Effects of α -Amanitin on the Stimulation of Prostatic Ribonucleic Acid Polymerase by Prostatic Steroid-Protein Receptor Complexes

By P. DAVIES and K. GRIFFITHS

Tenovus Institute for Cancer Research, Welsh National School of Medicine,
Heath Park, Cardiff CF4 4XX, U.K.

(Received 22 March 1974)

Stimulation of prostatic RNA polymerase *in vitro* by prostatic 17 β -hydroxy-5 α -androstan-3-one (5 α -dihydrotestosterone)-receptor complexes has been previously reported. By use of the selective inhibitor, α -amanitin, we have shown that both nucleolar and extranucleolar RNA polymerase activities may be stimulated, but stimulation is abolished at high ionic strength.

The stimulation of prostatic DNA-dependent RNA polymerase (nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6) by androgens has been well documented (Liao *et al.*, 1965; Bashirelahi *et al.*, 1969; Davies *et al.*, 1972). The retention and relocation in hormone-responsive tissues, not only of androgens but of other steroid hormones, has been shown to be dependent on their binding to cytoplasmic protein receptor molecules (Mainwaring, 1970; Fang & Liao, 1971), and the transfer of the complex to the nucleus (Fang & Liao, 1971; O'Malley *et al.*, 1972). A direct stimulation of prostatic RNA polymerase by prostatic dihydrotestosterone (17 β -hydroxy-5 α -androstan-3-one)-receptor complexes has been shown, that is dependent on the presence of a specific prostatic chromatin template (Davies & Griffiths, 1973*a,b*). The requirement for a specific template correlates with the association of steroid-receptor complexes specifically with chromatin from target tissues (Mainwaring & Peterken, 1971; Steggle *et al.*, 1971). Further studies were done on the stimulation of prostatic RNA polymerase by prostatic steroid-receptor complexes, to elucidate the exact nature of the stimulated enzyme and of the type of RNA produced as a result of the stimulation. This involved the evaluation of the effects of ionic strength on the system and the use of the toxin α -amanitin. This substance has no effect on nucleolar RNA polymerase *in vitro*, but inhibits the activity of nucleoplasmic RNA polymerase (Stirpe & Fiume, 1967; Novello & Stirpe, 1969; Jacob *et al.*, 1970*a,b*; Kedinger *et al.*, 1970; Lindell *et al.*, 1970). Such studies are of value in elucidating the role of hormonal steroids in the modification of gene expression, and of the mechanism by which steroids instigate the chain of biochemical events in cells of hormone-responsive tissues.

Materials and methods

All preparative procedures were done on ventral prostatic tissue excised from 8–12-week-old male

Sprague-Dawley rats 48 h after bilateral castration. Methods of purification of all necessary subcellular components and procedures for measuring the incorporation of labelled precursor into RNA have been described by Davies & Griffiths (1973*a,b*). Modifications and details of concentrations of (NH₄)₂SO₄ for individual experiments are given in the text. These solutions were adjusted to pH 8.1 with aq. NH₃ solution before addition to enzyme assays. When added, α -amanitin (Calbiochem Ltd., London W.1, U.K.) was in 120 mM-Tris-HCl buffer, pH 8.1. Incubation mixtures were adjusted to constant volume with this buffer. The [³H]5 α -dihydrotestosterone-receptor complexes were added to assay mixtures at a final radioactive 5 α -dihydrotestosterone concentration of 250 fmol/ml. Additional control assays containing '5 α -dihydrotestosterone-free' receptor proteins (Davies & Griffiths, 1973*a,b*) did not show an appreciably different [¹⁴C]UMP incorporation from the basic enzyme systems.

DNA content of subcellular preparations was determined by the method of Burton (1956) with calf thymus DNA as standard. Protein was measured by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin [Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.] as standard.

Results and discussion

Preliminary investigations showed that maximum inhibition of soluble whole nuclear RNA polymerase was achieved with 60–80 ng of α -amanitin in assays containing up to 50 μ g of enzyme protein. Dose response was similar in assays with both Mg²⁺-low-salt medium (25–35% inhibition) and Mn²⁺-high-salt medium (70–90% inhibition). Similarly, maximum inhibition of RNA polymerase in intact nuclei and in nucleoplasmic fragments was obtained with the use of those amounts of toxin/25–30 μ g of DNA.

