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# EFFECT OF DEXAMETHASONE ON NUCLEOLAR CASEIN KINASE II ACTIVITY AND PHOSPHORYLATION OF NUCLEOLIN IN LYMPHOSARCOMA *P*1798 CELLS

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**Summary**—Ribosomal RNA (rRNA) synthesis in murine *P*1798 lymphosarcoma cells is reversibly inhibited by glucocorticoids. The effects of dexamethasone upon nucleolin phosphorylation and upon the amount and activity of casein kinase II have been examined. *P*1798 cells were exposed to 0.1 $\mu$M dexamethasone for 36 h. Cells were labeled *in vivo* with [$^{32}$P]orthophosphate followed by immunoprecipitation with anti-nucleolin antibody. Nucleolin phosphorylation was reduced by 60% in dexamethasone-treated cells. Nucleoli were isolated and labeled with [$\gamma$-$^{32}$P]ATP *in vitro*. Nucleolin protein was reduced to 40% of control in nuclei from dexamethasone-treated cells. Nucleolin phosphorylation was reduced to 20% of control. Nucleolar casein kinase II activity and protein were also reduced (30–55% and 35–50% of control, respectively) by treatment with dexamethasone. Cycloheximide (10 $\mu$g/ml for 3 h) reduced the amount and activity of casein kinase II, but did not cause a decrease in nucleolin protein. These observations are discussed relative to the hypothesis that glucocorticoids regulate the amount or activity of proteins of short biological half-life that are involved in the regulation of rRNA synthesis.

## INTRODUCTION

Synthesis of eucaryotic ribosomal RNA (rRNA) occurs in nuclear organelles known as nucleoli. Nucleolin, a major nucleolar phosphoprotein which is also known as C23, is thought to participate in the organization of nucleoli [1, 2] and maturation of the ribosome [2, 3]. Further phosphorylation of nucleolin is catalyzed by casein kinase II [4–7] may play a role in regulating the processing of newly synthesized rRNA [2].

Glucocorticoids stimulate rRNA synthesis in liver [8–10]. This is accompanied by enhanced phosphorylation of nucleolin [5]. Androgens also stimulate rRNA synthesis and nucleolin phosphorylation in prostatic tissue [11]. On the other hand, glucocorticoids inhibit rRNA synthesis in lymphoid cells [12, 13]. It was of interest to determine if inhibition of rRNA synthesis in lymphoid cells is accompanied by changes in nucleolin phosphorylation. However, the results of such studies could be difficult to interpret, since glucocorticoids kill most lymphoid cells. Murine *P*1798 cells do not die in the presence of glucocorticoids, although inhibition of proliferation and rRNA synthesis are observed [14]. This permits evaluation of glucocorticoid effects on nucleolin expression and phosphorylation without concern for the possibility that such effects are manifestations of cell death, rather than physiological changes in the state of nucleolin phosphorylation.

The present study was undertaken to test the hypothesis that inhibition of rRNA synthesis is accompanied by inhibition of nucleolin phosphorylation and/or inhibition of casein kinase II expression.

## MATERIALS AND METHODS

### *Materials*

[$\gamma$-$^{32}$P]ATP (4,500 Ci/mmol), [$^{32}$P]orthophosphate (carrier free) and [$^{3}$H]thymidine (90 Ci/mmol) were purchased from ICN Biochemicals, Inc.(CA, U.S.A.). [$^{3}$H]UTP was from the Radiochemical Centre (Amersham, Bucks., England). Fetal bovine serum and RPMI 1640 were from Gibco and Biocell Labs (CA, U.S.A.), respectively. *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES)

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and cycloheximide were obtained from Dojindo Labs (Kumamoto, Japan) and Wako Pure Chemicals (Osaka, Japan), respectively. An avidin–biotin peroxidase complex kit was from Vector Labs (U.S.A.). Anti-calf thymus casein kinase II anti-serum [15] was kindly donated by Dr M. E. Dahmus. Anti-rat nucleolin antibody was prepared as described previously [16]. All nucleoside triphosphate were from Yamasa Shoyu Co. (Chiba, Japan). Phenylmethylsulfonyl fluoride (PMSF) was from Sigma (St Louis, MO, U.S.A.). Leupeptin and antipain were from the Peptide Institute Inc. (Osaka, Japan). All other reagents were purchased from Wako Pure Chemicals and Nakarai Tesque (Osaka, Japan).

