The Androgenic Regulation of Prostate Proteins with a High Affinity for Deoxyribonucleic Acid

EVIDENCE FOR A PROSTATE DEOXYRIBONUCLEIC ACID-UNWINDING PROTEIN

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(Received 19 November 1975)

1. When testosterone is injected into castrated rats *in vivo*, a significant increase in the incorporation of [³⁵S]methionine into prostate proteins may be detected under conditions *in vitro*. 2. Studies based on DNA–cellulose chromatography show that the synthesis of prostate proteins with a high affinity for DNA is particularly enhanced by androgenic stimulation. 3. These changes in protein synthesis are negated when the anti-androgen, cyproterone acetate, is administered concomitantly with testosterone *in vivo*. 4. Two assays were developed for measuring the strand separation of prostate DNA; first, the retention of ³H-labelled native DNA on nitrocellulose membranes, and second, the activation of native DNA as a template for 9S prostate DNA polymerase. On the basis of these criteria, DNA-unwinding activity is present in the prostate gland and it is regulated by androgens in a steroid- and tissue-specific manner. 5. The results are discussed in the context of the mechanism of action of androgens, particularly since the changes provoked in DNA-unwinding activity by androgens precede the onset of DNA replication and mitosis.

The number of cells in the rat ventral prostate gland is depleted by castration to below that in animals of fully maintained androgenic status. The restoration of the growth of the prostate gland is uniquely dependent on androgenic stimulation, and hence the male accessory sexual glands provide good experimental systems for studying the hormonal regulation of mitosis and glandular proliferation. On restoration of the circulating concentrations of androgens in castrated animals, there is a protracted latent period of some 48 h before changes are evident in many processes associated with mitosis, including the incorporation of thymidine into DNA (Coffey *et al.*, 1968; Lesser & Bruchovsky, 1973) and DNA polymerase activity of the replicative type (Coffey *et al.*, 1968; Rennie *et al.*, 1975). All morphological and biochemical indicators of mitosis in the prostate gland attain their maxima after 96 h of androgenic stimulation and then decline. Except that androgens promote a certain measure of protein synthesis during the latent period (Chung & Coffey, 1971*a,b*), the molecular events necessary for initiating mitosis in the prostate gland remain essentially unknown.

In both prokaryotic organisms (Gilbert & Müller-Hill, 1966; von Hippel & McGhee, 1972; Gudis *et al.*, 1975) and eukaryotic cells (Salas & Green, 1971; Stein *et al.*, 1974) it has been demonstrated that proteins engaged in the regulation of the genetic apparatus, notably in terms of transcription and DNA

replication, possess a high affinity for DNA. It has also been reported that specific DNA-binding proteins are synthesized during distinct phases of the cell cycle and they are believed to be implicated in controlling the onset of mitosis (Fox & Pardee, 1971; Salas & Green, 1971). The major objective of this study was to assess the changes in proteins with a particular propensity for DNA during the latent period of the restoration of prostate growth and mitosis evoked by androgens. We have concentrated particularly on prostate proteins capable of facilitating the denaturation or strand separation of DNA. Rennie *et al.* (1975) reported that the most striking feature of mitosis in the prostate gland was an increase in the activity of a replicative 9S DNA polymerase, with a pronounced preference for utilizing denatured (single-stranded) DNA as template. However, the mechanism by which androgens promoted the formation of this favoured DNA template was not established unequivocally, although DNA-unwinding proteins have been detected in bacteria (Sigal *et al.*, 1972; Molineux & Gefter, 1974) and, more recently, in mammalian cells (Keller, 1975).

Materials and Methods

Animals

Adult male Sprague–Dawley rats (250–300 g body wt.) were castrated by the scrotal route under fluo-

thane anaesthesia. Beginning 7 days after castration, rats were injected daily with 2.5mg of testosterone phenylpropionate subcutaneously in 0.25 ml of arachis oil into the flank region. Control animals received injections of oil only. This regime of injections was based on the findings of Rennie *et al.* (1975) and modifications of this protocol are given in the legends to the Tables.

Chemicals

Many batches of [^{35}S]methionine (all of 150 Ci/mmol or greater) were from The Radiochemical Centre, Amersham, Bucks., U.K. Tris base (99.5% pure), highly polymerized calf thymus DNA, dithioerythritol, EDTA and NH_4HCO_3 were from Sigma (London) Chemical Co., London S.W.6, U.K. Thymus DNA was immobilized on cellulose (Macherey-Nagel, type 2200ff; from Camlabs Ltd., Cambridge, U.K.) as described by Mainwaring & Irving (1973). The anti-androgen, cyproterone acetate (17 α -acetoxy-6 α -chloro-1,2 α -methylene-4,6-pregnadiene-3,20-dione) was a gift from Schering Pharmaceuticals, Burgess Hill, Sussex, U.K. All chemicals for polyacrylamide-gel electrophoresis were from Eastman Kodak Ltd., Liverpool, U.K. Highly purified sodium dodecyl sulphate (Serva, Heidelberg, W. Germany) was from Camlabs Ltd. *Escherichia coli* DNA, labelled with [^3H]thymidine, was isolated as described by Rennie *et al.* (1975). DNA was denatured by heating DNA solutions in 0.1M-NaCl in a boiling-water bath for 15 min, followed by rapid quenching in crushed ice.

