Initiation and Elongation of Polyribonucleotide Chains on Rat Ventral-Prostate Chromatin Transcribed by Homologous Ribonucleic Acid Polymerase B

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The characteristics of initiation of RNA synthesis and the elongation of RNA chains on rat ventral-prostate chromatin by RNA polymerase B were investigated by two methods. 1. Initiation was carried out under low-salt conditions with three ribonucleoside triphosphates, and elongation was begun in the absence of reinitiation by the addition of the fourth ribonucleoside triphosphate and increasing the salt concentration. 2. Stable initiation complexes were formed by preincubation of enzyme with template at 37°C, elongation was started by the addition of all four ribonucleoside triphosphates and reinitiation or spurious RNA synthesis was prevented by rifamycin AF/013. The latter method gave more reliable results. The dependence of those parameters on the androgenic status of the animal was studied. During the first 24 h after castration, elongation was mainly affected, whereas after 72 h a smaller number of initiation sites for RNA polymerase B on chromatin was evident. Considerable diurnal variations in the various parameters were observed. Changes in the relative concentrations of the chromatin-associated proteins were also observed after castration. In the rat ventral-prostate gland androgenic steroids may not only influence one stage of the transcriptional process, but may affect many factors involved in the control of gene expression.

The regulation of macromolecular synthesis in the rat ventral prostate depends on the binding of androgens to intracellular proteins (King & Mainwaring, 1974; Liao & Liang, 1974; Minguell & Sierralta, 1975). Complexes formed between 5 α -dihydrotestosterone (17 β -hydroxy-5 α -androstan-3-one) and a specific cytoplasmic macromolecule are removed to the nucleus, where they are retained for a finite period of time, probably by selective components (Mainwaring *et al.*, 1976). Studies performed *in vitro* show a direct effect of androgen-receptor complexes on the transcription of prostate chromatin by RNA polymerase (EC 2.7.7.6; nucleoside triphosphate-RNA nucleotidyltransferase) (Davies & Griffiths, 1973*a,b*, 1974*a,b*; Davies, 1975; Mainwaring & Jones, 1975; Hu *et al.*, 1975). Although these studies establish the general effects, certain drawbacks are evident (Mainwaring, 1975). Extensive purification of the androgen receptor and complete understanding of hormonal effects on the several stages of the transcriptive process are essential.

Measurement of template activity reflects the amount of DNA in chromatin available to RNA polymerase but, as Tsai *et al.* (1975) emphasized, the complexity of the process renders precise understanding of the mechanism of hormone action

difficult. Template activity may be modified by (a) the binding to chromatin of RNA polymerase, (b) its dissociation and reassociation until formation of stable initiation complexes with DNA, (c) the initiation of RNA synthesis, (d) the rate of elongation of RNA chains and (e) termination and reinitiation of RNA synthesis. Each process should be studied without interference from the others.

The object of this work was to establish experimental conditions for the measurement of initiation sites for RNA polymerase on rat ventral-prostate chromatin and for the determination of the initial rates of elongation, and absolute lengths, of RNA chains. The application of these techniques to a study of androgen effects on transcription is described.

Methods and Materials

Experimental animals

Male Sprague-Dawley rats were bred in the Animal Unit of the Tenovus Institute, and used when 8–12 weeks old. Animals were castrated via a median scrotal incision under ether anaesthesia at various times before death. Ventral-prostate tissue was carefully dissected from animals killed when required (see the Results section), placed in vessels surrounded

with crushed ice, and used immediately for the preparation of subcellular fractions.

Chemicals

[5-³H]UTP (specific radioactivity 10–30 Ci/mmol), [5-³H]CTP (specific radioactivity 23 Ci/mmol), [γ -³²P]ATP (specific radioactivity 15–17 Ci/mmol) and [γ -³²P]GTP (specific radioactivity 11–16 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The disodium salt of ATP, the trisodium salts of CTP, GTP and UTP, and α -amanitin were purchased from Boehringer Corp. (London) Ltd., London, W.5, U.K. Crystalline bovine serum albumin, calf-thymus DNA, calf-thymus histone (type II-A), dithiothreitol, Triton X-100 and Tris base (99% pure) were from the BDH, Poole, Dorset, U.K. Rifamycin AF/013 was generously provided by Dr. R. Cricchio and Professor L. G. Silvestri of Gruppo Lepetit, Milan, Italy. All other substances were of 'AnalaR' grade (BDH) and were dissolved in water distilled from all-glass apparatus.

