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Bone marrow ribonucleic acid polymerase. Effect of testosterone on nucleotide incorporation into nuclear RNA.

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ABSTRACT

The incorporation of ^{3}H-UTP into RNA by isolated rat bone marrow nuclei is stimulated by testosterone. This effect is hormone and tissue specific. Using α-amanitine and different ionic strenght conditions it was found that testosterone enhances preferentially RNA polymerase I activity. The sedimentation pattern of RNA isolated from bone marrow nuclei shows that the synthesis of RNA species within the 14-30 S range is mainly stimulated by the hormone.

INTRODUCTION

It has been previously reported that the injection of testosterone stimulates total and nuclear RNA synthesis in rat bone marrow cells (1, 2).

Eukaryotic cells contain at least three classes of RNA polymerase whose enzymatic characteristics, intranuclear location and sensitivity to different concentrations of α-amanitine are well known (3). The activity of some of these enzymes is subjected to hormonal regulation (4).

The present experiments were designed to investigate the effect of androgens on RNA synthesis by isolated rat bone marrow nuclei and to establish whether the hormonal effect is mediated by changes on the activity of either class of RNA polymerases I, II and III.

MATERIALS AND METHODS

Preparation of the nuclear fraction

Female Sprague-Dawley rats (200-300 g) were used in all the experiments. The extraction of bone marrow cells and the preparation of the nuclear fraction were performed as previously described (2). The resulting nuclear pellet was suspended in 60 mM Tris-HCl buffer, pH 8.1.

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Aliquots were taken for measurement of RNA synthesis and for DNA content (5).

Measurement of RNA synthesis

RNA synthesis was measured at low ionic strength in a reaction mixture containing 60 mM Tris-HCl buffer. pH 8.1, 5 mM $MgCl_2$, 30 mM KCl, 10 mM mercaptoethanol, 0.1 mM GTP, ATP and CTP, 1 uM UTP, 2 uCi ^{3}H-UTP and a proper volume of the nuclear suspension (10-50 ug DNA) in 0.5 ml. For assays performed at high ionic strengh the mixture also contained 0.4 M ammonium sulphate and 5 mM $MnCl_2$.

Reaction (15 min at 37° C) was terminated by the addition of 2 ml of 10% (w/v) trichloroacetic acid containing 1 mM sodium pyrophosphate. Mixtures were chilled and yeast RNA (200 ug in 0.2 ml) was added as carrier. The precipitate obtained was filtered on nitrocellulose filters (HAMP, Millipore) and washed (5 ml x 4) with 10% trichloroacetic acid. The filters were dried and radioactivity was measured with a liquid scintillation spectrometer (Nuclear Chicago, Mark I) using as scintillator a mixture of 4 g PPO, 400 ml ethanol and 600 ml toluene, with an efficiency of 30%. Proper blanks were run for each determination. RNA synthesis was estimated in terms of dpm ^{3}H-UTP incorporated into acid-insoluble material/10 ug DNA.

Isolation and characterization of in vitro synthesized RNA

a) Isolation

The reaction mixture described above was used but GTP, UTP, ATP and CTP concentrations were 1 uM and 2 uCi each of the four labeled nucleosides triphosphates used, instead of ^{3}H-UTP.

Reaction was terminated by the addition of 5 ml of 1% sodium dodecylsulphate containing 0.1% diethyl pyrocarbonate and 1 mM $MgCl_2$ and 1 volume of phenol-cresol-H_2O (7:1:2) mixture containing 0.1% hydroxyquinoline. Newly synthesized RNA was isolated by the method described by Nicol et al. (6).

b) Characterization

RNA isolated by the above method was solubilized in 2 ml of 0.05 m sodium acetate buffer, pH 7.0 containing 0.05 M NaCl and 20 mg/ml of popyvinyl phosphate. An aliquot (15,000 dpm in 0.2 ml) of the solution was layered on top of a linear 5-20% sucrose gradient, containing 1% sodium dodecylsulphate and centrifuged in the SW 65 L Ti

rotor of the Beckman L2 65B ultracentrifuge for 4 hr at 45,000 rpm at 20 C. Fractions (0.19 ml) were collected and 0.1 ml of a 1% bovine serum albumine solution and 5 ml of 10% trichloroacetic acid solution were added in the cold. The acid-insoluble material was collected by centrifugation at 2,500 g for 15 min and washed (5 ml x 2) with 10% trichloroacetic acid. The pellet obtained was solubilized in 0.2 ml of Protosol (New England Nuclear) and radioactivity was measured using a dioxane scintillation solution with an efficiency of 40%.

