Harris MR, Sigournay CM, Mayes ELV, Bustin M (1984): A survey of H1- and H5-like protein structure and distribution in higher and lower eukaryote. Eur J Biochem 138:309–317.

Steuer B, Breuer B, Alonso A (1990): Differentiation of EC cells *in vitro* by the Fluorescent dye Hoechst 33342. Exp Cell Res 186:149–157.

Weintraub (1984): Histone-H1 dependent chromatin superstructures and the suppression of gene activity. Cell 38:17–27.

Zheng MH, Nicholson GC, Warton A, Papadimitriou JM (1991): What's new in osteoclast ontogeny (review). Path Res Pract 187:117–125.

Zheng MH, Wood DJ, Papadimitriou JM: What's new in the role of cytokines in osteoblast proliferation and differentiation (review). Pathol Res Pract (in press).