Centrifuges and rotors

High-speed centrifugation procedures were carried out in a Beckman L2-65B or L5-65B ultracentrifuge by using either the SW.50.1 (6×5 ml) swinging-bucket rotor (r_{av} 8.35 cm) or the SW.60 (6×4 ml) swinging-bucket rotor (r_{av} 9.17 cm).

Preparation of subcellular fractions

Nuclei were purified from homogenates of rat ventral-prostate tissue as previously described (Davies & Griffiths, 1973b). Chromatin devoid of endogenous RNA polymerase activity was prepared by the method of Mainwaring & Peterken (1971). Chromatin with associated enzyme activity was prepared as described by Butterworth *et al.* (1971). Preliminary chemical analysis of chromatin was performed as described by Marushige & Bonner (1966). In these studies prostate chromatin from normal animals had a protein/DNA ratio ($\pm$ s.d.) of $2.4 \pm 0.55:1$ (histone/non-histone protein/DNA, $1.77 \pm 0.49:0.54 \pm 0.22:1$).

Purification of rat ventral-prostate RNA polymerase B

RNA polymerase B was solubilized from rat ventral-prostate nuclei and purified by a method derived from a combination of published procedures (Roeder & Rutter, 1970; Mainwaring *et al.*, 1971; Keding *et al.*, 1972; Davies *et al.*, 1975). This involved sonication at high ionic strength, selective precipitation of contaminating nucleic acid with protamine sulphate, fractionation with (NH₄)₂SO₄, and chromatography on columns of DEAE-cellulose and phosphocellulose.

Determination of RNA polymerase activity

(a) *Routine assessment.* RNA polymerase activity was assessed as a routine during purification procedures or to determine the specific activity of samples at various periods after preparation. The reaction mixture (500 μ l) contained Tris/HCl buffer (120 mM), pH 7.9, KCl (30 mM), dithiothreitol (400 μ M), NaF (600 μ M), MnCl₂ (3 mM), ATP, GTP and CTP (all 600 μ M), [³H]UTP (0.5 μ Ci), UTP (40 μ M) and glycerol (10%, w/v). DNA template (5–60 μ g) and RNA polymerase (4–50 μ g of protein) were added in 100 and 50 μ l respectively. Certain assays contained (NH₄)₂SO₄ (40 mM) and α -amanitin (40 ng/assay). Incubations (20 min at 37°C) were terminated and acid-insoluble material was processed and assessed for incorporated radioactivity as described previously (Davies & Griffiths, 1973b, 1975).

(b) *Determination of RNA synthesis from rifamycin-resistant sites.* The method used for this study was based on that of Chesterton *et al.* (1974). RNA polymerase (4–50 μ g of protein in 100 μ l) was preincubated (15 min at 37°C) with template (5–60 μ g of DNA in 100 μ l), in a reaction mixture (250 μ l) containing Tris/HCl buffer (120 mM), pH 7.9, KCl (60 mM), dithiothreitol (800 μ M), NaF (1.2 mM), MnCl₂ (6 mM) and glycerol (10%, w/v). At the end of this period, a mixture (250 μ l) containing ATP or GTP, CTP (1.2 mM), [³H]UTP (0.5 μ Ci), UTP (80 μ M), Tris/HCl buffer (120 mM), pH 7.9, and rifamycin AF/013 (60 μ g) was added. In each tube, the respective unlabelled purine nucleoside triphosphate was replaced with [γ -³²P]ATP or [γ -³²P]GTP (0.5 μ Ci in each case). Rifamycin AF/013 was dissolved in a minimal volume of dimethyl sulphoxide before being taken up in the correct volume of Tris/HCl buffer to give the required concentration. The incubation was allowed to proceed for a further 20 min at 37°C, then terminated and acid-insoluble material processed as described above.