Preparation of prostate-gland constituents

Prostate DNA was isolated by the method developed by Mainwaring & Peterken (1971). The DNA did not contain detectable amounts of protein and had a sedimentation coefficient of 70S when examined in the Spinco model E analytical ultracentrifuge. In certain experiments, the DNA was sheared to a form of 35S by expelling the DNA solution three times from a hypodermic syringe fitted with a 26G needle. Aldolase was prepared and finally crystallized as reported by Mainwaring *et al.* (1974). Alkaline phosphatase was prepared as described by Mainwaring & Irving (1973) and purified further to approx. 80% purity by a combination of ion-exchange and gel-exclusion chromatography as described by Linden *et al.* (1974). Other components could only be satisfactorily isolated from the prostate glands of castrated animals that had subsequently received 4 days of androgenic stimulation. Such was the case with DNA labelled with [^3H]thymidine and the 4S and 9S forms of DNA polymerase (EC 2.7.7.7); full details of these isolation procedures may be found in Rennie *et al.* (1975). Pooled fractions from several sucrose gradients containing 4S or 9S DNA polymerase (volume 8.0ml) were concentrated to 3.0ml

by using the B15 semi-permeable membranes (Amicon Ltd., High Wycombe, Bucks., U.K.), mixed with 1.0ml of glycerol and stored in small lots at -20°C for 1 month without detectable loss of enzymic activity. In certain experiments where it was of critical importance that the sedimentation properties of the enzymes were not influenced by the conditions of storage, the DNA polymerases were analysed directly after isolation and without freezing and thawing. The 9S DNA polymerase was prone to partial disaggregation after storage in the frozen state. For the labelling of proteins with [^{35}S]methionine, prostate glands (0.5–1.5g wet wt.), pooled from five to ten rats, were sliced into sections 0.25 mm thick by using a tissue chopper (Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.) and placed in 4.0ml of the culture medium devised by Morton (1970). After temperature equilibration in a Grant water bath at 37°C , [^{35}S]methionine was added to the final concentrations given in the text and incubation continued for 1 h in air with shaking at 160 cycles/min. The slices were then washed twice in 10ml of ^{35}S -free medium (Morton, 1970) at 4°C .

Isolation of prostate proteins with a high affinity for DNA

Except when otherwise stated, this was conducted at temperatures as close to 0°C as possible. Slices of prostate gland were suspended in medium A (40mM-Tris/HCl buffer, pH 8.0, containing 1mM-2-mercaptoethanol, 2mM-EDTA and 10mM- MgCl_2) in the ratio of 5ml of medium A/g wet wt. of tissue. The suspension was frozen in an acetone–solid CO_2 freezing mixture and then rapidly thawed in a water bath at 37°C . Solid NaCl was added to an overall concentration of 2M and the cycle of freezing and thawing was repeated. After disruption with a motor-driven Potter–Elvehjem homogenizer operating at 500 rev./min for 30 s, 0.5 vol. of medium A containing 2M-NaCl and 42% (w/v) polyethylene glycol 600 (BDH Chemicals Ltd., Poole, Dorset, U.K.) was added with stirring, and, after standing for 2 h, the heavy precipitate was removed by centrifugation at 20000g for 20 min. The supernatant was dialysed overnight against 4 litres of medium B (20mM-Tris/HCl buffer, pH 8.0, containing 1mM-2-mercaptoethanol and 1mM-EDTA) and any precipitate was removed by centrifugation at 100000g for 1 h. Up to 12 ml of dialysed supernatant (maximum of 150 mg of protein) was applied to columns of DNA–cellulose (1.5 cm diam. $\times$ 4.0 cm) equilibrated with medium B. Native or thermally denatured calf thymus DNA was immobilized on cellulose as described by Mainwaring & Irving (1973). On the basis of the methods of Alberts *et al.* (1968), each g of cellulose contained 400–500 μg of immobilized DNA. The column was then washed with medium B until all non-adsorbed proteins were removed. Proteins retained on the

columns were eluted in a stepwise manner, first with 15ml of medium B containing 1M-NaCl and then 15ml of medium B containing 2M-NaCl. Peak protein fractions were dialysed overnight against 3 litres of 0.25% NH_4HCO_3 without any visible precipitation, and after freeze-drying, the samples were stored at -70°C .

Electrophoresis of proteins

This was conducted in 7.5% or 10.0% (w/v) polyacrylamide gels (0.8cm diam. $\times$ 8cm) in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969; Laemelli, 1970). When many samples were to be processed concomitantly, especially samples labelled with [^{35}S]methionine, we reverted to the slab gels advocated by Studier (1970). These gels (10cm $\times$ 16cm $\times$ 0.2cm thick) contained 0.1% sodium dodecyl sulphate and electrophoresis was conducted at 30V for 18h at room temperature (approx. 20°C). The gels were stained with 0.1% Amido Black 10B in 1.0M-acetic acid, destained by repeated washing in 1.0M-acetic acid, dried *in vacuo* on to Whatman no. 4 paper and photographed. With proteins labelled with [^{35}S]methionine, samples containing 4000–10000 d.p.m. of radioactivity were separated. The gels were stained and photographed as above, but after drying *in vacuo* radioautographs were prepared by using X-ray film (Kodirex; Kodak Ltd.). The exposure time varied between 2 and 4 weeks, depending on the amount of radioactivity present. Both photographic negatives of the stained gels and the radioautographs were scanned in a Joyce-Loebl microdensitometer. In control experiments with bovine serum albumin, staining intensity and the integrated areas of the peaks measured in the microdensitometer were proportional within the range 5–50 μg of protein. By using these techniques, up to 12 samples of protein could be analysed together and methodological variation was minimized. Proteins of known molecular weight were always run in parallel.

Assay for the denaturation or unwinding of DNA

(a) *By retention of radioactive DNA on nitrocellulose membranes.* This procedure has been described in detail by Rennie *et al.* (1975). In the present experiments, native prostate DNA labelled with [^3H]thymidine (12 μg of DNA; 7100c.p.m.) was mixed with prostate extracts (5–60 μg of protein) and made up to a total volume of 0.25ml with medium C (50mm-Tris/HCl buffer, pH7.4, containing 5mM-EDTA and 0.5mM-dithioerythritol). After incubation at 37°C for 2min, the samples were diluted with 0.5ml of medium C and filtered through Millipore membranes (Rennie *et al.*, 1975). In the absence of denaturation or localized areas of strand separation, the background retention of radioactivity was 280c.p.m. per membrane, and where DNA-unwinding activity was present, retention of ^3H -labelled

DNA was strictly proportional to the input of protein up to 60 μg .