*Cell culture of P1798* cells

Cells were cultured in RPMI 1640 supplemented with 25 mM HEPES, 10 mM glucose, 20 $\mu$M 2-mercaptoethanol, 4 mM glutamine and 3% fetal bovine serum under 95% air/5% $CO_2$ at 37°C (doubling time is about 14 h). All experiments were performed using the cells in mid-log phase (4 to 8 $\times$ $10^5$ cells/ml). Dexamethasone (0.1 $\mu$M) was added to the cell suspension, 36 h before harvest. Cycloheximide (10 $\mu$g/ml) was added to the culture medium and the cells were incubated for 3 h before harvest.

*Cell labeling with [$^{32}$P]orthophosphate and immunoprecipitation of nucleolin*

Control or dexamethasone-treated cells (each about 5 $\times$ $10^7$ cells) were washed with phosphate-free buffer [6.2 g NaCl, 0.4 g KCl, 0.2 g $CaCl_2$, 0.1 g $MgSO_4$ ($7H_2O$), 2.2 g $NaHCO_3$, 2.28 g HEPES (pH 7.4) and 1 g glucose per 1 l] and suspended in 30 ml of the buffer. After 10 min incubation at 37°C, carrier free [$^{32}$P]orthophosphate was added to a concentration of 50 $\mu$Ci/ml. Cells were labeled at 37°C for 1 h, rinsed with phosphate-buffered saline (PBS) [10 mM sodium phosphate (pH 7.4) containing 0.15 M NaCl] twice, and nuclei were obtained using the buffer as described previously [17]. Nuclear proteins were extracted from the isolated nuclei and immunoprecipitation of nucleolin was performed using anti-nucleolin antibodies as described previously [16]. The resulting pellet was analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and autoradiography [16].

*Preparation of nuclei and nucleoli*

Cells (3.7 to 8.2 $\times$ $10^8$) were pelleted by centrifugation at 250 *g* for 3 min and washed 3 times with cold PBS. Nuclear pellet obtained by the method described above was suspended in 10 ml of 0.32 M sucrose containing 10 mM $MgCl_2$ and 0.5 mM PMSF, and the suspension was underlayed with an equal volume of 0.88 M sucrose containing 0.05 mM $MgCl_2$ and 0.5 mM PMSF and centrifuged at 1200 *g* for 10 min at 4°C. The nuclear pellet was suspended in 3–5 ml of 0.34 M sucrose containing 0.05 mM $MgCl_2$ and 0.5 mM PMSF and sonicated according to the method described previously [5]. The nucleoli were examined by light microscopy after staining with azure C and by assay of RNA polymerases I and II. The activities of RNA polymerases I and II were measured in the presence and absence of $\alpha$-amanitin (1 $\mu$g/ml), respectively, by run-on assay according to the methods described previously [9].

*Phosphorylation of nucleolar protein*

A reaction mixture (100 $\mu$l) containing 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.32 M sucrose, 0.5 mM PMSF, 0.2–0.5 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (10 $\mu$M) and nucleoli (50–100 $\mu$g protein) was incubated at 37°C for 10 min. The phosphorylated proteins were separated by SDS–PAGE, followed by autoradiography. Details were described previously [5, 6].

*Preparation of the nucleolar casein kinase II fraction*

A fraction containing casein kinase II was extracted from isolated nucleoli as described previously [9]. This fraction was applied to a DEAE A-25 Sephadex column, and casein kinase II activity was eluted. Details were as described previously [5, 6].

