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ISOPROTERENOL-INDUCED SELECTIVE PHOSPHORYLATION IN VIVO OF THE 214,000 DALTON SUBUNIT OF RAT C6 GLIOMA CELL RNA POLYMERASE II

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SUMMARY The phosphorylation in vivo of RNA polymerase II after isoproterenol stimulation of confluent rat C6 glioma cell cultures has been investigated. Glioma cells were incubated in the presence of $Na_2H^{32}PO_4$ and stimulated for 1 hour with the β-adrenergic agonist isoproterenol. The phosphorylation pattern was analyzed after purification of RNA polymerase II by immunoprecipitation, SDS-polyacrylamide gel electrophoresis and autoradiography. Isoproterenol markedly increased [^{32}P]phosphate incorporation into the 214,000 dalton RNA polymerase subunit. Analysis of the phosphate acceptor amino acid revealed the presence of only [^{32}P]phosphoserine. The data demonstrates an isoproterenol-induced structural modification of RNA polymerase II.

The role of cyclic nucleotides in the regulation of transcriptional events remains a challenging unsolved problem. In analogy to the cAMP regulation of a number of enzymes (1,2), functional modulation by cAMP of the enzyme responsible for structural gene transcription, RNA polymerase II, constitutes an attractive model for the direct control of transcriptional events. Based on evidence demonstrating activity changes of RNA polymerase II following its incubation with cAMP-dependent protein kinase and ATP (3), we have postulated that the function of RNA polymerase II may be altered through covalent phosphorylative modification by nuclear protein kinases (4). Through in vitro studies, using highly purified calf thymus RNA polymerase II and homologous cAMP-dependent as well as -independent nuclear protein kinases, we were able to demonstrate the phosphorylation of the 180,000 and 25,000 dalton subunits with a concomitant activity change of the polymerase (5,6). Recently, Dahmus provided evidence for the phosphorylation of the 240,000, 214,000 and 20,500 dalton subunits of HeLa cell RNA polymerase II in vivo (7). The question whether these phosphoryla-

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tions are constitutive modifications or are subject to regulation by effector agents remains, however, open. We report here a modulation of the degree of phosphorylation of the 214,000 dalton subunit of glioma cell RNA polymerase II as a consequence of isoproterenol stimulation in vivo.

MATERIALS AND METHODS (-) Isoproterenol bitartrate and all reagents of analytical grade were purchased from Sigma Chemical Company. Carrier-free $Na_2H^{32}PO_4$ (285 Ci/mg) was from Amersham/Searle or ICN Chemical Corporation. The preparation and characterization of chicken anti-calf RNA polymerase II antiserum will be described elsewhere (8). Culture and in vivo labeling of glioma cells with $Na_2H^{32}PO_4$ were carried out as described previously (9). After labeling of glioma cells with $Na_2H^{32}PO_4$ for 2 hours, stimulation was carried out for 1 hour by the addition of isoproterenol (10^{-5} M) and 5 x 10^{-5} M MIX (3-isobutyl-1-methylxanthine). The cells were harvested by scraping into 50 mM Tris, 2.0 M sucrose, 3 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, pH 7.4. After 15 strokes in a glass-glass homogenizer, the homogenate was layered onto 2.0 M sucrose, 3 mM $MgCl_2$, 0.2 mM PMSF and centrifuged for 1 hour at 105,000xg. The pelleted nuclei were suspended in nuclear extraction buffer (50 mM Tris, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, 10 mM NaF, 25% glycerol (v/v), pH 7.4, containing 50 Kalligrein international units/ml of aprotinin (Sigma Chemical Company). After the addition of ammonium sulfate to 0.63 M final concentration, the suspension was homogenized, diluted with 2 volumes of nuclear extraction buffer, homogenized again, and centrifuged for 90 min at 34,000xg. Ammonium sulfate was added to the supernatant fraction to 50% final concentration and the fraction was kept overnight at 4°C. Precipitated protein was recovered by centrifugation and dissolved in immunoprecipitation buffer (50 mM Tris, pH 8.0, 0.5 mM PMSF, 10 mM NaF, 50 Kalligrein international units/ml of aprotinin, 0.5% NP-40, 0.5% Triton X-100, 0.75 M KCl). After the addition of 5 µg of highly purified calf thymus RNA polymerase II as carrier and chicken anti-calf RNA polymerase II antiserum (25 µl antiserum/ml immunoprecipitation buffer), immunoprecipitation was carried out for three days at 4°C. The incubation mixture was layered onto PBS containing 1 M sucrose, 0.01% sodium azide, 0.1 mM PMSF, 0.1 M ammonium sulfate, 1.0% NP-40, 1% sodium deoxycholate, and centrifuged for 15 min at 10,000xg. The purification procedure was repeated once, after which the immunoprecipitate was washed three times with PBS containing 0.01% sodium azide, 0.1 mM PMSF, 0.1 M ammonium sulfate, 0.5% NP-40, and once with 75 mM Tris buffer, pH 8.3. The immunoprecipitate was then dissolved in Laemmli sample buffer (10). The samples were heated at 95°C for 5 min and subjected to electrophoresis on 10% SDS-polyacrylamide gels according to Laemmli (10). Autoradiography was carried out as described (11). The optical density of autoradiographic film image was measured using a Canalco model K densitometer. For analysis of [^{32}P]phosphoserine and [^{32}P]phosphothreonine, the 214,000 dalton subunit in gel slices was subjected to partial acid hydrolysis in 2 N HCl and high voltage paper electrophoresis as described (12).