(c) *Measurement of initiation in low-salt followed by elongation in high-salt conditions.* A method similar to that described by Cedar (1975) was used. The final concentrations of the components of the reaction mixture were as in method (b). RNA polymerase was preincubated (15 min at 37°C) in a mixture (270 μ l) containing the same reactants as in method (a), but with [γ -³²P]ATP or [γ -³²P]GTP as well as [³H]UTP, and MnCl₂ (1 mM) and without CTP. The initiation reaction was stopped by the addition of 85 μ l of (NH₄)₂SO₄ (at various concentrations; see the Results section), and chain propagation begun by the addition of MnCl₂ (final concentration 3 mM) and CTP (final concentration 300 μ M). Incubation and processing was as for method (b) above.

All assays performed by any of these methods were corrected for zero-time controls, controls in the

absence of DNA and absence of enzymes. The product of the reaction was sensitive to ribonuclease digestion and to alkali (0.2M-NaOH), and the reaction was dependent on the presence of a DNA template and a bivalent cation (Mn^{2+}), and was inhibited by α -amanitin. Variations in the above assay were introduced to check certain aspects (see the Results section). The initial rate of elongation was measured by termination of the reaction at various periods up to 80s of incubation. The number of initiation sites was calculated from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ incorporated. The rate of elongation and RNA chain length were calculated from the amount of $[\text{H}^3]\text{UMP}$ incorporated, assuming that UMP represents 25% of total ribonucleoside monophosphate present in RNA.

Measurement of template availability

The amount of DNA in a template that was available to RNA polymerase was estimated by a method similar to that described for routine assessment of RNA polymerase activity, except that the amount of DNA added to the assay was varied, but the quantity of enzyme remained constant.

Chemical analyses

DNA content of fractions was measured by the diphenylamine procedure of Burton (1956) with modifications described by Giles & Myers (1965), with calf-thymus DNA as standard. Protein content,

including non-histone protein, was measured by the procedure of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard. Histone was measured as described by Lowry *et al.* (1951), with calf thymus histone as standard.

Results

Inhibition of prostate RNA polymerase B by α -amanitin and rifamycin AF/013

RNA polymerase B of rat ventral prostate (Mainwaring *et al.*, 1971; Davies & Griffiths, 1974a,b) and other tissues (Jacob, 1973) is sensitive to α -amanitin. The maximum degree of inhibition of the rat prostate enzyme and the human prostate enzyme (Davies & Griffiths, 1976) was about 70–90%, but after further purification inhibition of prostate RNA polymerase B was above 95%.

RNA polymerase B from rat liver (Butterworth *et al.*, 1971) and calf thymus (Meilhac & Chambon, 1973) are sensitive to rifamycin AF/013 under certain conditions, but the enzyme is resistant to the inhibitor after the formation of the first internucleotide linkage. The activity of exogenous prostate RNA polymerase B was inhibited completely by rifamycin AF/013 at a concentration of 50 μg per assay (Fig. 1a), for the enzyme concentrations used in this study. When the addition of the antibiotic was delayed, the extent of RNA synthesis was dependent on the period of delay (Fig. 1b). Endo-

Fig. 1. Effects of rifamycin AF/013 on the activity of rat ventral-prostate RNA polymerase B

The transcription of ventral-prostate chromatin from normal rats by homologous RNA polymerase B was measured in the presence of various concentrations of rifamycin AF/013: 12 μg of chromatin DNA and 34 μg of enzyme protein were used in (a) and (b). The activity of RNA polymerase was measured in the presence of concentrations of rifamycin AF/013 between 0 and 100 μg per assay (a), and in (b) when rifamycin AF/013 (50 μg) was added 0 min ($\bullet$), 1 min (Δ), 3 min ($\blacktriangle$), 6 min ($\square$) or not added ($\circ$) after the start of the assay. The effect of rifamycin AF/013 on the activity of chromatin-associated RNA polymerase (three different samples $\circ$, $\bullet$, Δ ; c) was also investigated. In all cases RNA polymerase B activity was monitored by the incorporation of $[\text{H}^3]\text{UMP}$; all activity was sensitive to α -amanitin (60 ng/assay).

genous RNA polymerase activity in chromatin was not inhibited by rifamycin AF/013, and could be stimulated (Fig. 1c), as previously observed (Butterworth *et al.*, 1971). Apparently uninitiated and re-initiated, but not preinitiated, polymerase was sensitive to the toxin. The formation of rifamycin-resistant enzyme-template complexes was temperature-dependent, since all RNA synthesis was sensitive to the antibiotic if preincubation of RNA polymerase and template took place at 0–4°C.