*Determination of the amount of nucleolin in nuclei using anti-nucleolin antibody*

Nuclei were isolated from the cell that had been grown for 36 h with or without 0.1 $\mu$M dexamethasone. The nuclei (100 $\mu$g protein) were solubilized in 250 $\mu$l of SDS-buffer, followed by boiling for 2 min. The proteins were resolved by SDS–PAGE and transferred to a nitrocellulose membrane using a semi-dry blotting apparatus (Nihon Eido Co., Tokyo). The membrane was washed several times with PBS containing 0.05% Tween 20 (PBS-T) and incubated for 1 h at room temperature in PBS-T

containing 3% bovine serum albumin. Thereafter, the membrane was incubated for 1 h with anti-nucleolin anti-serum diluted (1:1000) with PBS-T containing 0.3% bovine serum albumin. After several washes with PBS-T, the membrane was incubated with horseradish-conjugated anti-rabbit IgG anti-serum for 1 h at room temperature. Unbound antibodies were removed by washing extensively with PBS-T and the membrane was stained with 0.6 mg/ml 4-chloro-l-naphtol, 0.015% $H_2O_2$ in 10 mM Tris–HCl (pH 7.5). The densities of colored bands were quantified by densitometric scanning using a chromatoscanner (CS910, Shimazu Co., Kyoto, Japan).

*Determination of the amount of nucleolar casein kinase II in P1798 cells*

A casein kinase II fraction (1 mg protein) was prepared as described above and lyophylized. The dried materials were dissolved in 100 $\mu$l of SDS-buffer, boiled for 2 min and resolved by SDS–PAGE, and blotted to a nitrocellulose membrane. The membrane was stained with anti-casein kinase II anti-serum (1:1000 dilution) by the method described above, except that the second antibody was replaced by an avidin–biotin peroxidase complex kit. Densities of the stained bands corresponding to the $\alpha$-subunit of casein kinase II were quantified as described above.

*Assay of casein kinase II activity*

The activity of casein kinase II was determined by the methods described previously [5].

*Assay of DNA synthesis activity*

DNA synthesis was observed by incorporation of radioactivity into acid-insoluble materials after cells were labeled with [$^3$H]thymidine (12 $\mu$Ci/ml) for 2 h.

*Determination of protein concentration*

Protein concentration was determined by the dye-binding method of Bradford [18].

## RESULTS

*Effect of dexamethasone on DNA synthesis in P1798 cells*

DNA synthesis was estimated by measuring the incorporation of [$^3$H]thymidine into *P*1798 cells that had been exposed to 0.1 $\mu$M dexamethasone for 36 h. Thymidine incorporation was reduced to 35% of control, whereas viability (as determined by trypan blue exclusion) was not affected. When dexamethasone was removed from the culture medium, thymidine incorporation was restored to that of control cells (data not shown).

*Change in phosphorylation and amount of nucleolin after dexamethasone treatment of P1798 cells*

*P*1798 cells were treated with 0.1 $\mu$M dexamethasone for 36 h. Control and treated cells were labeled for 1 h with [$^{32}$P]orthophosphate, and phosphorylation of nucleolin was assayed by immunoprecipitation. As shown in Fig. 1(A), nucleolin phosphorylation was reduced to 40% of the control by the treatment with dexamethasone.

Although Coomassie Blue staining [Fig. 1(B)] seemed to show no significant effect of dexamethasone on the band at 110 kDa, this was disturbed by the fact that significant cross-reaction with other proteins than nucleolin occurred due to the use of a large amount of the antibody to precipitate mouse nucleolin. Therefore, in the experiments shown in Fig. 2, nucleolin was directly immunoblotted on nitrocellulose membranes obtained after SDS–PAGE for nuclei isolated from dexamethasone-treated cells. The results demonstrated that nuclei from dexamethasone-treated cells contain about 40% less nucleolin than do nuclei from control cells.