RESULTS Immunoprecipitation of glioma cell RNA polymerase II and analysis of the immunoprecipitate by SDS-polyacrylamide gel electrophoresis reveals the presence in the immunoprecipitate of several polymerase subunits together with immunoglobulin bands (Fig. 1, lane A). Due to the presence of rather large

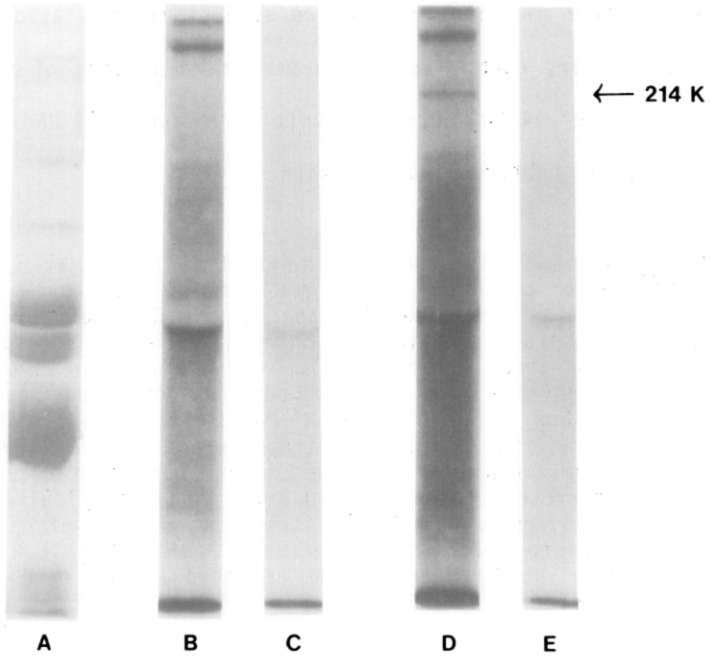

Figure 1. Autoradiograms of immunoprecipitated RNA polymerase II isolated from ^{32}P-labeled C6 glioma cells before and after isoproterenol+MIX stimulation. Immunoprecipitated RNA polymerase was subjected to SDS-polyacrylamide gel electrophoresis. Gels were either stained with Coomassie blue (lane A) or were subjected to autoradiography (lanes B-D). Lanes B and D show autoradiograms of RNA polymerase immunoprecipitated with chicken anti-calf RNA polymerase II antiserum; lanes C and E show autoradiograms of RNA polymerase II immunoprecipitated with pre-immune chicken serum.

amounts of immunoglobulins in the precipitate, some of the polymerase subunits cannot be visualized by Coomassie blue staining without grossly overloading the gel. When RNA polymerase II from control and isoproterenol-stimulated glioma cells is immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis, the autoradiographic patterns shown in Fig. 1, lanes B and D, are obtained. In spite of the relatively high background of ^{32}P in the lower two-thirds of the gels, it can be seen that a protein band corresponding to the 214,000 dalton subunit of RNA polymerase II is markedly phosphorylated in the polymerase obtained from isoproterenol-stimulated cells (Fig. 1, lane D). Immunoprecipitation of RNA polymerase II from control and stimulated cells using preimmune chicken serum electrophoresis of the immunoprecipitate

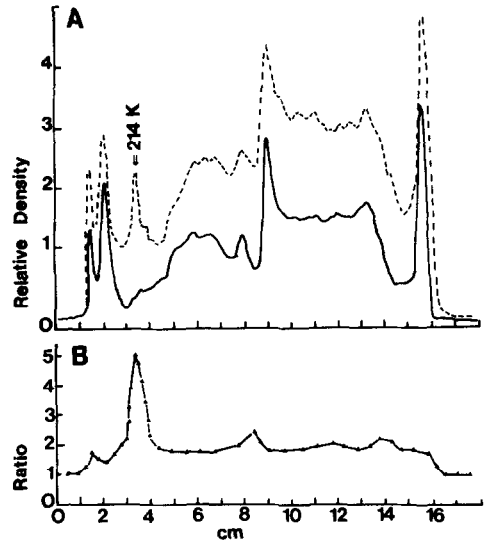

Figure 2. Panel A: Densitometric scans of immunoprecipitated RNA polymerase II from unstimulated cells (——) and from stimulated C6 glioma cells (---). These scans were taken from the autoradiograms shown in Fig. 1, B and D. The position of the 214,000 dalton subunit is indicated. Panel B: Ratio of optical densities (relative density of scan from stimulated cells/relative density of scan from unstimulated cells) calculated from the scans of panel A.

and autoradiography reveals no significant amounts of ^{32}P-labeled protein (Fig. 1, lanes C and E).