Titration of rat ventral-prostate RNA polymerase with template

The amounts of available DNA in various templates may be assessed by the titration of the template against a fixed amount of RNA polymerase (Cedar, 1975). The amount of template required to achieve maximum enzyme activity indicates the number of initiation, or binding, sites available on the template. As the amount of template is increased, the number of RNA chains synthesized is correspondingly increased until a plateau is reached. In the experiment shown (Fig. 2a), the titration point for a naked DNA template was 3 µg and that for the ventral-prostate chromatin template was approx. 40 µg. There are therefore 12–14 times as many sites on DNA as on ventral-prostate chromatin. By assuming maximum specific activity of RNA polymerase B and a mol. wt. 500 000 (Kedinger & Chambon, 1972), this would suggest 3.4×10^6 molecules of enzyme per pg of naked DNA or 0.24×10^6 – 0.28×10^6 molecules per pg of chromatin DNA. These values are probably an overestimate of actual initiation sites.

The titration curves of RNA polymerase with DNA and chromatin did not achieve the same plateau value (Fig. 2a) owing to a difference in the size of the

product synthesized from each template. Whereas the RNA synthesized from DNA had a number-average size of 810 ± 26 ($\pm$ s.d., $n = 12$) nucleotides, that synthesized from intact-rat prostate chromatin had 528 ± 75 ($\pm$ s.d., $n = 8$) nucleotides. This discrepancy was more obvious when titration curves of prostate chromatin from normal rats and from animals that had been castrated 24 h earlier were compared (Fig. 2b). Although chromatin appeared to bind RNA polymerase to the extent of approx. 0.3×10^6 molecules per pg of DNA, the number-average size of RNA synthesized from chromatin of castrated rats was 396 ± 25 ($\pm$ s.d., $n = 8$) nucleotides (see below). Apparent K_m values for the interaction between RNA polymerase and chromatin of normal and castrated rats were 115 and 112 pM respectively.

Initiation in low-salt conditions followed by chain propagation in high-salt conditions

This procedure was a modification of that of Cedar (1975). RNA polymerase B was allowed to initiate RNA synthesis in low salt with only ATP, GTP and [^{3} H]UTP present. After the preincubation period, the mixture was adjusted to 0.4M with $(\text{NH}_4)_2\text{SO}_4$ to prevent further initiation and CTP was added to permit elongation to proceed. The preincubation time of 15 min was sufficient to allow subsequent synthesis from all initiation sites; in fact, initiation was complete after 8 min preincubation (Fig. 3). Initiation sites were measured by inclusion of [γ - ^{32} P]ATP or [γ - ^{32} P]GTP in the preincubation mixture. These experiments were carried out under conditions of enzyme excess. Increasing the RNA polymerase concentration had no effect on the incorporation of [^{3} H]UMP, but doubling the amount of template doubled RNA synthesis.

Fig. 2. Titration of RNA polymerase B with DNA and chromatin

Increasing amounts of DNA (○), chromatin from ventral prostates of normal rats (●) or chromatin from ventral prostates of rats castrated 24 h previously (△) were incubated under standard assay conditions of 8.56 µg of RNA polymerase B. Enzyme activity was monitored by incorporation of [^{3} H]UMP into acid-precipitable material. (a) Comparison of DNA (○) and chromatin from prostates of normal rats (●); (b) comparison of prostate chromatin from normal (●) and castrated (△) rats.

Fig. 3. Kinetics of initiation in low salt

RNA polymerase B ($9\mu\text{g}$ of protein) was incubated with rat ventral-prostate chromatin ($56\mu\text{g}$ of DNA) under low-salt conditions to allow initiation, as described in the Materials and Methods section, for various periods of time. After the initiation period, the assay mixture was adjusted to the required concentrations of CTP, MnCl_2 and $(\text{NH}_4)_2\text{SO}_4$, and the rate of chain elongation measured as described in the Materials and Methods section. The activity of RNA polymerase B was monitored by incorporation of $[^3\text{H}]\text{UMP}$ into acid-precipitable material.

