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Effect of Testosterone on Nuclear Phosphoproteins of Rat Ventral Prostate

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SUMMARY

^{32}P from [γ-^{32}P]-ATP was rapidly incorporated into phosphoproteins of rat ventral prostate nuclei *in vitro*. At least 80% of the radioactivity (expressed per milligram of protein) was present in the non-histone phosphoproteins. The incorporation of ^{32}P into phosphoproteins of nuclei isolated from orchiectomized rats was reduced greatly compared with nuclei isolated from orchiectomized rats treated with testosterone propionate or from normal control rats. The effect of testosterone on the incorporation *in vitro* of ^{32}P from [γ-^{32}P]-ATP into non-histone phosphoproteins of nuclei from castrated rats could be demonstrated as early as 30 min following a single injection of testosterone propionate. This effect could be explained in terms of increased activity of protein phosphokinase(s) in the nuclei. A possible role of nuclear protein phosphokinase(s) and the phosphorylation of non-histone phosphoproteins in the events related to the control of gene action in the prostate in response to testosterone is suggested.

INTRODUCTION

The molecular mechanisms underlying the regulation or control of transcription in cells are obscure. In this connection, however, considerable attention has been given to nucleoproteins for their possible involvement in the control of RNA transcription *in vivo* (see, for example, the reviews cited in refs. 1–3). Suggestions have been made that enzymatic modifications such as acetylation or phosphorylation of nucleoproteins (both histones and non-histones) may be related to their possible roles in the control of gene activity (1–5). For example, Kleinsmith *et al.* (5) have demonstrated that one of the earliest responses of phytohemagglutinin-stimulated lymphocytes is an increase in the phosphorylation of nucleoproteins in these cells. However, for several reasons it is thought unlikely that histones (or the basic proteins of nuclei) alone are capable of complete regulatory control of gene action (2, 3). Non-histone proteins (or acidic proteins) are also excellent candidates for such a role, and hence correlation of their metabolism with gene activity is of considerable interest. Recently Benjamin and Goodman (6) have reported the active phosphorylation of non-histone proteins of the giant chromosomes of dipteran salivary glands, in which puff regions of the polytene chromosomes are considered to be actively engaged in gene activity.

In the present communication, additional evidence is presented to suggest a relation between gene activity and phosphorylation of nuclear proteins, especially non-histone phosphoproteins. Rat ventral prostate is an excellent model for such a study, since gene activity in this tissue can be physiologically

controlled by deprivation or administration of testosterone to the rats. That the development and activity of rat ventral prostate are under the strict control of androgenic hormones is well documented (7–10). Orchiectomy of adult rats leads to rapid involution of the prostate, which can be prevented or reversed by administration of androgenic steroids, and one of the earliest effects of administration of testosterone to orchiectomized rats is an increase in the activity of nuclear DNA-dependent RNA polymerase activity (10, 11).

A preliminary report of part of this work has been given (12).

METHODS

Sprague-Dawley rats, weighing 250–300 g, were orchiectomized via the scrotal route. They were killed by cervical dislocation, and ventral prostates were pooled (from 8–16 rats) to isolate the nuclei according to the methods used by Coffey *et al.* (13). The nuclear pellet was washed twice in a medium consisting of 0.25 M sucrose and 1 mM $MgCl_2$, and then suspended in a small volume of the same medium. Routine examination of the preparations by light microscopy revealed well-preserved nuclei. In control experiments, the extent of cytoplasmic contamination was checked and found to be minimal by the criteria employed by other workers (14). The RNA:DNA ratio was less than 0.30. Nuclei (0.2–0.8 mg of protein) were incubated in a reaction medium consisting of $MgCl_2$, 6 mM; NaCl, 115 mM; Tris-HCl, pH 7.5, 30 mM; and ATP (containing trace amounts of [γ-^{32}P]-ATP), 3 mM, in a final volume of 2 ml. This reaction medium gave optimal incorporation of ^{32}P into nuclear phosphoproteins. The specific activity of the ATP mixture in the reaction was generally 2000 dpm/mμmole of ATP. Histone and non-histone proteins were separated from nuclei after the reaction by the procedures detailed previously (15, 16). In brief, nuclei were washed successively with various organic solvents and then extracted with 0.25 N HCl (pH $\sim$ 0.6) for 60 min with continuous mixing in the cold. The HCl-insoluble fraction was separated by centrifugation. The HCl extract was mixed with 20 volumes of acetone to precipitate histone proteins. This procedure has been shown to remove histones quantitatively (4, 15, 16) with minimal contamination. Repeated extraction of the HCl-insoluble proteins (non-histones) with 0.25 N HCl did not significantly alter their specific activity (counts per minute per milligram of protein). In our experience, about 95% of the radioactivity in the histone fraction was extracted in the first extraction of the nuclear proteins with 0.25 N HCl. The proteins thus separated were used to isolate phosphoproteins by the methods described in detail previously (17, 18). This included the washing of the fractions with 10% (w/v) trichloracetic acid (containing 1 mM P_i and 1 mM ATP as carriers) several times to remove the contaminating radioactivity, followed by removal of lipids and nucleic acids. When total nuclear phosphoproteins were to be studied, the reaction was terminated by the addition of trichloracetic acid (10% final concentration). The rest of the procedures for isolating the phosphoprotein fraction were the same as described above. The protein fractions (histone, non-histone, or total nuclear phosphoproteins) were finally hydrolyzed in 1 N NaOH for 17 hr. Each experimental sample was assayed for protein content according to Lowry *et al.* (19), and the radioactivity of the phosphate released was measured (17, 18).