Table 1. *Effect of ionic strength and α -amanitin on stimulation of nuclear RNA polymerase by [3 H]5 α -dihydrotestosterone-receptor complexes*

The activity of RNA polymerase in intact rat ventral-prostatic nuclei or in preparations solubilized from such nuclei prepared from rats castrated 48 h previously was measured in standard assay mixtures with and without various substances. Cytoplasmic (8S) and nuclear (4.5S) [3 H]5 α -dihydrotestosterone-receptor complexes were added to certain assays at a final [3 H]steroid concentration of 0.25 pmol/ml. α -Amanitin was added to some assays in quantities of 60 ng/assay. RNA polymerase activity of nuclei is expressed as pmol of [14 C]UMP incorporated/100 μ g of DNA and that of soluble preparations as pmol of [14 C]UMP incorporated/100 μ g of enzyme protein. Values are the means $\pm$ s.d. of at least quadruplicate determinations. *P* values for stimulation of activity by [3 H]steroid-receptor complexes versus controls: * *P* < 0.001; † *P* > 0.10. *P* values for stimulation of α -amanitin-sensitive activity, as calculated from relative inhibition of activity by α -amanitin versus inhibition of controls: ‡ *P* > 0.1; § *P* < 0.1; || *P* < 0.01.

Conditions of assay	RNA polymerase activity (pmol of [14 C]UMP incorporated)				
	Control	Control + α -amanitin	8S complex	8S complex + α -amanitin	4.5S complex + α -amanitin
(1) Intact nuclei					
Mg ²⁺ (5 mmol/litre), no (NH ₄) ₂ SO ₄	3.60 $\pm$ 0.34	2.47 $\pm$ 0.28	*7.22 $\pm$ 0.62	‡5.78 $\pm$ 0.76	‡4.54 $\pm$ 0.58
Mn ²⁺ (5 mmol/litre), (NH ₄) ₂ SO ₄ (0.4 mol/litre)	16.72 $\pm$ 0.92	1.63 $\pm$ 0.92	†15.78 $\pm$ 3.15	1.35 $\pm$ 0.47	1.60 $\pm$ 0.14
Mg ²⁺ (5 mmol/litre), Mn ²⁺ (3 mmol/litre), (NH ₄) ₂ SO ₄ (0.04 mol/litre)	9.23 $\pm$ 0.09	4.45 $\pm$ 0.63	*15.09 $\pm$ 0.94	‡9.80 $\pm$ 0.27	§9.98 $\pm$ 1.12
(2) Solubilized nuclear RNA polymerase					
Mg ²⁺ (5 mmol/litre), Mn ²⁺ (3 mmol/litre), (NH ₄) ₂ SO ₄ (0.04 mol/litre)	1.43 $\pm$ 0.21	0.40 $\pm$ 0.08	*4.66 $\pm$ 0.21	0.80 $\pm$ 0.21	0.65 $\pm$ 0.12

Table 2. *Effects of α -amanitin and 5 α -dihydrotestosterone-receptor complexes on nucleoplasmic RNA polymerase activity*

Nucleoplasm was prepared by sonication from ventral-prostatic nuclei of rats castrated 48 h previously. RNA polymerase activity of nucleoplasmic material and preparations solubilized from such material was measured in assay media containing Mn²⁺ (3 mmol/litre) and (NH₄)₂SO₄ (0.04 mol/litre). Cytoplasmic (8S) and nuclear (4.5S) [3 H]5 α -dihydrotestosterone-receptor complexes were added to certain assays at a final [3 H]steroid concentration of 0.25 pmol/ml. α -Amanitin was added in quantities of 60 ng/assay. RNA polymerase activity of nucleoplasmic material was expressed as pmol of [14 C]UMP incorporated/100 μ g of DNA and that of solubilized nucleoplasmic enzyme as pmol of [14 C]UMP incorporated/100 μ g of protein. Values are the means $\pm$ s.d. of at least quadruplicate determinations. *P* values for stimulation of activity by [3 H]steroid-receptor complexes versus controls: * *P* < 0.001. *P* values for stimulation of α -amanitin-sensitive activity, as calculated from relative inhibition of activity by α -amanitin versus inhibition of controls: † *P* < 0.001; ‡ *P* < 0.01.

	RNA polymerase activity (pmol of [14 C]UMP incorporated)				
	Control	Control + α -amanitin	8S complex	8S complex + α -amanitin	4.5S complex + α -amanitin
Nucleoplasmic fragments					
Control	5.81 $\pm$ 0.09	1.16 $\pm$ 0.16	*10.57 $\pm$ 0.62	‡2.87 $\pm$ 0.50	‡4.15 $\pm$ 1.19
Solubilized nucleoplasmic RNA polymerase	2.92 $\pm$ 0.10	1.00 $\pm$ 0.56	*7.40 $\pm$ 0.24	‡2.75 $\pm$ 0.41	‡1.65 $\pm$ 0.49