Fig. 4. Incorporation of $[^3\text{H}]\text{UMP}$ in high salt after initiation in low salt

Ventral-prostate chromatin from normal rats (○; $5.1\mu\text{g}$ of DNA) or from rats castrated 24h previously (●; $5\mu\text{g}$ of DNA) were incubated with RNA polymerase B ($11.7\mu\text{g}$ of protein) under low-salt conditions, permitting initiation. Elongation was started by the addition of CTP, MnCl_2 and $(\text{NH}_4)_2\text{SO}_4$, and incorporation of $[^3\text{H}]\text{UMP}$ into acid-precipitable material was monitored.

Measurement of RNA chain initiation and elongation is complicated by the contamination of commercial nucleoside triphosphates with small amounts of the other nucleoside triphosphates, resulting in the synthesis of oligonucleotide chains during the low-salt preincubation procedures. Considerable incorporation of $[^3\text{H}]\text{UMP}$ occurred before propagation of RNA chains in high-salt media (Fig. 4). In a similar experiment, using $10\mu\text{g}$ of chromatin DNA and $23\mu\text{g}$ of RNA polymerase, low-salt incorporation of $[^3\text{H}]\text{UMP}$ was $146.8\text{pmol}/100\mu\text{g}$ of protein and, in parallel experiments, 15.44pmol of $[^3\text{H}]\text{CMP}$ was incorporated per $100\mu\text{g}$ of protein. The degree of contamination can vary from preparation to preparation, and from one nucleoside triphosphate to another. This oligoribonucleotide formation may not affect the absolute length of RNA chains, but will influence the rates of elongation measured in the first minute of propagation.

Comparison of RNA chain initiation and elongation as measured by low-salt/high-salt technique and by rifamycin AF/013 challenge

The initiation and elongation of RNA chains synthesized from normal rat ventral-prostate chromatin was studied by these two methods. The number of initiated sites per pg of DNA was 10057 ± 866 ($\pm\text{s.d.}$, $n = 8$) measured by the low-salt/high-salt technique and 11429 ± 1089 ($\pm\text{s.d.}$, $n = 8$) measured by the rifamycin-challenge assay. The number-

Fig. 5. Comparison of rate of elongation of RNA chains on rat ventral-prostate chromatin transcribed by homologous RNA polymerase B under different conditions of initiation

RNA polymerase B ($46\mu\text{g}$ of protein) was incubated with ventral-prostate chromatin ($5\mu\text{g}$ of DNA) from normal rats either in the absence of nucleotides (●) or in low-salt conditions in the presence of three nucleotides (○). Elongation was started at zero time either by addition of all four nucleotides plus rifamycin AF/013 (●) or by addition of CTP, MnCl_2 and $(\text{NH}_4)_2\text{SO}_4$ (○). Incorporation of $[^3\text{H}]\text{UMP}$ into acid-precipitable material over the first 80s of chain propagation was monitored.

Fig. 6. Effect of salt concentration on initiation

RNA polymerase B (23 μg) was incubated with prostate chromatin from normal rats (10 μg of DNA) in low-salt conditions in the absence of nucleoside triphosphates. Chain elongation was started by the addition of all four nucleoside triphosphates plus $(\text{NH}_4)_2\text{SO}_4$ to give final concentrations between 0.01 and 0.1 M. The amount of ^{3}H -nucleoside monophosphate (NMP) incorporated (a, ^{3}H UMP; b, ^{3}H CMP) into acid-precipitable material in a 20 min incubation was measured.