Densitometric scanning of the autoradiographs allows a semi-quantitative assessment of the extent of phosphorylation as shown in Fig. 2. The peak corresponding to the 214,000 dalton subunit is clearly visible in the densitometric scan of RNA polymerase from stimulated cells (Fig. 2A) but not in control cells. Determination of the ratio of the optical densities of polymerase from stimulated cells versus nonstimulated cells (Fig. 2B) indicates a relatively unchanged ratio throughout the scans with the exception of the ratio determined for the 214,000 dalton subunit which is selectively increased by about 2 to 3-fold.

Recovery of the gel section corresponding to the 214,000 dalton subunit and partial acid hydrolysis of the ^{32}P-labeled protein reveals the presence of only [^{32}P]phosphoserine and no [^{32}P]phosphothreonine was identified (Fig. 3). Under identical experimental conditions the amount of [^{32}P]phosphoserine identified

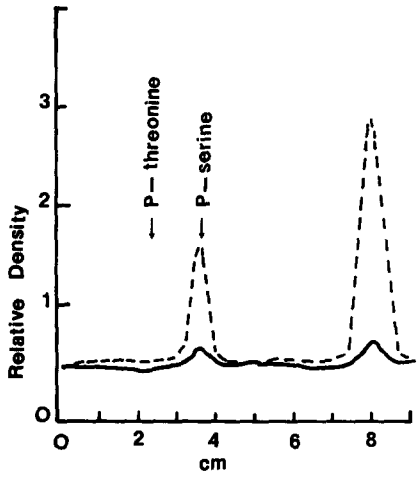

Figure 3. Analysis of [^{32}P]phosphoamino acids by paper electrophoresis. Partial acid hydrolysates of gel slices containing the 214,000 dalton subunit were fractionated by paper electrophoresis (12) at pH 2.4 (1% formic acid - 4% acetic acid; 500 volts; 16 hours). ^{32}P acceptor amino acids were visualized by autoradiography and identified by co-migration of authentic phosphoserine and phosphothreonine. The figure depicts densitometric scans of the autoradiographs obtained from samples of unstimulated cells (——) and from stimulated cells (---).

in the subunit isolated from stimulated cells is about two-fold higher than from control cells.

DISCUSSION We have demonstrated the in vivo phosphorylative modification of the 214,000 dalton subunit of glioma cell RNA polymerase II as the result of β-adrenergic receptor stimulation by isoproterenol. The increased level of ^{32}P radioactivity was identified as phosphoserine. These results establish the covalent structural modification of RNA polymerase II and the regulation of this modification by a β-adrenergic agent in glioma cells. If the modification has functional significance, it follows that the polymerase function can be regulated as well. Although the functional significance remains to be established, stimulation of glioma cells with isoproterenol has been shown to lead to increased transcriptional activity and increased levels of lactate dehydrogenase mRNA (13-15). Under our present experimental conditions (2 hour labeling with $Na_2H^{32}PO_4$), little or no ^{32}P labeling of RNA polymerase subunits was observed in unstimulated cells (some ^{32}P radioactivity was associated

with the 214,000 dalton subunit which, however, is not visible on the autoradiograph, Fig. 1), whereas Dahmus observed ^{32}P incorporation into several subunits of HeLa cell RNA polymerase II after a 5-hour labeling period (7). Our labeling conditions were chosen to allow optimal visualization of the effect of isoproterenol which is best seen at shorter labeling times.

Based on previous findings that isoproterenol stimulation of glioma cells leads to increased intracellular cAMP levels and increased nuclear cAMP-dependent protein kinase activity (16), it is likely that the phosphorylative modification of the subunit is a cAMP-mediated event. Previously, we have demonstrated the *in vitro* phosphorylation of the 180,00 and 25,000 dalton subunits of calf thymus RNA polymerase II by highly purified homologous cAMP-dependent protein kinase (5). The failure to identify *in vitro* phosphorylation of the 214,000 dalton subunit can best be explained by available evidence indicating that the 180,000 dalton subunit may arise after proteolytic modification of the larger subunits of the enzyme (17, 18, 19), especially during extensive purification procedures. Immunoprecipitation of the polymerase from nuclear protein extracts is fast and may eliminate or reduce proteolysis. Although no selective phosphorylation of the 25,000 dalton subunit of glioma RNA polymerase II was observed under the present experimental conditions, preliminary results indicate its selective *in vivo* phosphorylation under slightly different conditions. (8).

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