Chemicals

^{3}H-CTP (28.6 Ci/mmol), ^{3}H-UTP (36.0 Ci/mmol), ^{3}H-GTP (8.1 Ci/mmol), ^{3}H-ATP (26.2 Ci/mmol) were obtained from New England Nuclear. ATP, CTP, GTP, UTP, testosterone, estradiol-17β, 5α-dihydrotestosterone (17β-hydroxy-5α androstan-3 one) and 5β-dihydrotestosterone (17β-hydroxy-5α androstan-3 one) were from Sigma Chemical Co. α-amanitine was from Boehringer Sohn. All other chemicals were reagent grade.

RESULTS

Purified bone marrow nuclei actively synthesize RNA in an in vitro assay which includes ^{3}H-UTP and the unlabeled nucleoside-triphosphates. In this condition a linear relationship is established between DNA in the reaction mixture (10 to 50 ug) and ^{3}H-UTP incorporated into RNA both at high and low ionic strength conditions.

To study the effect of testosterone on RNA synthesis by the nuclear fraction of bone marrow cells, experiments were done at two ionic strengths and results are presented in Table 1. Testosterone increases the incorporation of ^{3}H-UTP into acid insoluble material at both conditions, however the stimulatory effect of the hormone is more notorius at the low ionic media. In this case an enhancement of 43% on the ^{3}H-UTP incorporation into RNA was observed as compared to the control. Table 1 also shows that when liver nuclei are used, testosterone does not increase the incroporation of ^{3}H-UTP into RNA in none of the salt conditions used.

The effect of several steroids on the ^{3}H-UTP incorporation into RNA by bone marrow nuclei was studied and results in Table 2 show that 5α-dihydrotestosterone, 5β-dihydrotestosterone and estradiol-17β do not mimic the effect of testosterone on the incorporation of ^{3}H-UTP into acid-insoluble material.

The ionic dependence for the effect of testosterone on RNA synthesis

Table I

Effect of testosterone on RNA synthesis by bone marrow and liver nuclei.

Conditions	dpm ^{3}H-UTP incorporated/10 ug DNA
A.- Low ionic strength	
bone marrow nuclei	8,460 ± 600*
+ testosterone	12,000 ± 750
liver nuclei	15,000 ± 1,200
+ testosterone	16,230 ± 1,260
B.- High ionic strength	
bone marrow nuclei	32,600 ± 650
+ testosterone	41,500 ± 1,050
liver nuclei	44,800 ± 2,160
+ testosterone	46,000 ± 2,500

*The data represent the mean ± S.D. of four determinations.
Testosterone was added to the reaction mixture at a final concentration of 5 x 10^{-9} M.
Conditions for incubation and measurement of ^{3}H-UTP incorporation into RNA were done as described in Materials and Methods.

Table II

Effect of different steroids on RNA synthesis by bone marrow nuclei at low ionic strength.

Addition	dpm ^{3}H-UTP incorporated/10 ug DNA
none	7,980 ± 560 *
testosterone	11,450 ± 630
5α-dihydrotestosterone	8,500 ± 660
5β-dihydrotestosterone	8,300 ± 580
estradiol-17β	8,100 ± 710

*The data represent the mean ± S.D. of four determinations.
Testosterone, 5α-dihydrotestosterone, 5β-dihydrotestosterone and estradiol-17β were added to the reaction mixture at a final concentration of 5 x 10^{-9}M. Reaction mixture and measurement of ^{3}H-UTP incorporation into RNA were done as described in Materials and Methods.

suggests that different RNA polymerases might be involved in the process. In order to gain insight into the types of RNA polymerases engaged in RNA synthesis under the effect of testosterone, experiments were done in the presence of α-amanitine at two ionic strength conditions.

The results in Figure 1-A show that at low ionic strength condition, testosterone stimulates ^{3}H-UTP incorporation up to 50% as compared with controls. In the presence of α-amanitine at low (0.1 ug/ml) or high (100 ug/ml) concentration, there is an inhibition in the incorporation of the radioactive precursor of 22% and 37% respectively, in comparison to controls. However, when bone marrow nuclei are incubated in the presence of testosterone and α-amanitine, there is an increase of 76% at low α-amanitine concentration (Figure 1-B) and 86% at high α-amanitine concentration in the ^{3}H-UTP incorporation into RNA, in comparison to controls containing α-amanitine alone (Figure 1-C).

