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THE EFFECTS OF DEXAMETHASONE ON HISTONE PHOSPHORYLATION IN L CELLS[†]

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Summary: Phosphorylation of the histones of L cells was examined during early G1 and S phases after exposure of the cells to 1 μM dexamethasone. H2A and H1 were the only histones phosphorylated during either phase, and the extent of phosphorylation was greater during S phase. The steroid hormone increased the amount of phosphorylation of H2A during both phases, but had no effect on that of H1. This increase in H2A phosphorylation due to the steroid hormone could be part of the mechanism by which the hormone exerts its effects on genetic expression.

INTRODUCTION

The glucocorticoid hormones influence the transcriptional activities of sensitive cells. They stimulate the synthesis of certain species of RNA, induce specific enzymes, and suppress vital functions in a variety of tissues (1,2,3,4). They inhibit the proliferation of strain L mouse cells by interfering with growth-related processes at an early stage of the G1 traverse (5). Among the unanswered questions about steroid action are how the hormone-receptor complex interacts with chromatin and what events follow that interaction to influence genetic expression.

The histones are considered to be important determinants of the structure of chromatin (6), but any possible direct involvement in other functions has not been shown. They are structurally stable proteins *in situ*, capable of undergoing selective covalent modifications which, in some instances, have been linked to the proliferative state of the cell (7,8) and to specific phases of the cell cycle (9,10). Transient covalent modifications of proteins are known to affect the expression of certain enzymes (11) and, consequently,

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are central to metabolic regulation. Similar modifications of histones could affect chromatin structure so as to influence genetic expression.

We report here that the synthetic glucocorticoid, dexamethasone, causes an increase in the phosphorylation of histone H2A of L cells during G1 and S phases.

METHODS

NCTC clone 929 mouse cells, strain L (Earle), were grown in half-gallon disposable glass roller bottles in a synthetic medium which is a modification of Waymouth's MD 705/1 (5). Mitotic cells were collected by selective mitotic detachment (5). Synchrony achieved by this method was 99 percent.

The synchronized cells thus obtained were plated in 75 cm^2 Falcon flasks at a density of 2.6 x 10^5 cells cm^{-2} using five flasks per variable. The cells were incubated at 37°C. for 1 3/4 h for early G1 phase cells or 14 h for S phase cells. At these times the medium was replaced with 10 ml of 5% phosphate medium (0.18mM), with or without 1 μM dexamethasone. Hormone pretreatment lasted for 30 min, at which time {^{32}P}phosphate (New England Nuclear, Boston, MA) was added to a final concentration of 100 μCi ml^{-1} (562 Ci $mole^{-1}$). The cells were then incubated further at 37°C for 1 1/2 h.

The cells were harvested by scraping with a rubber policeman and collected in ice-cold saline. They were pelleted by centrifuging at 800 x g for 2 min. Nuclei were isolated by the method of Penman (12), with the modification that before detergent treatment they were soaked twice for 10 min each in 0.14M NaCl, 10mM Tris, 1mM $MgCl_2$, pH 8.0. The nuclei were suspended in 5mM EDTA, 0.1mM dithiothreitol, 1mM glycine, 0.05M sodium bisulfite, pH 8.0 and sheared at 0°C at top speed in a Sorvall Omnimixer for 5 min. After centrifugation at 800 x g for 2 min the supernatant fluid, which contains sheared chromatin, was collected and made 0.4N in H_2SO_4. This extraction mixture was incubated at 0°C for 30 min, then centrifuged at 17000 x g for 20 min and the supernatant fluid kept.

The acid-extracted material was dialyzed at 4°C against 1% acetic acid followed by deionized distilled water. Histone concentration was determined by the method of Lowry et al. (13) or by turbidity assay (14). The histone solutions were lyopholized and the powder dissolved in 0.9N acetic acid, 20% sucrose for electrophoresis. Polyacrylamide gel electrophoresis was performed using either the acid-urea gels of Panyim and Chalkley (15) or the Triton gels of Zweidler and Cohen (16) as adapted by Gurley and Walters (17). Gel dimensions were 100 mm x 5 mm. Fifty μg of histone was applied to each gel. Electrophoresis conditions are given in the figure legends. Gels were stained for 2 min with 0.75% Amido Black in 6% acetic acid and 47% methanol and destained in the same solvent. They were scanned at 600 nm on a Gilford model 240 spectrophotometer with linear transport. Positions of histone migration on Triton gels were determined by fractionation of whole histone using the modified Johns technique of Oliver et al. (18).