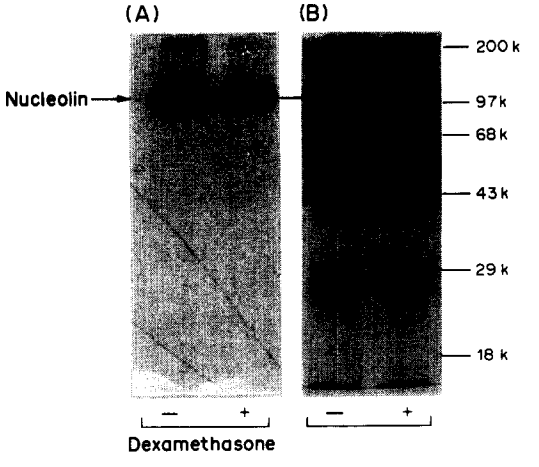


Fig. 1. Effect of dexamethasone on the phosphorylation of nucleolin in *P*1798 cells. *P*1798 cells were cultured for 36 h in the presence (0.1 $\mu$M) or absence of dexamethasone and with [$^{32}$P]orthophosphate for 1 h before harvest. From the cells, nuclei were isolated, and nucleolin was immunoprecipitated with anti-rat nucleolin antibody and protein-A Sepharose. The precipitates were solubilized with SDS-buffer and subjected to SDS–PAGE. (A) Autoradiography; (B) Coomassie Brilliant Blue staining.

The reduction of nucleolar nucleolin phosphorylation after treatment with dexamethasone was also observed when isolated nucleoli were labeled *in vitro* with [$\gamma$-$^{32}$P]ATP [Fig. 3(B)] The result shown in Fig. 3 is representative of several experiments. These experiments indicated that the phosphorylation of nucleolin in nucleoli isolated from dexamethasone-treated cells was reduced to approximately 20% of control. The purity of nucleoli isolated in these experiments was checked by measuring the activities of RNA polymerases I and II: no significant activity of RNA polymerase II was found in the isolated nucleoli. Although the change in the amount of nucleolin protein in nucleoli was not checked by an immunological method, the density of the protein band of 110 kDa [Fig. 3(A)] seemed to be reduced to the same degree as seen in the immunoblotting experiments of Fig. 2.

From these results, it is likely that the phosphorylation of nucleolin in nucleoli is reduced to 20% of the control by treatment of the cells with dexamethasone, and that the reduction can not be solely explained by a reduction in the amount of nucleolin protein in the nucleoli.

### *Casein kinase II activity in nucleoli from dexamethasone-treated cells*

Evidence was presented indicating that phosphorylation of nucleolin is thought to be catalyzed by casein kinase II in Chinese hamster ovary cells [4], liver cells [5, 6] and adult bovine aortic epitheial cells [7]. It was found that this is the case in lymphoid cells, since the phosphorylation of nucleolin by nuclei from *P*1798 cells