Experiments done at high ionic strength conditions indicate, as

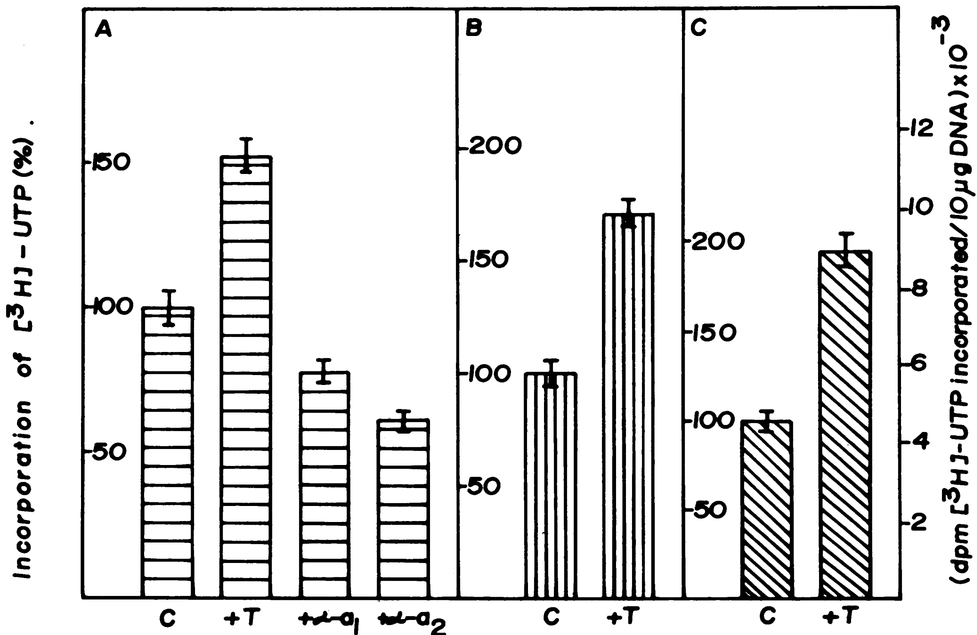

Figure 1. The effect of testosterone and α-amanitine on RNA synthesis by bone marrow nuclei at low ionic strength.

A. bone marrow nuclei incubated in the the presence of: none(C), testosterone (T) (5 x 10^{-9} M), α-amanitine (α-a$_1$) (0.1 ug/ml) or α-amanitine (α-a$_2$) (100 ug/ml)

B. bone marrow nuclei incubated in the presence of α-amanitine (C) (0.1 ug/ml) to which testosterone (T) was added.

C. bone marrow nuclei incubated in the presence of α-amanitine (C) (100 ug/ml) to which testosterone (T) was added.

Each bar represents the mean ± SD of 3 determinations

For experimental conditions, see Material and Methods.

shown in Figure 2-A, that testosterone stimulates up to 20% the ^{3}H-UTP incorporation into RNA, as compared to controls. In the presence of α-amanitine at low (0.1 ug/ml) and high (100 ug/ml) concentration there is an inhibition of 22% and 85% respectively, in comparison to controls. When bone marrow nuclei are incubated at high ionic strength conditions in the presence of testosterone and α-amanitine, there is and increase in the ^{3}H-UTP incorporation into RNA of 11% at low α-amanitine concentration (Figure 2-B) and 84% at high α-amanitine concentration (Figure 2-C) in comparison to the respective controls containing the toxine alone.

The partial characterization of the RNA synthesized in the presence of testosterone was obtained after sucrose density gradient analysis. In Figure 3, the sedimentation pattern of the RNA extracted from the reaction mixture is presented. It can be seen that among the RNAs species with higher radioactivity are those having sedimentation coefficients within the range of 14 and 30 S.