(b) *By using 9S DNA polymerase.* It was established by Rennie *et al.* (1975) that the 9S form of prostate DNA polymerase had a marked preference for denatured (single-stranded) DNA as template and this was exploited as a sensitive means for monitoring the strand separation of native (helical) prostate DNA. The only departure from our published procedure for the assay of DNA polymerase activity (Rennie *et al.*, 1975) was that the concentration of [^3H]dGTP was raised to 0.5 μCi per standard assay mixture (volume 0.30ml). DNA synthesis by the 9S form of DNA polymerase with native prostate DNA as template was measured in both the presence and the absence of extracts with putative DNA-unwinding activity. The incorporation of [^3H]dGTP into DNA was absolutely dependent on the provision of complementary 2-deoxyribonucleoside 5'-triphosphates, thus eliminating the possibility that we were measuring terminal addition rather than replicative synthesis of DNA. The results were corrected for the marginal DNA polymerase activity present in the protein extracts under scrutiny for potential DNA-unwinding proteins.

Other procedures

Analysis for deoxyribonuclease activity was based on the method of Zöllner *et al.* (1974) as modified by Rennie *et al.* (1975). Accordingly, each gel contained 5% (w/v) polyacrylamide, 0.7% each of ampholytes [LKB (U.K.), London S.W.12, U.K.] of pH ranges 5–8 and 8–9.5 and 50 μg (2.1×10^6 – 2.4×10^6 c.p.m.) of ^3H -labelled *E. coli* DNA. After the completion of isoelectric focusing, replicate gels were sliced and incubated under conditions which discriminated between deoxyribonuclease activities types I and II, with pH optima at 8.0 and 5.0 respectively (Rennie *et al.*, 1975). Studies by Sharp *et al.* (1973) provided a novel means for detecting proteins with the ability to promote subtle changes in the tertiary structure of DNA. DNA from simian 40 and polyoma viruses can exist in either the superhelical or relaxed circular forms (Champoux & Dulbecco, 1972) and these may be distinguished by differences in their rate of electrophoretic migration (Sharp *et al.*, 1973). After incubation with prostate proteins, changes in the structure of superhelical polyoma-virus DNA were monitored by the electrophoretic procedure devised by Keller (1975) in slab gels (10cm $\times$ 16cm $\times$ 0.2cm thick) containing 1.4% (w/v) agarose.

Chemical analyses

DNA was determined by the method of Burton (1956) with calf thymus DNA as the standard. The procedure of Lowry *et al.* (1951) was used in the determination of protein; to increase sensitivity, the assay volume was scaled down to only 0.5ml.

Counting of radioactivity

Measurement of radioactivity was performed in a Nuclear-Chicago mark II liquid-scintillation spectrometer. Radio-labelled compounds retained on Millipore membranes or Whatman DE 81 discs were counted in 10ml of a toluene-based phosphor [15g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/litre] in which the counting efficiency for ^3H was 54%. Radioactive proteins were collected and washed on Whatman GF/A discs (Mainwaring & Wilce, 1972) and counted in 10ml of a water-miscible phosphor [15g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole dissolved in 1770ml of toluene, 110ml of water and 625ml of Triton X-100]. Counting efficiency for ^{35}S in this phosphor was 78%.

Results

Incorporation of [^{35}S]methionine into prostate proteins in vitro

In a series of initial experiments, optimum conditions for the labelling of prostate proteins *in vitro* were established. Under the conditions of incubation described in the Materials and Methods section, the incorporation of [^{35}S]methionine into the protein fraction of the prostate gland isolated by the use of polyethylene glycol 600 and 2M-NaCl was linear with respect to the concentration of radioactive precursor within the range 0.4–1.5 nM. With [^{35}S]methionine at a specific radioactivity of approx. 150 Ci/mmol, this corresponds to the addition of 200 μCi of tracer amino acid to 4.0 ml of standard incubation medium. In addition, incorporation of [^{35}S]methionine into polypeptide linkage was proportional to the duration of incubation between 30 and 90 min and to the amount of tissue present within the range 0.5–2.0 g wet wt. DNA-cellulose chromatography provided a simple and reproducible means for isolating prostate proteins with a high affinity for DNA. A representative elution profile from columns containing immobilized DNA is illustrated in Fig. 1. The vast majority of labelled prostate proteins were not retained by DNA-cellulose. Proteins with a high affinity for DNA were eluted in 1M-NaCl, although a minor proportion of strongly adsorbed protein was eluted only in 2M-NaCl. The overall recovery of the radioactivity applied to the columns was always greater than 90%. The selective retention of prostate proteins was totally dependent on the presence of immobilized DNA, for negligible amounts of radioactivity were adsorbed to a matrix of cellulose alone. Provided that the input of protein was below 150 mg per DNA-cellulose column, the binding capacity of the columns was sufficient to retain all the proteins with a high affinity for DNA. The failure to saturate the DNA-cellulose by our standard procedure is demonstrated in Fig. 1. The incorporation of [^{35}S]methionine into

Fig. 1. Binding of soluble prostate proteins to columns of DNA-cellulose

At 7 days after castration, prostate glands pooled from eight animals were incubated as described in the Materials and Methods section with 200 μCi of [^{35}S]methionine (180 Ci/mmol). A soluble protein extract was prepared by treating the homogenized tissue with 2M-NaCl in the presence of polyethyleneglycol 600. The dialysed extract (8.0 ml; 120 mg of protein) was applied to a native DNA-cellulose column (1.5 cm diam. $\times$ 4.0 cm) in medium B. Where indicated by the arrows, the column was eluted with 1M- and 2M-NaCl in medium B. Profile of radioactivity under standard conditions, $\bullet$. Non-adsorbed proteins in the standard procedure (fractions 1–20) were concentrated by using an Amicon B15 apparatus and chromatographed on a second DNA-cellulose column, $\circ$. The fractions were of 1 ml each.

proteins with a high affinity for DNA (or more simply, DNA-binding proteins) was also linear with respect to three parameters of the initial incubations *in vitro*: the concentration of tracer amino acid; the amount of tissue; the duration of incubation. These results illustrate the reproducibility and authenticity of the procedure for the labelling and isolation of DNA-binding proteins. Such considerations are particularly important in comparative experiments on prostate glands from animals of different androgenic status.