Stimulation of RNA polymerase in intact nuclei occurred when cytoplasmic (8S) or nuclear (4.5S) 5 α -dihydrotestosterone-receptor complexes were included in a low-salt medium containing Mg²⁺ (5mmol) (Table 1). This apparently was stimulation of nucleolar RNA polymerase, since it was not sensitive to α -amanitin. In a high-salt [(NH₄)₂SO₄; 400mmol/litre] medium containing Mn²⁺ (3mmol/litre), a considerable increase in α -amanitin-sensitive activity was observed. This probably represented salt-stimulation of nucleoplasmic RNA polymerase, but inclusion of receptor complexes in the assay caused no further stimulation. However, when nuclei were incubated in a medium containing (NH₄)₂SO₄ (40mmol/litre), a condition promoting Mn²⁺-dependent activity but not inhibiting Mg²⁺-dependent activity, enzyme stimulation by 5 α -dihydrotestosterone-receptor complexes was observed. The stimulated activity appeared to be partly α -amanitin-sensitive and partly insensitive. Further studies were carried out with solubilized nuclear enzyme (Davies & Griffiths, 1973b) in an attempt to resolve the nature of the stimulated enzyme. Incubations were carried out in a medium containing (NH₄)₂SO₄ (40mmol/litre), with chromatin as template. The enzyme was stimulated to a great extent by 5 α -dihydrotestosterone-receptor complexes, and most of the activity was α -amanitin-sensitive. This suggests that, under favourable ionic conditions, steroid-receptor complexes stimulate both nucleolar and extranucleolar RNA polymerase activity. To demonstrate this further, nucleoplasmic fragments and solubilized nucleoplasmic enzyme were incubated with steroid-receptor complexes in a medium containing (NH₄)₂SO₄ (40mmol/litre), with chromatin from prostatic nuclei as template. Again part of the stimulated RNA polymerase activity was α -amanitin-sensitive (Table 2). Apparently, stimulation of RNA polymerase activity by 5 α -dihydrotestosterone-receptor complexes is inhibited under conditions of high ionic strength. This cannot be due entirely to inhibition of nucleolar enzyme (Raynaud-Jammet *et al.*, 1971), since the activity of nucleoplasmic RNA polymerase, which was increased by high salt, was not stimulated by addition of the complexes. Any such stimulation caused by increased template activity would be

countered, however, by the removal of chromatin-associated proteins at high salt concentrations. Further, binding of more polymerase molecules to initiation sites could be inhibited at high ionic strength or alternatively the high salt concentration may interfere with the essential interaction between the steroid-protein complex and chromatin.

We are grateful to the Tenovus Organization for their generous financial support.

- Bashirelahi, N., Chader, G. T. & Vilee, C. A. (1969) *Biochem. Biophys. Res. Commun.* **37**, 976-981
- Burton, K. (1956) *Biochem. J.* **62**, 315-323
- Davies, P. & Griffiths, K. (1973a) *Biochem. Biophys. Res. Commun.* **53**, 373-382
- Davies, P. & Griffiths, K. (1973b) *Biochem. J.* **136**, 611-622
- Davies, P., Fahmy, A. R., Pierrepont, C. G. & Griffiths, K. (1972) *Biochem. J.* **129**, 1167-1169
- Fang, S. & Liao, S. (1971) *J. Biol. Chem.* **246**, 16-24
- Jacob, S. T., Sajdel, E. M., Muecke, W. & Munro, H. N. (1970a) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 681-691
- Jacob, S. T., Sajdel, E. M. & Munro, H. N. (1970b) *Biochem. Biophys. Res. Commun.* **38**, 756-770
- Kedinger, C., Gniazdowski, M., Mandel, J.-L., Gissinger, F. & Chambon, P. (1970) *Biochem. Biophys. Res. Commun.* **38**, 165-171
- Liao, S., Leininger, K. R., Sagher, D. & Barton, R. W. (1965) *Endocrinology* **77**, 763-765
- Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G. & Rutter, W. J. (1970) *Science* **170**, 447-449
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-276
- Mainwaring, W. I. P. (1970) in *Some Aspects of the Aetiology and Biochemistry of Prostatic Cancer* (Griffiths, K. & Pierrepont, C. G., eds.), pp. 109-114, Alpha Omega Alpha Publishers Ltd., Cardiff
- Mainwaring, W. I. P. & Peterken, R. M. (1971) *Biochem. J.* **125**, 285-295
- Novello, F. & Stirpe, F. (1969) *Biochem. J.* **112**, 721-727
- O'Malley, B. W., Spelsberg, T. C., Schrader, W. T., Chytil, F. & Steggle, A. W. (1972) *Nature (London)* **235**, 141-144
- Raynaud-Jammet, C., Bierei, F. & Baulieu, E.-E. (1971) *Biochim. Biophys. Acta* **247**, 355-360
- Steggles, A. W., Spelsberg, T. C., Glasser, S. R. & O'Malley, B. W. (1971) *Proc. Nat. Acad. Sci. U.S.A.* **68**, 1479-1482
- Stirpe, F. & Fiume, L. (1967) *Biochem. J.* **105**, 779-782