Fig. 7. Diurnal variations in transcription of rat ventral-prostate chromatin by homologous RNA polymerase B

Chromatin was prepared from the ventral prostates of rats killed at various times during a 24 h period. Initiation was allowed to proceed by incubation of template with an excess of RNA polymerase in the absence of nucleoside triphosphates, which were added after 15 min with rifamycin AF/013 to allow elongation without reinitiation (see the Materials and Methods section). (a) Initiation sites were measured by incorporation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (●), and number-average chain length from the incorporation of ^{3}H UMP (○), assuming this to be 25% of total nucleotide incorporation. The initial rate of elongation (b) was measured by incorporation of ^{3}H UMP into acid-precipitable material over the first 80 s of propagation. Preliminary chemical analysis of chromatin (c) was performed by the method of Marushige & Bonner (1966). Hours of darkness are indicated by the stippled bar.

average chain length of synthesized RNA was 546 ± 60 and 515 ± 165 ($\pm$ s.d., $n = 8$) nucleotides respectively. The ratio $[\gamma\text{-}^{32}\text{P}]\text{ATP}/[\gamma\text{-}^{32}\text{P}]\text{GTP}$ incorporated was in the range 2.11 ± 0.65 ($\pm$ s.d., $n = 16$) for both methods. The methods therefore give reasonable agreement for chain length and for the number of initiation sites per pg of DNA, although this number is considerably less than the estimated number of molecules of RNA polymerase that may associate

with the template. However, when the initial rate of elongation was measured over the first 80 s of propagation time (Fig. 5), a discrepancy was observed. In this particular experiment, the initial rate of elongation in the rifamycin assay was 8.23 nucleotides/s, but using a low-salt/high-salt procedure, the apparent rate was 27.1 nucleotides/s, a high value which may be attributed to the extent of preincubation incorporation of ^{3}H UTP. Moreover,

Table 1. *Effect of castration on template characteristics of rat ventral-prostate chromatin*

Chromatin was prepared from the ventral prostate glands of unoperated rats or rats castrated 4, 8, 16 or 24h previously. In the Table, the extreme left-hand column represents no castration, and movement through the columns from left to right represents increased time after castration. Values in parentheses are the assessments of the various parameters for the correct control to allow for diurnal variations. Thus one value is given for unoperated animals, which act as their own controls. Initiation and elongation were measured under conditions of enzyme excess, by using the rifamycin-challenge assay, and elongation was measured over the first 80s of propagation (see the Materials and Methods section). Analysis of chromatin was performed by the method of Marushige & Bonner (1966).

	Day 2				Day 1			
	Time of operation	16:00 h	12:00 h	08:00 h	24:00 h	16:00 h		
Post-castration period (h)	...	0	4	8	16	24		
Parameter measured								
Initiation sites (no./pg of DNA)		11185	9053 (12937)	8915 (12155)	8354 (11889)	8009 (10346)		
Number-average chain length (nucleotides)		211	196 (398)	174 (294)	159 (247)	132 (233)		
Rate of elongation (nucleotides/s)		10.6	8.4 (14)	7.6 (12.2)	7.4 (8.5)	7.2 (10.4)		
Chromatin constituents								
Protein/DNA		2.41:1	2.18:1 (1.48:1)	1.6:1 (3.42:1)	1.75:1 (2.09:1)	2.05:1 (2.30:1)		
Histone/non-histone protein/DNA		1.51:0.85:1	1.36:0.78:1 (0.94:0.52:1)	0.99:0.59:1 (2.17:1.23:1)	1.08:0.4:1 (1.32:0.73:1)	1.27:0.72:1 (1.46:0.8:1)		

elongation was completed more rapidly in high-salt incubation.

Preincubation of RNA polymerase with template in the absence of all nucleotides allows the formation of rifamycin-resistant complexes at specific initiation sites (Chesterton *et al.*, 1974; Fig. 1). Similar preincubation does not render complexes resistant to salt. Initiation was inhibited by 0.1M-(NH₄)₂SO₄ (Fig. 6). As reinitiation will be prevented by this concentration, it would be more effective in the measurement of elongation *in vitro*, as a concentration of 0.15M is necessary for dissociation of chromatin-associated proteins (Butterworth *et al.*, 1971).

Both methods described could be used to determine the number of specific initiation sites on chromatin and the number-average nucleotide length of RNA chains. If, however, the rate of elongation is to be considered, then the rifamycin-challenge method, which precludes propagation until after the preincubation period, should be the method of preference.