Androgenic stimulation of the incorporation of [^{35}S]methionine into prostate proteins

Testosterone was administered to castrated rats for various times *in vivo* and the pooled prostate glands were incubated with [^{35}S]methionine *in vitro*. Soluble protein extracts were prepared and subjected to DNA-cellulose chromatography in order to isolate selectively the prostate proteins with a high affinity for DNA. These experiments are summarized

Table 1. *Incorporation of [³⁵S]methionine into prostate proteins after androgenic stimulation*

Beginning 7 days after castration, groups of eight rats were injected daily with 2.5 mg of testosterone phenylpropionate; control animals were injected with arachis oil only. After timed periods of androgenic stimulation, the prostate glands were pooled and incubated for 1 h with 200 μ Ci of [³⁵S]methionine *in vitro*. Soluble protein extracts were analysed directly or after DNA-cellulose chromatography to separate the proteins with a high affinity for DNA. The latter proteins were those selectively eluted from columns of native DNA-cellulose in medium B containing 1 M-NaCl. Details of all procedures are given in the Materials and Methods section. All preparations were analysed in triplicate and the results are quoted as the means $\pm$ S.E.M.

Period of androgenic stimulation (days)	Incorporation of [³⁵ S]methionine (c.p.m./mg of protein)	
	Total soluble proteins	DNA-binding proteins
None (castrated controls)	9400 $\pm$ 1800	3200 $\pm$ 700
1	24400 $\pm$ 3900	14000 $\pm$ 1800
2	28900 $\pm$ 2400	9600 $\pm$ 1200
3	12100 $\pm$ 1400	4000 $\pm$ 600
4	7500 $\pm$ 1100	1000 $\pm$ 200

in Table 1. Androgenic stimulation conspicuously enhanced the incorporation of [³⁵S]methionine into polypeptide linkage within the soluble protein fraction of the prostate gland; this process attained a maximum after 48 h of androgenic stimulation and then declined. Restoration of the circulating concentrations of androgens evoked similar changes in the labelling of the DNA-binding proteins, but in this case the maximum was reached earlier, after 24 h of hormonal stimulation. Our observations cannot simply be attributed to changes in the endogenous metabolic pools of methionine during the phase of prostate growth mediated by androgens. Such a conclusion would be at variance with the work of Shimazaki *et al.* (1973); these workers reported that the intracellular concentration of methionine remained essentially constant during regeneration of the prostate gland. It should also be emphasized that the incorporation of [³⁵S]methionine showed a similar trend after androgenic stimulation irrespective of whether the data were calculated with reference to DNA or protein, as reported in Table 1.

The critical importance of androgens in regulating the synthesis of DNA-binding proteins in the prostate gland was substantiated further by the experiments presented in Table 2. The anti-androgen, cyproterone acetate, provides an invaluable means for delineating the metabolic processes ultimately under control of the specific androgen-receptor system

(Fang & Liao, 1969; Liao & Fang, 1971). Cyproterone acetate must be administered in relatively high doses to elicit its anti-androgen effect, but with a minor modification to our regimen of androgen injections, it was possible to demonstrate unequivocally that the synthesis of DNA-binding proteins in the prostate gland was subject to modulation by androgens (Table 2). Further, the rapid response in the synthesis of DNA-binding proteins relative to the protracted delay in the enhancement of DNA polymerase and other enzyme activities (Rennie *et al.*, 1975) suggested that the DNA-binding proteins were representative of the important regulatory processes enhanced by androgens before the onset of cell division and DNA replication.

To determine whether the synthesis of a particular species of DNA-binding protein was specifically enhanced during the androgenic response, analyses of high resolution were conducted by electrophoresis in polyacrylamide slab gels. In initial experiments, prostate proteins retained by columns of native DNA-cellulose and denatured DNA-cellulose were compared. In general terms, the electrophoretic profiles of the DNA-binding proteins were similar, the majority of proteins being in the mol.wt. range of 40000 and below. However, some specificity was evident in the secondary structure of the DNA

Table 2. *Suppression of the incorporation of [³⁵S]methionine into prostate proteins by the anti-androgen, cyproterone acetate*

Beginning 7 days after castration, rats were injected with 0.3 mg of testosterone phenylpropionate twice over a 2 h period into the left flank; control animals received injections of arachis oil only. Cyproterone acetate (9.0 mg dissolved in oil) was administered where indicated, into the right flank. Precisely 24 h later, prostate glands pooled from four animals per experimental group were incubated with 200 μ Ci of [³⁵S]methionine *in vitro* and the incorporation of radioactivity into soluble proteins and DNA-binding proteins was determined. The results, quoted as means $\pm$ S.E.M., are taken from three separate experiments, with correction for changes in the specific radioactivity of different batches of [³⁵S]methionine to a reference of 150 Ci/mmol.

Hormonal treatment	Incorporation of [³⁵ S]methionine (c.p.m./mg of protein)	
	Total soluble proteins	DNA-binding proteins
None (castrated controls)	4500 $\pm$ 400	1700 $\pm$ 100
Testosterone alone	11600 $\pm$ 1200	8900 $\pm$ 500
Cyproterone acetate alone	4100 $\pm$ 300	1500 $\pm$ 200
Testosterone plus cyproterone acetate	8500 $\pm$ 400	3400 $\pm$ 300

immobilized on cellulose, for certain polypeptide moieties showed a preference for helical (or native) DNA. Therefore, in comparative experiments on the DNA-binding proteins in animals of different androgenic status, proteins were labelled with [^{35}S]methionine and subjected to fractionation on columns of native DNA-cellulose. These results are summarized in Fig. 2. In all preparations, a considerable amount of radioactivity was recovered close to the ion front of the polyacrylamide gels and this may represent functional peptides, degradation products or incompletely nascent polypeptide chains. Notwithstanding this, three DNA-binding proteins of approx. mol.wts. 27000, 40000 and 45000 were labelled far more extensively after androgenic treatment (see protein peaks I, II and III in Fig. 2*b* as compared with Fig. 2*a*). The increase in labelling 24 h after the restoration of the circulating concentrations of androgens is entirely harmonious with our findings presented above in Table 1. Although not reported in great detail here, experiments were performed after other periods of androgenic stimulation than those presented in Fig. 2. Overall, these results suggest that certain prostate DNA-binding proteins are rapidly and selectively synthesized during the early phase of the androgenic response and then decline at the time when DNA replication and mitosis are proceeding at maximum rates.