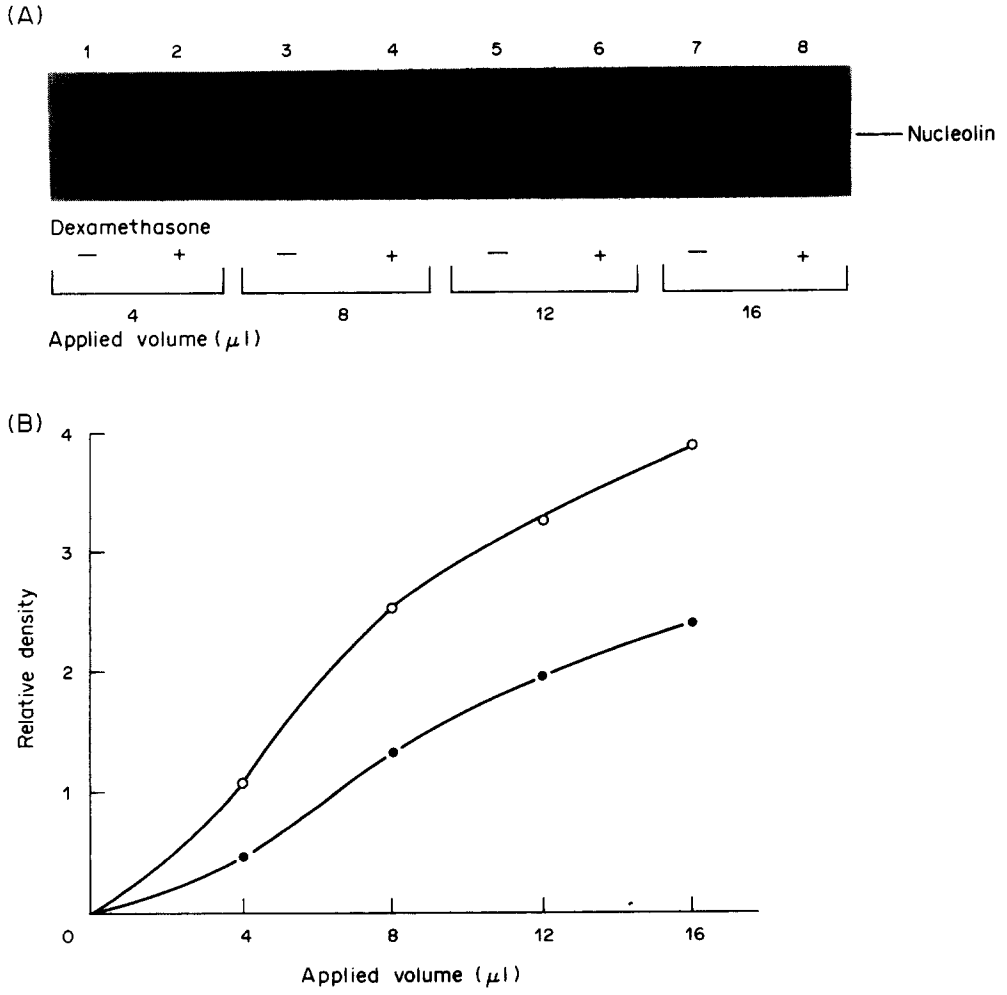


Fig. 2. Change in the amount of nucleolin in nuclei of *P*1798 cells after treatment with dexamethasone. (A) Nuclei isolated from the cells incubated for 36 h in the presence (0.1 $\mu$M) or absence of dexamethasone were directly solubilized in SDS-buffer and applied to SDS–PAGE. After that, proteins in the gel were blotted onto nitrocellulose membrane and immunostaining were carried out using anti-rat nucleolin anti-serum and horseradish peroxidase-conjugated anti-serum as the second antibody. (B) The stained bands were quantified by densitometric scanning. ○, control; ●, +dexamethasone.

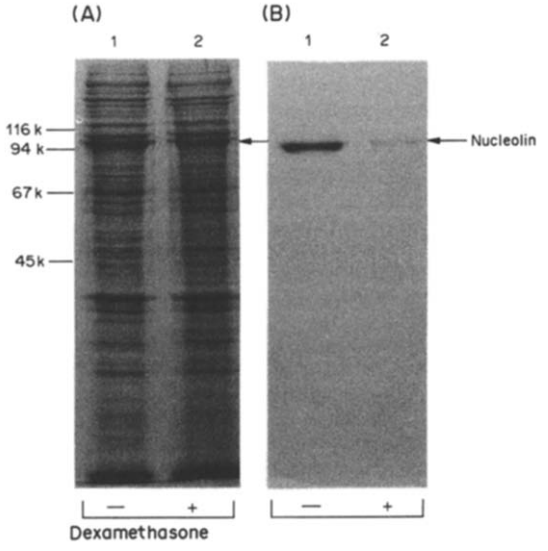


Fig. 3. *In vitro* phosphorylation of nucleolin isolated from cells cultured with or without dexamethasone. Nucleoli isolated from the cells treated or untreated with dexamethasone (0.1 $\mu$M) were phosphorylated with 10 $\mu$M [$\gamma$-$^{32}$P]ATP (2 $\mu$Ci/ml) for 10 min at 37°C. After the incubation, the phosphorylated nucleolar proteins were subjected to SDS–PAGE. (A) protein staining; (B) autoradiography.

was inhibited by GTP and heparin and stimulated by spermine. The casein kinase II was extracted from control- and dexamethasone-treated nucleoli and separated from casein kinase I by chromatography on DEAE-Sephadex A-25 [5, 6]. The identity of casein kinase II was confirmed by inhibition by heparin and GTP and activation by spermine (data not shown). The results are shown in Fig. 4, in which casein kinase II activity is given as a function of cell number (left panel) or nucleolar protein (right panel). The data indicate that glucocorticoids reduced the level of casein kinase II activity to 30–45% of control level.