Effect of castration on the number of initiation sites on rat ventral-prostate chromatin, the rate of elongation of RNA chains, and the number-average chain length

This study used rifamycin AF/013 to determine specific initiation sites and to prevent reinitiation, for reasons stated above. Two periods of castration were investigated: (a) a 'short' term, to study effects produced in the first 24h of castration, and (b) a 'long' term, to observe changes over a 3-day post-castration period. Since diurnal variations in RNA polymerase B activity, reported for this enzyme in other tissues (Glasser & Spelsberg, 1972; Spelsberg & Cox, 1976), could interfere with the short-term study, the number of initiation sites, the rate of elongation and the chain length were measured by using prostate chromatin in intact rats killed at various times over a 24h period (Fig. 7). A considerable variation in all three parameters occurs. Values for initiation sites and chain length were lowest at 16:00h and highest at 12:00h (Fig. 7a), but the elongation rate was lowest at 24:00h, but again highest at 12:00h (Fig. 7b). The diurnal variations in these parameters were sufficiently significant ($\pm 8.4\%$ for initiation sites, $\pm 27\%$ for chain length and $\pm 18\%$ for elongation rate) to warrant rigorous control systems during the first 24 h after castration, so that results for animals castrated at various times during the day could be compared with average normal values. When animals were castrated at daily intervals, 12:00h was taken as the time-point for experimentation.

Over the first 24h after castration (Table 1), the number of chromatin initiation sites and the rate of elongation were slightly decreased. The length of the synthesized RNA chains was most affected, but elongation was more severely impaired than is usual in

Table 2. *Changes in template characteristics of rat ventral-prostate chromatin after castration*

Chromatin was prepared from the ventral prostate glands of unoperated rats and rats castrated 24, 48 or 72 h previously. The various parameters were measured as described in the legend to Table 1.

Time after castration (days)	Initiation sites (no./pg of DNA)	Number-average chain length (nucleotides)	Elongation rate (nucleotides/s)	Protein/DNA	Histone/non-histone protein/DNA
0	10640	520	11	2.90	2.06:0.7:1
1	10066	418	7	2.94	1.84:0.98:1
2	7707	379	—*	1.53	1.10:0.31:1
3	6214	349	—*	1.56	1.08:0.46:1

* Could not be determined accurately.

these experiments. The data in Table 2, on changes in the various parameters over a 3-day period after castration, suggest that over the first 24 h elongation of RNA chains is most affected, whereas initiation sites are decreased 48 or 72 h after castration. Within the limits of the experimental procedures used, it appears that androgen withdrawal results in decreased transcription and that more than one of the stages of transcription are ultimately affected.

The chromatin-associated proteins may be involved in the regulation of transcription of prostate chromatin (Couch & Anderson, 1973*a,b*; Davies & Griffiths, 1974*b*). Histone and non-histone protein content of prostate chromatin were monitored during the post-castration periods (Tables 1 and 2). It is noteworthy that the highest degree of transcription during the day coincides with the lowest protein content of chromatin (Fig. 7*c*). Changes in protein content and composition of chromatin occur during the post-castration periods, but greater comprehension of their role and modifications awaits more detailed analysis.

Discussion

Gross effects of steroids on the activity of RNA polymerases (Mainwaring *et al.*, 1971; Davies & Griffiths, 1973*a,b*; 1974*a,b*) only give a slight indication of the processes concerned with gene activation. Independent assessment of the interaction of the steroid-receptor complex with the genome and the various stages involved in the production of the polyribonucleotide chain by RNA polymerase associated with the template is necessary. Our laboratory has concerned itself first with the latter problem.

Homologous RNA polymerase B was used in these studies as differences in transcription characteristics of eukaryotic and bacterial RNA polymerases have been observed (Butterworth *et al.*, 1971; Reeder, 1973; Cedar, 1975), and differing responses of *Escherichia coli* RNA polymerase to androgen-receptor complexes have been reported (Davies & Griffiths, 1973*a*; Mainwaring & Jones, 1975).

Although the bacterial enzyme has been used successfully in other steroid-hormone-dependent systems (Schwartz *et al.*, 1975; Tsai *et al.*, 1976), it may have an impaired or decreased ability to recognize 'activated' sites on the prostate template.