Androgens and a prostate DNA-unwinding protein

We next pursued the possibility that a DNA-unwinding protein was present among the complement of prostate proteins with a propensity for binding to DNA. The local denaturation or strand separation of prostate DNA was unequivocally established as an important prerequisite for DNA replication and mitosis by Rennie *et al.* (1975), and proteins performing this function have been termed DNA-unwinding or DNA-relaxing components. In our search for such a DNA-unwinding activity in the prostate gland, we used both enzymic and physicochemical procedures, as originally advocated in the innovative work of Sigal *et al.* (1972), Molineux & Gefter (1974), Germond *et al.* (1975) and Vogel & Singer (1975).

The various DNA polymerases display a pronounced preference with respect to the secondary structure of the obligatory DNA template (Sigal *et al.*, 1972; Molineux & Gefter, 1974) and this may be exploited as a particularly sensitive assay of a putative DNA-unwinding protein. From the evidence presented in Table 3, the 9S form of prostate DNA polymerase provides a potential assay for DNA-unwinding activity because of its marked preference for denatured DNA as template. This property was not shared by the 4S prostate DNA polymerase or the purified DNA polymerase (type I) of *E. coli*.

Fig. 2. Analysis of prostate DNA-binding proteins by electrophoresis

Beginning 7 days after castration, groups of rats (eight animals per experimental group) were injected daily with testosterone phenylpropionate (2.5 mg per animal). The pooled prostate glands from each group were incubated with 200 μCi of [^{35}S]methionine (180 Ci/mmol) and labelled proteins with a high affinity for native DNA-cellulose were isolated. Samples of the DNA-binding proteins (200 μg of protein) from animals of different androgenic status were analysed concurrently in 7.5% (w/v) polyacrylamide gel slabs containing sodium dodecyl sulphate, with crystalline proteins of known molecular weight serving as electrophoretic markers. Photographic negatives of gels stained with Amido Black were first prepared; then the gels were dried *in vacuo* and radioautography was carried out. The photographic negatives and radioautographs were scanned in a microdensitometer; the scale on the ordinate is in arbitrary units, but all samples were analysed throughout under identical conditions. DNA-binding proteins from (a) castrated (control) animals, and (b) after 24 h and (c) after 96 h of androgenic stimulation. The protein bands designated I, II and III are those most conspicuously labelled after the administration of androgens. Protein (stained gels), —; ^{35}S (radioautographs), ----.

By using an assay based on native prostate DNA and the 9S form of prostate DNA polymerase, it was possible to demonstrate DNA-unwinding activity in

Table 3. *Specificity of various DNA polymerases with respect to the structure of the DNA template*

The 9S form of prostate DNA polymerase is present in significant amounts only during mitosis (Rennie *et al.*, 1975). Accordingly, beginning at 7 days after castration, rats received daily injections of testosterone phenylpropionate and after 96 h of hormonal stimulation, soluble (100000g supernatant) extracts were sedimented in sucrose gradients to resolve the 4S and 9S forms of prostate DNA polymerase. Highly purified *E. coli* DNA polymerase (type I) was from Boehringer Corp., London W.5, U.K. *E. coli* enzyme (10 µg of protein), 4S and 9S prostate DNA polymerases (225 µg and 60 µg of protein respectively) were then incubated with 50 µg of native or denatured prostate DNA as template [or 20 µg of poly(dG,dC) from Miles-Seravac Ltd., Maidenhead, Berks., U.K.] and the incorporation of [³H]dGTP into DNA was determined. Complete experimental details are given in the Materials and Methods section. The results are quoted as means ± s.e.m. of assays performed in triplicate.

DNA polymerase	Template	[³ H]dGTP incorporated (c.p.m./assay)
<i>E. coli</i> , type I	Native DNA	150000 ± 6500
	Denatured DNA	28100 ± 2100
	Poly(dG,dC)	9300 ± 600
4S Prostate enzyme	Native DNA	2010 ± 210
	Denatured DNA	1820 ± 140
	Poly(dG,dC)	210 ± 20
9S Prostate enzyme	Native DNA	1210 ± 120
	Denatured DNA	5160 ± 300
	Poly(dG,dC)	70 ± 10
None	Denatured DNA	60 ± 10

the fraction of prostate proteins that characteristically possess a high affinity for DNA (Table 4). This highly selective stimulation of DNA synthesis could not be attributed to the contamination of the DNA-binding proteins with either DNA or DNA polymerase activity. An enhancement of DNA synthesis with native DNA as template was not observed with the 4S prostate enzyme, illustrating the specificity of this activation process. Another important feature of the results presented in Table 4 was that even when denatured DNA served as the template for the 9S prostate DNA polymerase, the presence of the DNA-binding proteins still enhanced DNA synthesis to a significant extent. This implied that the DNA-binding proteins may also have a direct stimulatory effect on the 9S DNA polymerase in addition to promoting denaturation or strand separation in the DNA template. In additional experiments, not reported in detail, it was established that a correlation existed between the activation of native DNA and the input of DNA-unwinding protein; this was maximal with the input of protein used in the experiments presented in Table 4.