Preparation for liquid scintillation counting followed the method of Balhorn and Chalkley (19) with the modification that 1 mm gel slices were used. Radioactivity measurements were made using a Packard model 3310 scintillation counter. Specific activity determinations were made by triangulating areas of the curves.

RESULTS

Dexamethasone Effects During Early G1 Phase

Histones of L cells labeled during early G1 with {^{32}P}phosphate were resolved on acid-urea gels. Significant amounts of radioactivity were found

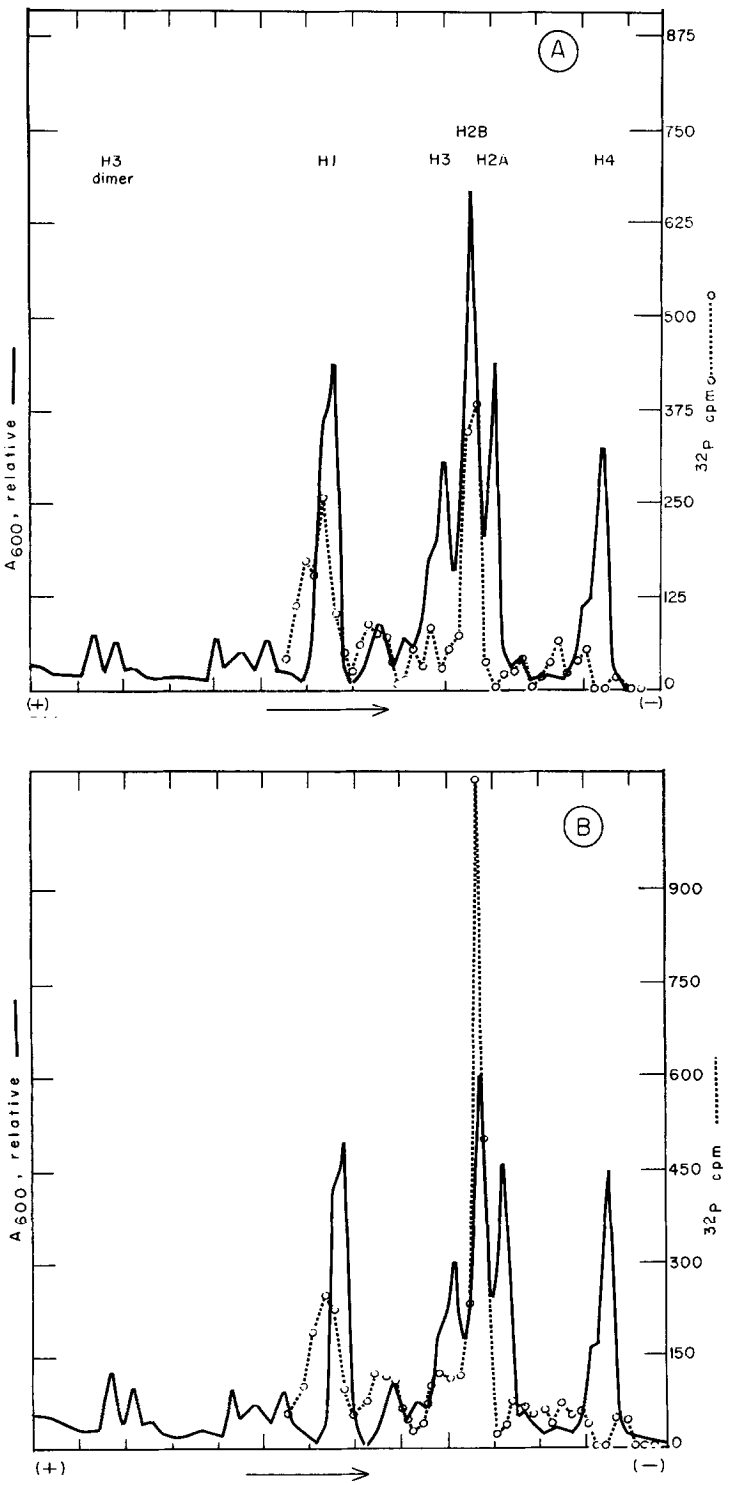


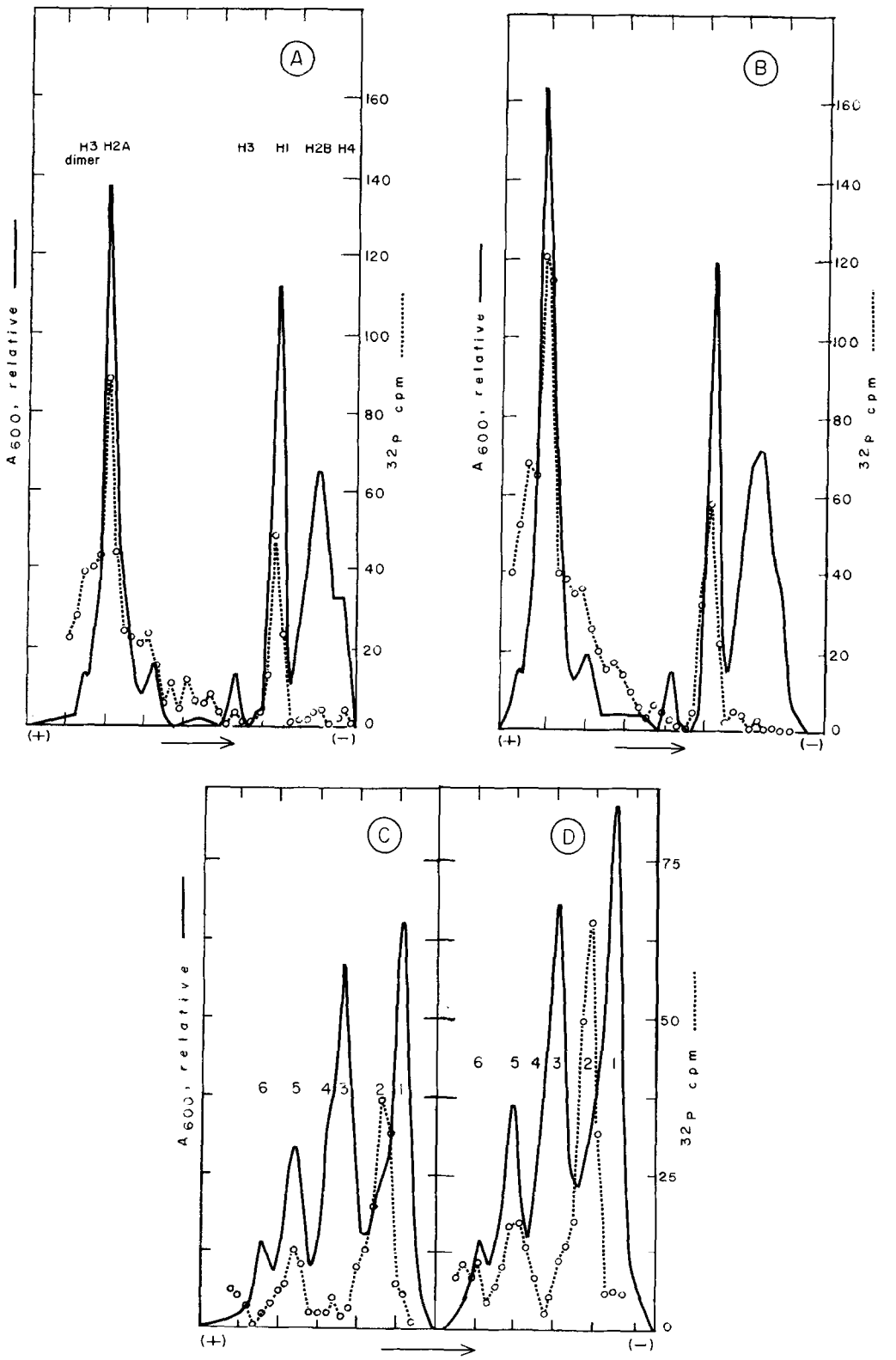
Fig. 1. G1 phase histones, acid-urea gels. (A) Control, showing positions of histone migration in these gels. (B) Dexamethasone-treated. Electrophoresed for 8 h at 2 mA/gel.

to be associated only with the H1 and H2B regions of the gels (Fig. 1A). If the cells were exposed to dexamethasone the only observable change was an increase in the amount of radioactivity associated with the H2B region (compare Figs. 1A and 1B). The results of Gurley and Walters (17) indicated that in acid-urea gels the H2B-associated radioactivity was due to a phosphorylated form of H2A. To resolve H2A and H2B definitively polyacrylamide gels containing Triton X-100 were used (16,17). In these gels significant amounts of radioactivity were found to be associated only with H1 and H2A (Fig. 2A). Thus, the radioactivity observed in the H2B region of the acid-urea gels was actually due to phosphorylated H2A. The specific radioactivity of H2A from dexamethasone-treated cells was 41 percent greater than that of the non-treated cells (compare Figs. 2A and 2B).