Preliminary experiments (Fig. 2) showed that more sites were available for RNA polymerase on naked DNA than on a native chromatin template. The calculated numbers of polymerase molecules bound per unit of DNA vastly exceeded the actual number of initiation sites. A number of regions on the template may retain RNA polymerase but do not provide the correct conditions for the formation of a specific initiation complex. Similar amounts of RNA polymerase associate with the prostate chromatin from both normal and castrated rats, although shorter RNA chains are synthesized in the latter case.

There are two principal means by which the measurement of initiation and elongation can be separated. These involve either rifamycin AF/013 inhibition of reinitiation and initiation at non-specific sites or alteration of salt concentration and nucleotide availability thereby preferentially controlling conditions to allow initiation or elongation. Rifamycin AF/013 was an effective inhibitor of non-preincubated prostate RNA polymerase B (Fig. 1). Delayed addition of rifamycin allowed an increased synthesis of RNA, probably because only reinitiation was being inhibited and the number of reinitiations became less as time progressed. As illustrated in Fig. 1(*c*) preinitiated RNA polymerase B could not be inhibited. This may be due to the insensitivity of the enzyme-DNA complex to the drug or the rapid rate of initiation by the complex (Chamberlin, 1974). No reinitiation in the presence of rifamycin occurred during the incubation period.

The rifamycin-challenge assay has distinct advantages over the low-salt/high-salt technique which can produce incorrect calculations of chain length, possibly owing to dissociation of chromatin-associated proteins as well as oligonucleotide formation in low-salt conditions. The introduction of high salt is essential to inhibit reinitiation, but dissociation of

chromatin-associated proteins could be decreased by using a final salt concentration of 0.1M. Moreover, similar chain lengths were observed using either the rifamycin-challenge assay or the low-salt/high-salt technique. Oligonucleotide formation is dependent on the extent of contamination of nucleotide preparations and could probably be overcome only by stringent purification. As the rifamycin assay allows addition of all nucleotides after preincubation, spurious elongation rates caused by nucleotide contamination or salt would not be observed (Fig. 5).

Transcription of chromatin was impaired *in vitro* in relation to transcription of DNA. This could be attributed to the chromatin-associated proteins, but, since equally short chain lengths were observed by using rifamycin and 0.4M salt, which might be expected to promote elongation, other factors might possibly be involved. These problems should be taken into consideration, together with castration-induced effects.

The observed diurnal variations (Fig. 7) in the number of initiation sites, number-average chain length and elongation rate necessitated the use of controls over the first 24h post-castration period. During this time, the major effects of castration were on chain length. This agrees with previous observations (Mainwaring & Jones, 1975; Davies, 1975) that initiation sites were not significantly decreased 24h after castration. However, 48 and 72h after castration the numbers of initiation sites were decreased, with further decreases in the number-average nucleotide size of RNA chains.

The decreased length of RNA chains transcribed from chromatin *in vitro* can be attributed to the loss of elongation factors from the template during preparation (Cox, 1976). The results from castration studies suggest that androgen withdrawal may further decrease the number or efficiency of such factors. These substances may themselves be dependent on androgens for their maintenance or synthesis.

The absolute length of RNA chains, as well as the rate of elongation, may be controlled by certain chromatin-associated proteins (Solage & Cedar, 1976). Alterations in the proportion of non-histone and histone proteins are evident during post-castration periods (Table 2) and may be related to variations in transcription (Couch & Anderson, 1973a,b; Davies & Griffiths, 1974a; Nyberg & Wang, 1976).

An important feature of the transcription of chromatin *in vitro* is the involvement of ribonucleases. Inhibitors of these enzymes vary in efficiency (Jones, 1976). The decrease in chain length may be independent of nuclease action (Davies *et al.*, 1976) but the production of RNA is susceptible to numerous degradative enzymes.

The androgenic status of the animal influences more than one aspect of the transcription of prostate

chromatin by homologous RNA polymerase B. The present data provide a basis for a more detailed appraisal of the many interactions involved in these events. A relationship exists between the concentration of nuclear receptors and transcription (Davies *et al.*, 1976), but it is uncertain whether the androgen-receptor complex acts by displacement of chromatin-associated proteins to reveal initiation sites, by assisting in the unwinding of DNA, by promoting elongation factors or by inactivating termination factors.

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