Using sucrose density gradients, we found that the DNA-unwinding protein was able to form a discrete complex of sedimentation coefficient 10.6S when mixed with the 9S prostate DNA polymerase (Fig. 3). This interaction was quite specific, because it could not be evoked by highly purified prostate enzymes, aldolase or alkaline phosphatase, neither of which has a necessary functional requirement for interacting with DNA or DNA polymerase. The specificity of the formation of this complex was further illustrated by the fact that under identical conditions, the DNA-unwinding protein did not change the sedimentation behaviour of the 4S prostate DNA polymerase. A

Table 4. *Detection of DNA-unwinding activity in the prostate protein fraction isolated by DNA-cellulose chromatography* DNA-binding proteins were isolated from the prostate glands of normal (non-castrated) animals and samples (40 µg of protein) were added to the system for DNA synthesis described in the legend to Table 3. To accommodate the DNA-binding proteins, however, the amount of DNA polymerases present was halved, but the incubations were continued for 60 min rather than 30 min as in Table 3. Controls were run to eliminate the possibility that the enhancement of 9S DNA polymerase with native DNA as template could be attributed to contamination of the DNA-binding proteins with DNA or DNA polymerase. Assays were conducted in triplicate and the results are expressed as means ± s.e.m.; all results are corrected for a background of 60 c.p.m. for the standard assay system. The ratio of DNA synthesis in the presence and absence of DNA-binding proteins for each combination of template and enzyme is given in parentheses.

DNA polymerase	DNA template	DNA-binding proteins	[³ H]dGTP incorporated (c.p.m./assay)
4S Enzyme	Native DNA	Absent	1620 ± 100
		Present	1820 ± 70 (1.13)
	Denatured DNA	Absent	1640 ± 130
		Present	1810 ± 60 (1.10)
9S Enzyme	Native DNA	Absent	1370 ± 100
		Present	4370 ± 120 (3.19)
	Denatured DNA	Absent	4980 ± 200
		Present	6010 ± 190 (1.20)
None	Denatured DNA	Present	60 ± 10
9S Enzyme	None	Present	40 ± 6

Fig. 3. Interaction between prostate DNA polymerases and other proteins

DNA-unwinding protein was prepared by DNA-cellulose chromatography of prostate extracts from normal (non-castrated) animals. Samples of 4S and 9S DNA polymerases were mixed with other proteins, incubated at 37°C for 2 min and sedimented in linear (4.5 ml) 5–20% (w/v) sucrose gradients, prepared in medium B, at 100000*g*_{av}, for 16 h at 4°C in the SW52Ti rotor of a Christ ultracentrifuge. Samples (100 µl) of each gradient fraction were supplemented with 50 µg of native or denatured DNA template (for the 4S and 9S DNA polymerases respectively) and the incorporation of [³H]dGTP into DNA was determined. DNA-unwinding activity was measured either by the activation of native prostate DNA with respect to 9S DNA polymerase or by the retention of native ³H-labelled prostate DNA on nitrocellulose filters. Details of all procedures are given in the Materials and Methods section. (a) 9S DNA polymerase (280 µg), sedimented alone or after mixing with 80 µg of aldolase or alkaline phosphatase, ○, or after mixing with 80 µg of DNA-unwinding protein, ●. (b) 4S DNA polymerase (950 µg) sedimented alone, ○, or after mixing with 240 µg of DNA-unwinding protein, ●. (c) DNA-unwinding protein (480 µg) sedimented alone and assayed by the retention of ³H-labelled DNA, ▲, or by the enzymic procedure, ●. Sedimentation markers catalase (C; 11.4S) and haemoglobin (H; 4.6S) were run in a parallel gradient. The fractions were of 0.25 ml each.

Fig. 4. Influence of DNA-unwinding protein on the sedimentation behaviour of DNA and the interaction between native DNA and 9S DNA polymerase

Two experiments are summarized here. (a) The interaction of DNA with DNA-unwinding protein. Native prostate DNA was sheared to a sedimentation coefficient of 35S and analysed directly or after thermal denaturation for 15 min at 95°C. Samples of DNA (100 µg) were sedimented alone or after mixing with a DNA-unwinding protein fraction (80 µg) in linear (4.5 ml) 8–32% (w/v) sucrose gradients, prepared in medium B, at 280000*g*_{av}, for 3 h at 4°C in the SW65Ti rotor of a Spinco ultracentrifuge. The DNA was located by extinction analysis at 254 nm. Native DNA alone, ●; denatured DNA alone, ○; native DNA plus DNA-unwinding protein, △. The fractions were of 0.25 ml each. (b) The interaction of native DNA, 9S DNA polymerase and DNA-unwinding protein. DNA (native or denatured; 100 µg) and 9S DNA polymerase (140 µg) were mixed and sedimented in 4.0 ml gradients as described for (a) or in the additional presence of DNA-unwinding protein (80 µg). Samples (100 µl) of each gradient fraction were supplemented with 50 µg of denatured DNA and the incorporation of [³H]dGTP into DNA was determined. As a sedimentation marker, one gradient contained native DNA alone; this was located by scanning at 254 nm. Native DNA and DNA polymerase, ●; denatured DNA and DNA polymerase, ○; native DNA, DNA polymerase and DNA-unwinding protein, △; *E*₂₅₄, --- (arbitrary units). All mixtures were incubated at 37°C for 2 min before sedimentation analysis. The fractions were of 0.2 ml each.

EXPLANATION OF PLATE I

Influence of prostate DNA-unwinding proteins on the structure of supercoiled polyoma-virus DNA

Supercoiled DNA (1.5 μ g) was mixed with 15 μ g of prostate proteins containing DNA-unwinding activity and incubated for 5 min at 37°C. After the addition of sodium dodecyl sulphate to 1% (w/v), the samples were analysed by electrophoresis in 1.4% (w/v) agarose gels for 16 h. The gel was stained with ethidium bromide and photographed under u.v. light. Samples 1 and 2, supercoiled DNA only; 3–6, DNA and DNA-unwinding protein; 7 and 8, controls where the DNA-unwinding protein was incubated at 90°C for 1 min before admixture with DNA.

very important control is presented in Fig. 3(c), where the DNA-binding proteins were sedimented in sucrose gradients and DNA-unwinding activity was monitored by two independent procedures, the retention of native ^3H -labelled prostate DNA on nitrocellulose membranes or the enzymic assay based on the 9S prostate DNA polymerase. Both activities were superimposable in the 4.2S region of the gradient profile, suggesting that both functions are attributable to a similar if not identical protein.