To resolve the H2A region even further, the Triton gels were electrophoresed much longer, running H4, H2B, H1, and monomeric H3 off the end of the gels. The single "H2A" peak (Fig. 2A) resolved into six distinguishable components, labeled 1 to 6 in Fig. 2C. Unphosphorylated H2A (peak 1) is followed by its phosphorylated derivative (peak 2), which can be seen as a shoulder on the main absorption band. Peaks 3, 4, and 6 were identified as forms of H3 dimer using the Oliver et al. modification of the Johns procedure for obtaining marker histones (18). Peak 5, which contains radioactive phosphate, was not positively identified.

The "H2A" peak from dexamethasone-treated cells (Fig. 2B) also resolved into six components (Fig. 2D). The amount of phosphorylated H2A (peak 2) was 55 percent greater than that of the control. The greater increase in phosphorylation observed with the high-resolution gels over that seen with the lower-resolution gels was due to the separation of the H3 components which co-migrated with H2A in the lower-resolution gels.

Fig. 2. G1 phase histones, Triton gels. (A) Control, showing positions of histone migration. (B) Dexamethasone-treated. Electrophoresed for 8 h at 1 1/2 mA/gel. (C) Control, high-resolution gel. (D) Dexamethasone-treated, high-resolution gel. Electrophoresed for 16 h at 1 1/2 mA/gel.


A
B
C
D
H3 H2A dimer
H3
H1
H2B H4
A600, relative
32P cpm
(+)
(−)
6 5 4 3 2 1

Dexamethasone Effects During S Phase

For the mitotically-synchronized culture, 60 to 70 percent of the cells were actually in S phase at the 14 h labeling time while the remainder were in G1 phase. Histones from this S phase-enriched population had electrophoretic absorption and radioactivity patterns similar to those from early G1 (compare Figs. 1A and 3A). As before, dexamethasone increased the phosphate incorporation in the H2B region of acid-urea gels (compare Figs. 3A and 3B). When the histones were separated on Triton gels, the radioactivity was clearly associated with H2A and H1 (Fig. 4A). Histone H1 showed a 138 percent increase in phosphorylation over that seen in early G1, while H2A showed an increase of 66 percent. This large increase in phosphorylation during S phase, at least for H1, may be due in part to the immediate post-translational modification of newly-synthesized histone (20), although some of the increase could be related to S phase-specific modification of pre-existing histone. When the S phase cells were exposed to dexamethasone, there was little change in the level of phosphorylation of H1, while H2A phosphorylation increased 16 percent (compare Figs. 4A and 4B). When the histones from untreated cells were separated on high-resolution Triton gels, the single "H2A" band (Fig. 4A) resolved into six components (Fig. 4C). The high-resolution gels of histones from hormone-treated cells revealed an increase in phosphorylation of 53 percent (compare Figs. 4C and 4D). This increase in phosphorylation with hormone treatment in S phase is approximately equal to that seen in early G1 (55 percent).

DISCUSSION

Histone H2A of L cells exhibits a minor phosphorylated species which migrates closely behind the parent species in both acid-urea and Triton gels. A similar phosphorylated H2A species was seen by Balhorn *et al.* (21). Treatment of the L cells with 1 μM dexamethasone during either early G1 or S phase results in an increase in phosphorylation of H2A. The other phosphorylated histone, H1, appears to be unaffected by the hormone.

Little is known about the chromatin-associated events induced by steroid hormones which alter the transcriptional output of a cell. We submit that