### *Effect of cycloheximide on casein kinase II activity and nucleolin phosphorylation*

Cycloheximide was used to determine the relationship among protein synthesis, casein kinase II activity, and nucleolin phosphorylation. Casein kinase II activity was reduced by 35–50% within 3 h after addition of cycloheximide, as shown in Fig. 4. Nucleolin phosphorylation was also reduced by cycloheximide [Fig. 5(B)], the mean reduction from three experiments being 50%, while the 110 kDa protein was not changed so much [Fig. 5(A)]. The data indicate that decreased casein kinase II acitivity accompanies decreased nucleolin phosphorylation.

### *Effect of dexamethasone and cycloheximide on the amount of casein kinase II*

Anti-casein kinase II anti-serum was used to determine if dexamethasone and/or

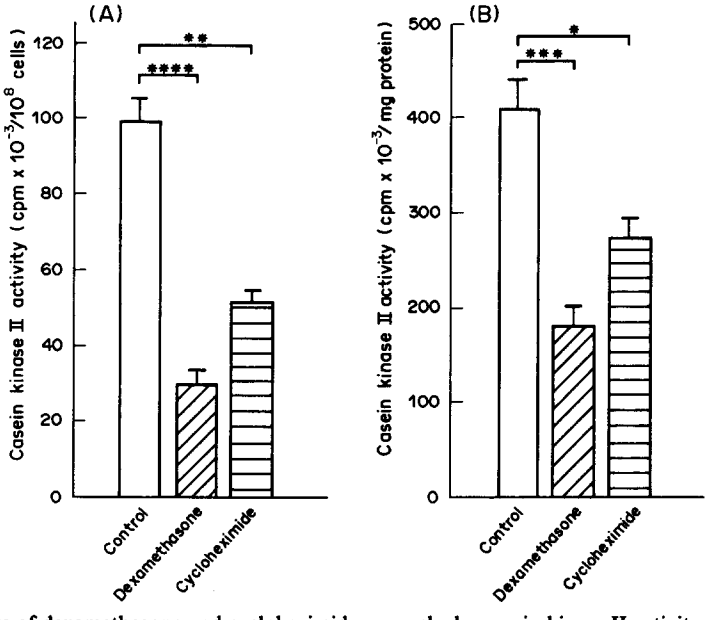


Fig. 4. Effects of dexamethasone and cycloheximide on nucleolar casein kinase II activity of P1798 cells. The cells were treated with dexamethasone (0.1 $\mu$M) for 36 h or cycloheximide (10 $\mu$g/ml) for 3 h. Nucleolin were isolated from these cells and the casein kinase II fraction was solubilized, followed by partial purification by DEAE-chromatography. Casein kinase II activity in the fraction was then determined. Enzyme activity was expressed at cpm per $10^8$ cells (A) or per mg protein (B). Each bar represents the mean ± SE of three experiments. *, $P < 0.005$; **, $P < 0.01$; ***, $P < 0.05$; ****, $P < 0.01$ vs control.

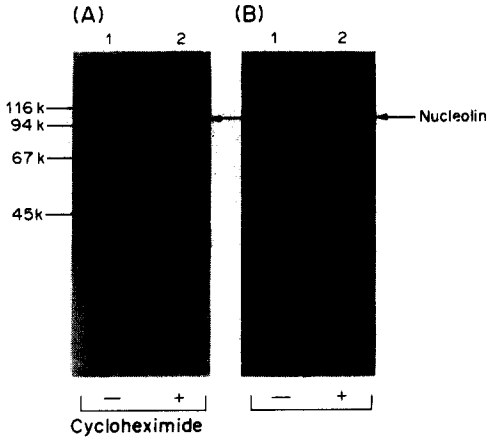


Fig. 5. Effect of cycloheximide on the phosphorylation of nucleolin. Cycloheximide (10 μg/ml) was added to the culture medium of *P*1798 cells. After 3 h, cells were harvested and nucleoli were isolated. The nucleoli were then phosphorylated with [γ-$^{32}$P]ATP and then subjected to SDS–PAGE, followed by protein staining (A) and autoradiography (B).