The influence of the DNA-unwinding protein on the structure of DNA and the interaction of native DNA with 9S DNA polymerase was investigated. Studies on the interaction between native prostate DNA and the DNA-unwinding protein are presented in Fig. 4(a). In marked contrast with the profound change promoted by heating at 95°C , the DNA-unwinding protein did not evoke a detectable change in the sedimentation behaviour of native prostate DNA. Nevertheless, additional experiments presented in Fig. 4(b) indicate that the DNA-unwinding protein had changed the structure of DNA in a very subtle manner. In the absence of DNA-unwinding protein, the 9S prostate DNA polymerase failed to co-sediment with native DNA; conversely, when the DNA-unwinding protein was present, the enzyme could bind to DNA and a discrete DNA-enzyme complex was identified. We cannot rigorously exclude the possibility that DNA polymerase is retained in this complex during sedimentation solely by its interaction with the DNA-unwinding protein; binding of the enzyme to DNA need not necessarily have occurred. By contrast, the enzyme bound to denatured

DNA in the absence of the DNA-unwinding protein. Thus, although our evidence supports the view that the term DNA-unwinding protein may describe the structural changes promoted in native DNA, these are probably very localized and may only be detected, for example, by taking advantage of the specificity of DNA polymerase enzymes in recognizing their favoured form of DNA template.

By using the elegant procedure of electrophoresis in dilute agarose gels, a change in the physicochemical properties of the supercoiled form of polyoma-virus DNA was promoted by the prostate DNA-unwinding protein (Plate 1). The relaxation of the structure of polyoma-virus DNA was not evident when the protein fraction was subjected to conditions of thermal denaturation before analysis. On this evidence, relaxation of the structure of supercoiled DNA is a specific function of the DNA-unwinding protein and cannot simply be attributed to the random adsorption of proteins with a high affinity for DNA. Taken in concert, our evidence suggests that the DNA-unwinding protein enhances the process of DNA replication in two ways; first, by combining directly with certain types of DNA polymerase, and secondly, by promoting functionally significant but structurally discrete changes in the secondary structure of native DNA.

Androgenic regulation of prostate DNA-unwinding activity

Since it was established that the incorporation of [^3S]methionine into prostate proteins with a high affinity for DNA was conspicuously stimulated by

Table 5. *Regulation of DNA-unwinding activity in the prostate gland by androgens*

Beginning 7 days after castration, groups of eight rats were injected daily with 2.5 mg of testosterone phenylpropionate; eight animals served as controls and were injected with arachis oil only. At timed intervals groups of rats were killed and proteins with a high affinity for DNA were isolated from the prostate gland and spleen by DNA-cellulose chromatography. Samples (40 μg of protein) were added to the DNA-synthesizing system described in the legend to Table 4 and containing prostate 9S DNA polymerase and 50 μg of native prostate DNA. Assays were conducted in triplicate and the results are expressed as the means $\pm$ S.E.M.; all results were corrected for a background incorporation of [^3H]dGTP into DNA in the absence of added DNA polymerase. In all cases, the DNA-binding proteins contained minimal amounts of DNA polymerase (60–110 c.p.m. of [^3H]dGTP incorporated/assay). The relative DNA-unwinding activity, expressed as the enhancement of DNA synthesis over controls containing native DNA and 9S polymerase only, is given in parentheses.

Source of DNA-binding proteins	Period of androgenic stimulation (days)	[^3H]dGTP incorporated (c.p.m./assay)
None	—	1210 $\pm$ 120
Prostate gland	None; castrated controls	1290 $\pm$ 70 (1.06)
	1	3070 $\pm$ 140 (2.53)
	2	5720 $\pm$ 200 (4.73)
	3	3020 $\pm$ 40 (2.49)
	4	1400 $\pm$ 70 (1.14)
Spleen	None; castrated controls	4100 $\pm$ 170 (3.38)
	1	3940 $\pm$ 100 (3.26)
	2	3990 $\pm$ 200 (3.27)
	3	3840 $\pm$ 110 (3.17)
	4	4200 $\pm$ 240 (3.41)

androgens (see Tables 1 and 2), a logical extension of this work was to monitor the time-course of the induction of prostate DNA-unwinding protein directly. Such an experiment is summarized in Table 5 and two points warrant particular comment. First, DNA-unwinding activity in the prostate gland is stringently regulated by androgens; secondly, the induction of DNA-unwinding activity is relatively rapid and, under rigidly defined experimental conditions (see also Rennie *et al.*, 1975), certainly precedes the onset of DNA replication and mitosis. The DNA-unwinding protein is therefore a particularly attractive candidate for inclusion in the group of important regulatory proteins postulated by Chung & Coffey (1971*a,b*) and Sufrin & Coffey (1973); the distinctive features of these proteins include a critical dependence on circulating concentrations of androgens and a rapid rate of induction before prostate growth and cell division.

Probable absence of deoxyribonucleases from prostate extracts containing DNA-unwinding activity

Many of our findings could be attributed to the presence of deoxyribonucleases in our preparations containing DNA-unwinding activity. Since the DNA-unwinding activity is prepared by DNA-cellulose chromatography, heavy contamination with conventional deoxyribonucleases would not be expected; Alberts *et al.* (1968), for example, reported that these enzymes had a limited ability to bind to immobilized DNA. More rigorous proof of this supposition is presented in Fig. 5. Under analytical conditions where

1 μ g of pancreas deoxyribonuclease I or spleen deoxyribonuclease II may be detected after isoelectric focusing in polyacrylamide gels containing highly polymerized *E. coli* DNA labelled with [3 H]thymidine, no deoxyribonuclease activity of either type I or II could be detected in preparations containing DNA-unwinding activity, even at a high input of protein to each analytical gel. Although this enzyme assay will not detect traces of other deoxyribonucleases with anomalous requirements with respect to pH and bivalent cations, the authenticity of the assay is illustrated by the excellent agreement in the *pI* of commercial deoxyribonucleases with those established by other investigators, namely deoxyribonuclease I, *pI* 4.7–5.0 (Price *et al.*, 1969) and deoxyribonuclease II, *pI* 10.2 (Bernardi *et al.*, 1965).