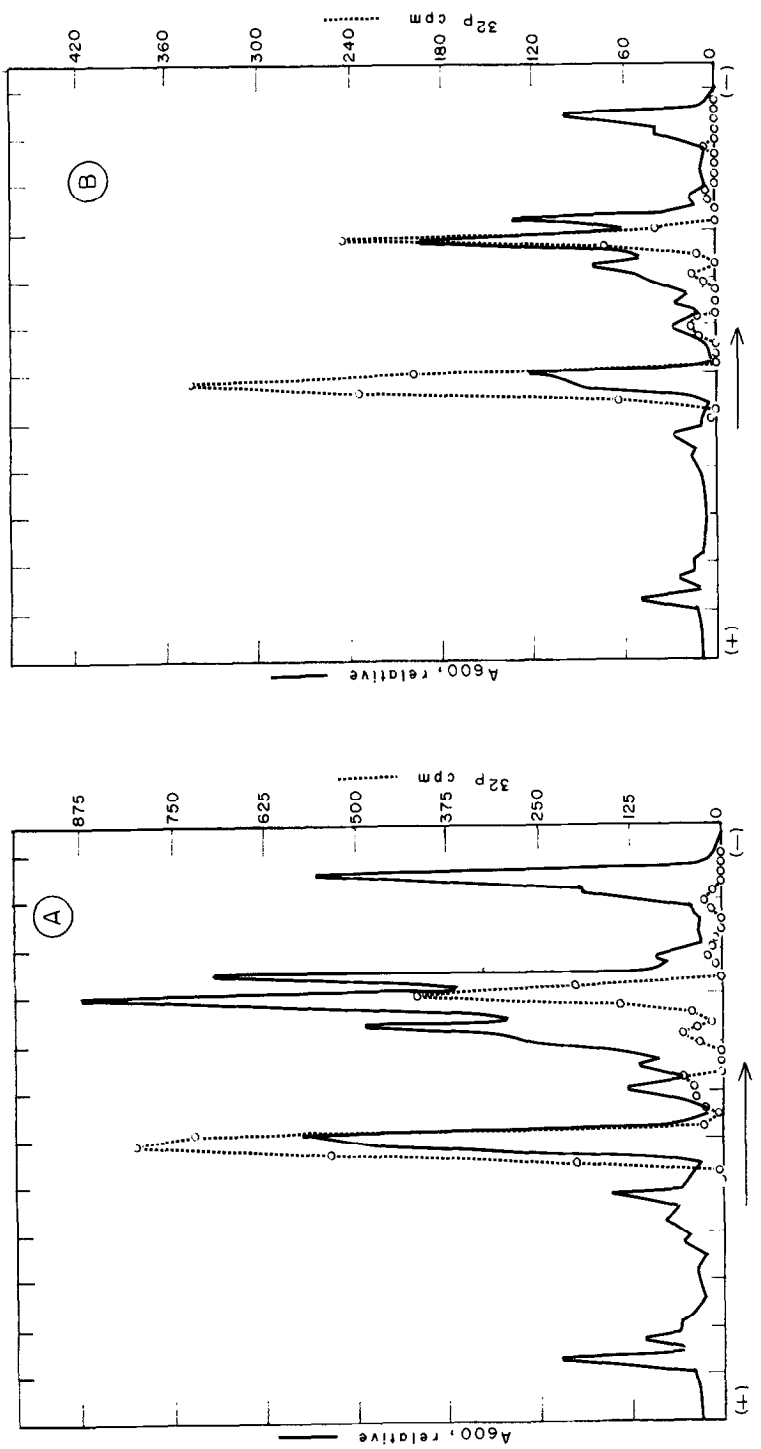
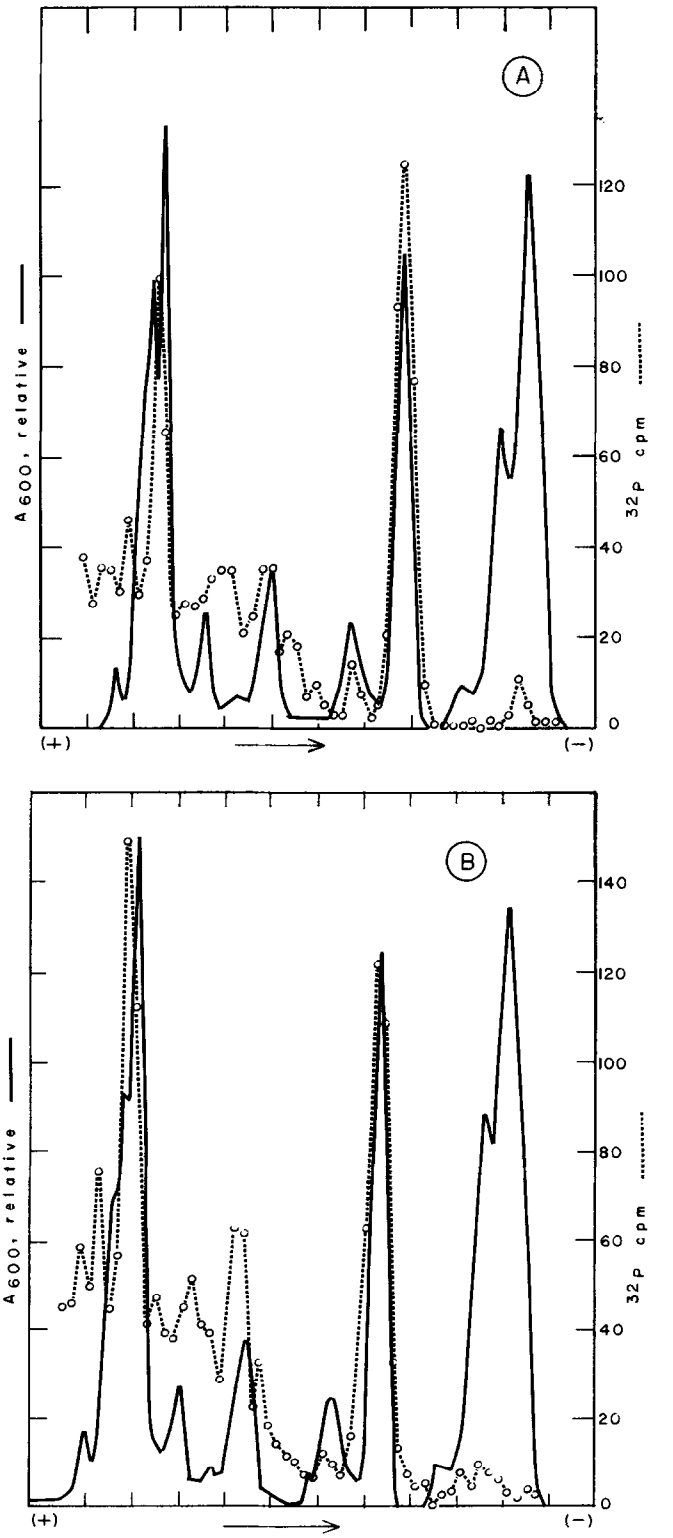