The results were expressed as millimicromoles of ^{32}P per milligram of protein and were calculated from the specific radioactivity of ATP in the reaction.

RESULTS AND DISCUSSION

When rat ventral prostate nuclei were incubated *in vitro* with [γ-^{32}P]-ATP, rapid incorporation of the label into phosphoproteins was observed. Separation of the total nuclear phosphoproteins into two major fractions, non-histones and histones, revealed that the preponderant proportion of radioactivity was present in the non-histone fraction (Fig. 1A). Time course studies showed that the rates of incorporation of ^{32}P from [γ-^{32}P]-ATP into the non-histone and histone phosphoprotein fractions were linear for 2 and 3 min, respectively. Similar results for

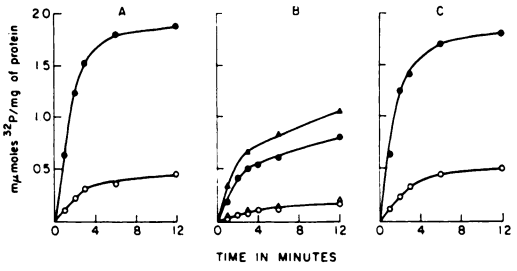

FIG. 1. *Time course of incorporation of ^{32}P into non-histone and histone phosphoproteins*

A. Normal rats: ●, non-histone phosphoproteins; ○, histone phosphoproteins.

B. Castrated control rats given a single injection of 0.2 ml of sesame oil: ●, non-histone phosphoproteins; ○, histone phosphoproteins. At 60 and 120 min, the values (not shown) for the non-histone phosphoproteins in the castrated control rats were 1.1 and 1.3 mμmoles, respectively. Daily treatment of castrated rats with 0.2 ml of sesame oil gave essentially the same result obtained with a single injection of sesame oil reported here. Castrated rats given a single injection of 2 mg of testosterone propionate:▲, non-histone phosphoproteins;△, histone phosphoproteins.

C. Castrated rats given daily injections of 1 mg of testosterone propionate dissolved in 0.2 ml of sesame oil: ●, non-histone phosphoproteins; ○, histone phosphoproteins.

Each point represents the mean of three experiments, with 36 rats in each group. All other experimental details were the same as described in the text and in the legend to Table 1.

the initial rates were observed with nuclei isolated from rats of varying androgenic status (Fig. 1B and C). In normal and testosterone-treated rats, the incorporation of ^{32}P essentially reached equilibrium within 8 min of the reaction (Fig. 1A and C). However, the nuclei from castrated rats continued to incorporate ^{32}P over a long period of time (see legend to Fig. 1B), and the radioactivity tended to approach the control values. This observation strongly suggests that the decline in ^{32}P incorporation as a result of castration could be due to a decline in the protein phosphokinase(s) activity associated with nuclei. If the decline observed were due solely to the loss of acceptor proteins for ^{32}P, the incorporation, though reduced, should have reached equilibrium in a manner similar to that observed with nuclei from prostates of normal rats (Fig. 1A). Furthermore, we

TABLE 1

Effect of single injection of testosterone on initial rate of ^{32}P incorporation from [$\gamma^{32}P$]-ATP into phosphoproteins of nuclei isolated from rat ventral prostate

Castrated control rats (118 hr after orchiectomy) received subcutaneous injections of 0.2 ml of sesame oil; the testosterone-treated castrated rats each received 2 mg of testosterone propionate dissolved in 0.2 ml of sesame oil. The animals were killed after 30 min following the injections, and the prostates were pooled from each group of rats to isolate nuclei. The duration of the reaction was 60 sec at 37°. The results below are based on five different experiments. Each group had 14–16 rats in an experiment. The values in the table are means ± standard deviations.