Discussion

Throughout this paper we have adopted the term, DNA-unwinding protein, to describe a constituent of the prostate gland capable of modifying the properties of DNA. For the main part, this description is acceptable, because this protein mimics the strand separation achieved in DNA by thermal denaturation, at least in the activation of DNA for certain replicative DNA polymerases and in the retention of DNA on nitrocellulose membranes. On the other hand, the limited change evoked in the structure of helical prostate DNA compared with thermal denaturation exposes the limitations in the term. Therefore DNA-

Fig. 5. Analysis of prostate extracts containing DNA-unwinding activity for deoxyribonucleases

Prostate proteins containing DNA-unwinding activity were derived from normal (non-castrated) animals and isolated by DNA-cellulose chromatography. Prostate proteins (300 μ g) or commercial deoxyribonucleases (1 μ g of pancreas enzyme I or spleen enzyme II) were separated by isoelectric focusing in the pH range 3–10 in polyacrylamide gels containing 50 μ g of 3 H-labelled native *E. coli* DNA (2.4×10^6 c.p.m.). At the completion of focusing, the gels were cut into slices (2.5 mm thick) and incubated under optimum conditions for either deoxyribonuclease I (pH 8.0; in the presence of 5 mM-MgCl₂) or deoxyribonuclease II (pH 5.0; absence of added cations). Enzymic activity is located by the release of 3 H into a soluble form. (a) Deoxyribonuclease I; (b) deoxyribonuclease II. Commercial enzyme, ●; prostate extracts, ○; pH, ----.

unwinding protein will suffice only until its precise function has been unequivocally established.

Relaxation or uncoiling of the structure of supercoiled viral DNA can be achieved by interaction with histone F1 (Vogel & Singer, 1975) or certain relaxing factors (Keller, 1975; Germond *et al.*, 1975). Despite the basic pI of our purified DNA-unwinding protein (W. I. P. Mainwaring, unpublished work), there is strong evidence against its identification as a histone. First, the sedimentation coefficient of 4.2S is incompatible with a histone (Hnilica, 1972). Secondly, the synthesis of histones is coincident with DNA replication in many systems (Gurley & Hardin, 1968; Mueller, 1969), yet the appearance of DNA-unwinding activity clearly precedes the onset of DNA synthesis and mitosis under the conditions of androgenic stimulation used in the present study (Coffey *et al.*, 1968).

In an earlier publication from this laboratory, Rennie *et al.* (1975) described the presence of DNA-unwinding proteins of sedimentation coefficients 4S and 6S in the prostate gland. In the present study, both activities were presumably present in our unpurified preparations of prostate proteins that are characterized by a high affinity for DNA. This does not necessarily cast doubt on the interpretation of our findings, because the 4S and 6S moieties could be oligomeric forms of a fundamental subunit.

The temporal appearance of the prostate DNA-unwinding activity after the restoration of the circulating concentrations of androgens in castrated animals is a particularly important feature of the present study. Although it remains true that the mitotic response mediated by androgens is not necessarily synchronized, essentially all of the prostate epithelial cells are provoked to undergo at least one cycle of cell division (Lesser & Bruchovsky, 1974a,b). The hormone-responsive cells may enter and leave the S phase of the cell cycle at slightly different rates, but it has been widely documented that a co-ordinated surge of DNA synthesis and mitosis occurs only after 3 or 4 days of sustained androgenic stimulation (Coffey *et al.*, 1968; Rennie *et al.*, 1975). Before this essentially synchronized phase of the mitotic response, the prostate cells must mandatorily proceed through a period of intense protein synthesis, which is similarly subject to stringent androgenic regulation (Sufrin & Coffey, 1973). Prostate proteins with a high affinity for DNA, including the DNA-unwinding protein and the proteins designated as I, II and III, are among the early manifestations of the androgenic response and the synthesis of this important class of proteins may be decisive in the entry of cells into the process of mitosis. This postulate is totally consistent with the important studies of Salas & Green (1971), but the distinctive features of the prostate DNA-unwinding protein are as follows: first, its synthesis is regulated by androgens and

countered by anti-androgens, and secondly, its regulatory function has been partially elucidated.

Taking an overall survey of the properties of the DNA-unwinding protein, it facilitates the formation of a stable complex between a template of native DNA and the 9S replicative form of DNA polymerase. This is seemingly achieved by two distinct mechanisms, because the DNA-unwinding protein is not only capable of introducing local areas of strand separation in native (helical) DNA, thereby promoting a relaxation in the secondary structure of the DNA template, but also interacts directly with the 9S DNA polymerase, forming a discrete and stable protein-enzyme complex. This interpretation is in accord with the findings of Molineux & Geffer (1974) on a DNA-unwinding protein derived from *E. coli*.

One reservation concerning the present study is the use of relatively impure preparations of DNA polymerases and DNA-unwinding protein. This stricture applies to many studies on the regulatory mechanisms in eukaryotic cells, and the problem may only be circumvented to a certain extent by the use of judiciously selected experimental controls. Extension of the present findings demands more purified components in our reconstituted cell-free systems and possibly the wider use of the supercoiled form of polyoma-virus DNA as a simpler model for DNA-DNA polymerase interactions.

We are grateful to Mrs. Margaret Barker for her assistance in the preparation of the manuscript, to Miss Susan North and Miss Jean Carsons for performing the experimental castrations and to Dr. Beverly Griffin and Dr. Robert Kamen for assistance in the electrophoretic analysis of polyoma-virus DNA.

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