Fig. 3. S phase histones, acid-urea gels. (A) Control. (B) Dexamethasone-treated. Electrophoresed for 8 h at 2 mA/gel.


A
A600, relative
32P cpm
120
100
80
60
40
20
0
(+)
(−)
B
A600, relative
32P cpm
140
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100
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60
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Fig. 4.

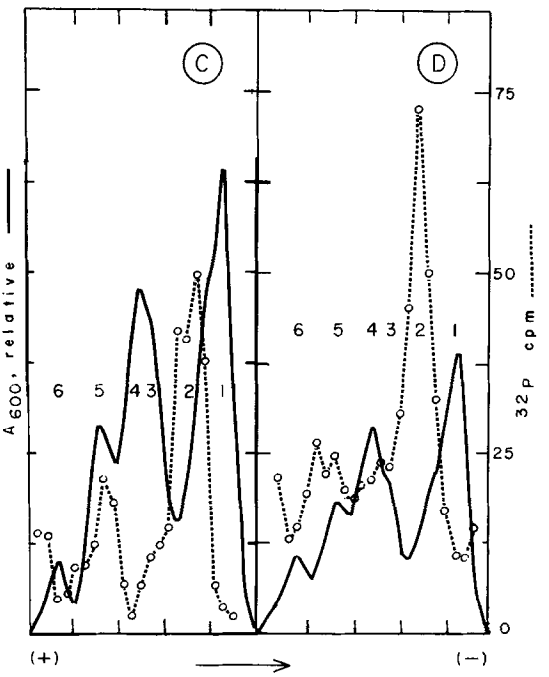

Fig. 4. S phase histones, Triton gels. (A) Control. (B) Dexamethasone-treated. Electrophoresed for 9 h at 1 1/2 mA/gel. (C) Control, high-resolution gel. (D) Dexamethasone-treated, high-resolution gel. Electrophoresed for 16 h at 1 1/2 mA/gel.

covalent modifications of certain histones, probably mediated by non-histones, may be early events in gene expression. As a model, we suggest that the steroid-receptor complex activates gene-specific non-histone proteins on the chromatin. The activated non-histone proteins, in turn, mediate the covalent modification of certain histones in the region of the targeted sequences, altering the expression of those genes. The amount of histone modified would be only a small fraction of the total because affecting expression of only a few genes would produce the hormone-specific results. Similarly, histone modification brought about by factors other than steroids could change the genetic expression of the cell. The small fraction of H2A that is phosphorylated and the increased phosphorylation seen in both G1 and S phase in L cells exposed to dexamethasone support this role of histone modification in the control of genetic expression.

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