cycloheximide caused a decrease in casein kinase II protein. The immunostaining data are shown in the upper panels of Fig. 6(A and B). The α- and β-subunits of casein kinase II were detectable, and the amount of both subunits was decreased by dexamethasone [Fig. 6(A)] and cycloheximide [Fig. 6(B)]. Quantitative data concerning the α-subunit are shown in the lower panels. Dexamethasone caused a 70% decrease in the amount of the subunit, whereas cycloheximide caused a 40% decrease.

## DISCUSSION

Glucocorticoids inhibit proliferation of *P*1798 cells [14]. This is accompanied by G1 arrest [19]. Expression of thymidine kinase is associated with G1 arrest and 50% inhibition of thymidine kinase expression is observed 14–20 h after addition of dexamethasone [20]. Synthesis of rRNA is more rapidly inhibited in *P*1798 cells, with 50% inhibition observed with in 6 h [12, 21]. The data presented in this report indicate that glucocorticoid inhibition of rRNA synthesis is accompanied by inhibition of nucleolin phosphorylation and a decrease in the expression of nucleolin protein. Glucocorticoids also cause a decrease in casein kinase II activity, which likely accounts for decreased nucleolin phosphorylation. It has been previously shown that stimulation of rRNA synthesis in liver is accompanied by an increase in casein kinase II