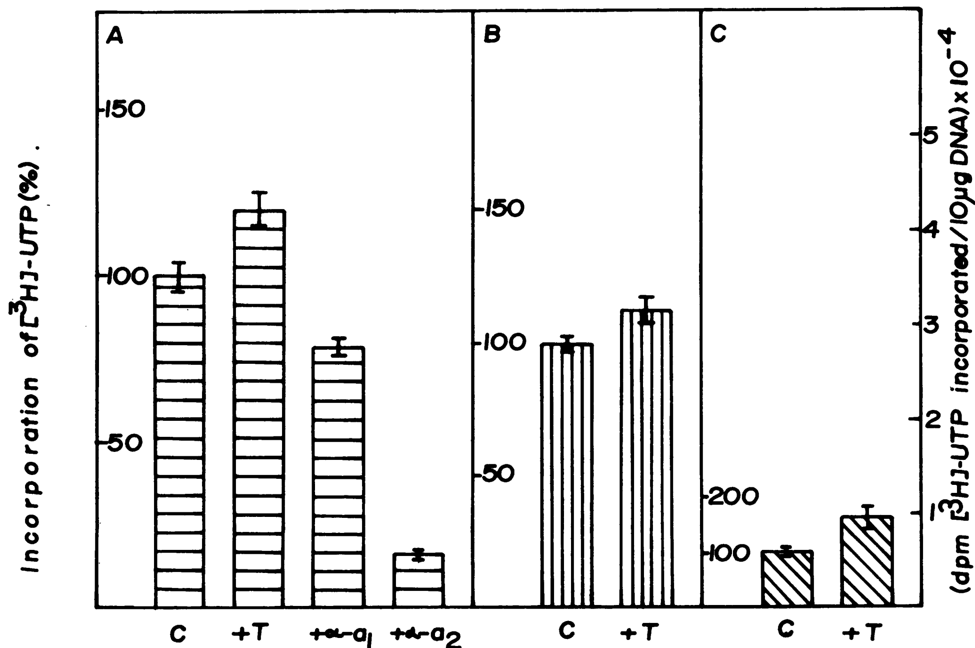

Figure 2. The effect of testosterone and α-amanitine on RNA synthesis by bone marrow nuclei at high ionic strength.
See legend to figure 1 for details.

DISCUSSION

The present data demonstrate that testosterone stimulates the incorporation of ^{3}H-UTP into RNA in isolated rat bone marrow nuclei and confirm previous reports of the in vivo effect of the hormone on nuclear RNA synthesis (2).

In a previous communication we have reported that the entry of testosterone to bone marrow nucleus does not requires cytoplasmic components and it depends only on the presence of a nuclear receptor (8). The stimulatory effect of testosterone on RNA synthesis in bone marrow nuclei and the lack of effect of the hormone in liver nuclei give further support to the above conclusion. We assume that this tissue-specificity is due to the fact that in liver cells the entry of androgens to the cell nucleus requires a cytoplasmic receptor (9).

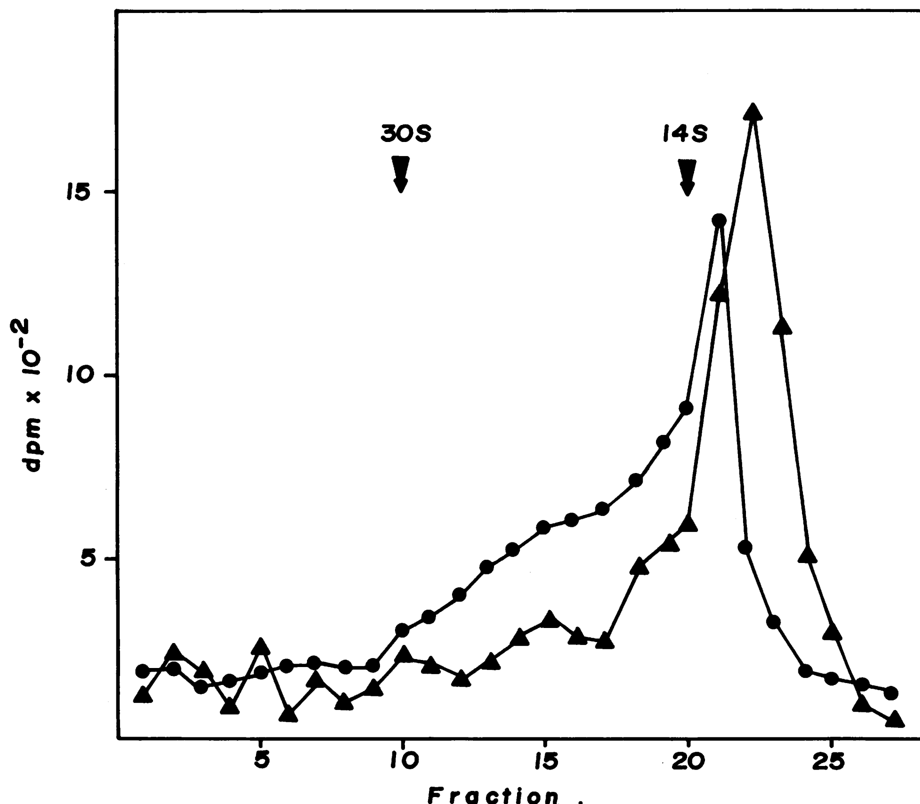

Figure 3. Centrifugation patterns of RNA extracted from bone marrow nuclei. Control (▲ - ▲) and testosterone-treated nuclei (● - ●). The arrows show calculated sedimentation coefficients (7). For details, see Materials and Methods.