Rats	Histone phospho-proteins	Non-histone phospho-proteins
	mμmoles ^{32}P/mg protein/hr	
Castrated controls	2.8 ± 1.0	9.9 ± 2.2
Castrated, treated with testosterone	3.5 ± 0.8	17.8 ± 3.5
Increase due to testosterone treatment	25%	80%

observed that not all the phosphate groups of the non-histone phosphoproteins were capable of rapid exchange. This was established by first allowing the nuclei to react with unlabeled ATP for 8 min; [γ-^{32}P]-ATP was then added, and the time course of incorporation of ^{32}P was studied. The result was qualitatively similar to that shown in Fig. 1A. However, the initial rate of incorporation of ^{32}P and the extent of labeling were about 35% of those observed without prior incubation with unlabeled ATP.

In order to determine whether the phosphorylation of nuclear proteins was related to gene action in the prostate in response to testosterone, we investigated the early effect of a single injection of testosterone. It is clear from Table 1 that as early as 30 min after the injection (earliest time studied by us) there was a large increase in the initial rate of phosphorylation of non-histone phosphoproteins; the increase in the histone phosphoprotein was relatively small. The effect of a single injection of testosterone on the time

course of ^{32}P incorporation in the non-histone and histone phosphoproteins was also investigated (Fig. 1B). Not only was there an increase in the initial rate, but the phosphorylation also continued to increase with time. The break in the time course curve might suggest more than one site of phosphorylation. Again, the nature of this time course suggests that the result could be due to increased enzyme activities.

The phosphoprotein nature of the non-histone and histone phosphoprotein fractions was established by isolating *O*-phosphorylserine from acid hydrolysates of the labeled proteins (17, 18). Nuclei were labeled with ^{32}P in the usual reaction medium for 1 min. Non-histone and histone phosphoproteins were separated, and each fraction was analyzed for radioactivity in the phosphoamino acids in acid hydrolysates of the protein fractions. Most of the radioactivity was found in *O*-phosphorylserine. The yield of *O*-phosphorylserine from the non-histone protein fraction was 0.85 mμmole/mg of protein, whereas it was 0.13 mμmole/mg of histone phosphoprotein. These values are of the same order as those observed using alkali-labile phosphate as a measure of the phosphoprotein fractions. Furthermore, with 8-^{14}C-labeled ATP in the reaction, the rate of incorporation of the label into the nucleoproteins was less than 4% in comparison to that with [γ-^{32}P]-ATP.

It may be argued that the radioactivity in the "non-histone" fraction might have been due to the presence of a highly phosphorylated histone. Such a histone would have had to be present in very small amounts and be resistant to repeated extractions with HCl in the procedures used by us. The histone and non-histone proteins extracted by the procedures outlined above gave the same proportions of two types of proteins in zero-time samples (which would not be phosphorylated) as well as in the experimental samples. Thus, it is unlikely that the presence of a large amount of a particular histone would account for the radioactivity in the "non-histone" fraction described above. We hope to fractionate these non-histone proteins further to isolate the phosphorylated components.

The effect of testosterone on nuclear phosphoproteins is "target tissue"-specific. No changes were observed in the ability of liver nuclei, isolated from castrated or testosterone-treated castrated rats, to incorporate radioactivity into phosphoprotein fractions. Therefore, we suggest that the activity of some nuclear protein phosphokinase(s) is increased early during the action of testosterone in the prostate. Also, there may be a possible relationship between the increased phosphorylation of some proteins (in particular, those of the non-histone type) and gene action in the prostate in response to testosterone. In this regard, our results corroborate the findings of Benjamin and Goodman (6) on the phosphorylation of non-histone proteins of the giant chromosomes of dipteran salivary glands.

The addition of testosterone, dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), cyclic 3′,5′-AMP, or dibutyryl cyclic 3′,5′-AMP at various concentrations *in vitro* did not stimulate the phosphorylation reactions in nuclei isolated from prostates of either normal or castrated rats. It is noteworthy that Rosenfeld and O'Malley (20) observed no stimulation of the adenyl cyclase of rat prostate as a result of injections of testosterone into the animals. However, several cyclic AMP-stimulated protein phosphokinases capable of phosphorylating histones are known to be present in the cell cytoplasm, and they may be of importance in special types of gene control (21–23).

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