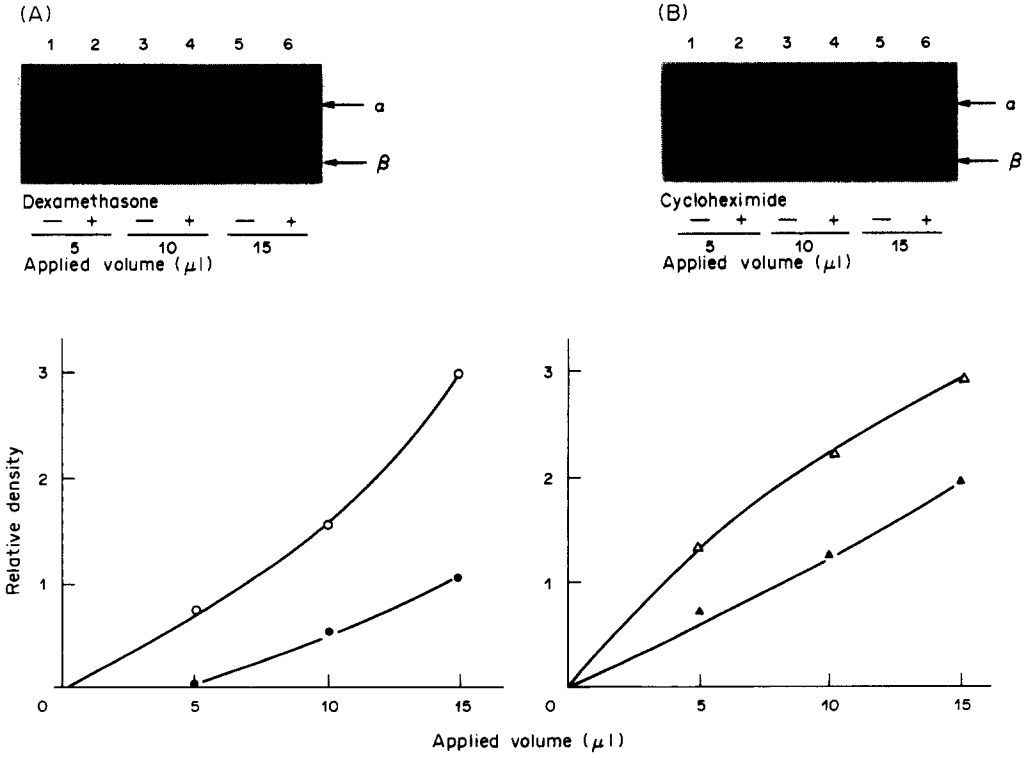


Fig. 6. Change in the amount of nucleolar casein kinase II after treatment with dexamethasone (A) or cycloheximide (B). Partially purified nucleolar casein kinase II fractions which were obtained in the experiments of Fig. 4 were applied to SDS–PAGE and immunoblotted using anti-casein kinase II anti-serum and avidin–biotin complex kit as the second antibody (upper panels). The stained bands (corresponding to the α-subunit) were quantified by densitometric scanning (lower panels). ○ and △, control; ● and ▲, treated with dexamethasone and cycloheximide, respectively.

activity and a corresponding increase in nucleolin phosphorylation [5, 9]. All of these observations indicate that casein kinase II, nucleolin phosphorylation, and rRNA synthesis are tightly linked; and the data are consistent with the hypothesis that casein kinase II may play a significant role in regulation of rRNA synthesis.

Previous studies with casein kinase II have not indicated whether glucocorticoids regulate the amount or activity of the enzyme. The data presented in the present report indicate that nucleoli from glucocorticoid-treated cells contain less casein kinase II protein. It is not known if this is due to inhibition of synthesis of casein kinase II, stimulation of degradation of the protein, or inhibition of nucleolar uptake.

Protein synthesis is known to be required in order to sustain rRNA synthesis [9, 22] and inhibition of protein synthesis blocks glucocorticoid stimulation of rRNA synthesis in liver [9, 23]. These data suggest that a short-lived protein may play a role in hormonal regulation of rRNA synthesis. Data in support of this hypothesis have been reported in prostate [24], uterus [25] and liver [9, 10]. Studies with *P*1798 cells indicate that glucocorticoids regulate the activity of an RNA polymerase I transcription factor, called TFIC [12, 22, 26]. Inhibition of protein synthesis also leads to rapid loss of TFIC activity and inhibition of rRNA synthesis in *P*1798 cells [22]. These data indicate that some protein of short biological half-life plays a crucial role in transcription of rRNA genes, but the identity of this protein remains to be established. It is interesting that the same protein (or two proteins of similar properties) appear to be involved in glucocorticoid stimulation of rRNA synthesis in liver and glucocorticoid inhibition of rRNA synthesis in lymphoid cells.

The data presented above indicate that cycloheximide causes a rapid and significant decrease in casein kinase II protein located within the nucleolus. It is tempting to speculate that casein kinase might be the hypothetical short-lived protein that is involved in regulation of rRNA synthesis. A number of observations are consistent with the conclusion that a phosphotransferase could be involved in transcription by RNA polymerase I. Casein kinase II co-purifies with the polymerase I [27] and can phosphorylate RNA polymerase I *in vitro* [28]. Phosphorylation of RNA polymerase I or an ancillary transcription factor has been implicated in initiation of transcription of the rRNA genes [29]. Finally, it appears that glucocorticoid regulation of rRNA synthesis is due to a loss of activity of the transcription factor TFIC [E. A. Thompson, preliminary data]. The data suggest that a decrease in casein kinase II activity could result in a decrease in phosphorylation of some transcription factor, such as TFIC, leading to inhibition of initiation of transcription of RNA polymerase I. However, no direct test of this hypothesis has been undertaken to date.

Alternatively, it may be that rRNA synthesis is directly effected by the amount of phosphorylated nucleolin within the nucleolus. It is not known to what extent transcription of rRNA genes is influenced by the amount of nucleolin or the extent of nucleolin phosphorylation. Dexamethasone causes a decrease in both nucleolin protein and nucleolin phosphorylation. This is consistent with the obseravtion that nucleolin mRNA decreases in glucocorticoid-treated *P*1798 cells [30]. Cycloheximide has little effect on total nucleolin proteins, but inhibits nucleolin phosphorylation. This suggests that if nucleolin plays a role in transcription, the active principle must be the phosphorylated form of the protein.

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