Comparative studies on the effect of different steroids on RNA synthesis by bone marrow nuclei suggest the existence of hormone-specificity. Thus, steroids such as 5α-dihydrotestosterone, 5β-dihydrotestosterone and estradiol-17β are not as effective as testosterone to stimulate RNA synthesis. This specificity seems to be due to the interaction of testosterone with a highly specific nuclear receptor which shows low binding affinity for others steroids (10).

The maximun effect of testosterone on RNA synthesis is observed when nuclei are incubated at low salt conditions, even at high concentrations of α-amanitine (100 ug/ml). At this concentration of the toxine, RNA polymerases II and III are known to be completely inhibited whereas RNA polymerase I is not affected (11). Therefore, the increased ^{3}H-UTP incorporation into RNA induced by the hormone suggests that the hormonal effect might be mediated by a modification on the activity of RNA polymerase I. This assertion was confirmed by the results obtained after measuring RNA synthesis conduced at a high salt medium. Thus, the addition of α-amanitine (100 ug/ml) produces on inhibition of 85% on the incorporation of ^{3}H-UTP into RNA; this result seems to be the consequence of the inhibitory action of the toxin on RNA polymerases II and III. Since in these conditions testosterone induces a 2 fold increase in the ^{3}H-UTP incorporation into RNA, in comparison to controls with α-amanitine but lacking testosterone, these results might suggests a specific effect of testosterone on the activity of RNA polymerase I. This enzyme is not affected by high concentrations of toxine.

According to the differential sensitivity of RNA polymerases to variable concentrations of α-amanitine it is posible to determine the relative contribution of each class of RNA polymerase to synthesize RNA in bone marrow nuclei. In control nuclei, RNA polymerase I activity accounts for the synthesis of approximately 15% of RNA being formed, but in the presence of testosterone the enzyme seems to be more actively engaged in RNA synthesis and it accounts for 22% of the RNA formed. After the addition of testosterone RNA polymerase II and III activity accounts for 86% of RNA being formed; in the absence of the hormone the enzyme activity accounts for 76% of RNA. Thus, there was not a marked difference in the activity of RNA polymerase II and III, while there was a significant difference in the activitv of RNA polymerase I in the presence of testosterone.Therefore,these results allow to conclude that the activity of RNA polymerase I is hormone-dependent.

It is worth mentioning the high activity of RNA polymerase III, sensible to high dosis of α-amanitine. Several reports have revealed that the cellular level of this enzyme is higher in tissues with apparently greater rates of growth and proliferation (12, 13) as is the case of rat bone marrow.

The increased incorporation of nucleoside triphosphates seems to be a direct effect of the hormone on the transcriptional activity of chromatin rather than an effect on the entry rate of nucleotides to the nuclei or to changes produced on the activity of bone marrow nuclear endonucleases (14). Experiments with broken nuclei have shown that testosterone stimulates RNA synthesis to the same extent as it occurs when intact nuclei are used, excluding thus an effect at the nuclear membrane level. It has been shown that testosterone, at the *in vitro* conditions used in this work, does not modify the activity of nuclear ribonucleases (unpublished results)

Although it was clear that testosterone stimulates RNA synthesis, it was not determined whether changes on RNA polymerase I reflects modulation of the activity of a constant nuclear population of enzymes molecules or a greater availability to the DNA template due to conformational changes on bone marrow chromatin.

The rate of synthesis of all species of RNA seems to increase after the administration of testosterone to rats (2), however according to our findings it is expected that the rate of synthesis of rRNA should increase to a greater extent than does the rate of DNA-like RNA or tRNA. The sedimentation pattern of RNA synthesized by bone marrow in the presence of testosterone shows that 14 - 30S RNA species were predominantly formed in these conditions. Accordingly, we assume that among the RNA stimulated by the hormone the ribosomal RNA may be present.

Thus, we propose that testosterone stimulates rRNA synthesis in rat bone marrow nuclei by a selective effect on the activity of the nucleolar Mg^{++}-dependent RNA polymerase I. Similar mechanism of action has been described in several others androgen-dependent tissues (15).

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