Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 1013–1020. Pergamon Press. Printed in Great Britain

FURTHER STUDIES ON THE DNA-UNWINDING PROTEIN OF THE RAT VENTRAL PROSTATE: EVIDENCE FOR LOCAL AREAS OF DENATURATION

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SUMMARY

Soluble extracts of the rat ventral prostate contain a protein capable of modifying the secondary structure of native (helical) DNA. Two assays for this activity have been developed; the selective retention of [^{3}H]-labelled prostate DNA on nitrocellulose membranes and the enhancement of DNA synthesis by the replicative form of prostate DNA polymerase using native DNA as template. Although not purified to homogeneity, the changes in the secondary structures of DNA promoted by this protein cannot be attributed simply to contamination of the preparations with deoxyribonucleases or other enzymes. Because the structural changes evoked in DNA are similar in nature but not extent to those achieved by thermal denaturation, we have termed this prostate constituent a DNA-unwinding protein. In keeping with this concept, three independent methods have been used to demonstrate a limited strand separation in native prostate after treatment with this protein; digestion with S1 nuclease, binding of mithramycin and immunological precipitation by antibodies raised against single-stranded (denatured) DNA.

INTRODUCTION

Ever since the demonstration by Gilbert and Müller-Hill [1] and Ptashne [2] that important proteins engaged in the regulation of bacterial metabolism could bind to DNA, the study of proteins with a high affinity for DNA has been of widespread interest. Many proteins share the distinctive property of being retained by chromatographic matrices containing immobilized DNA and these are generally termed simply as DNA-binding proteins. Studies on eukaryotes were somewhat slower to begin [3, 4], but DNA-binding proteins are now a ubiquitous feature of a remarkably wide spectrum of higher organisms, including mouse embryo cells [5], the fruit fly, *Drosophila melanogaster* [6], cultures of human AGMK cells infected with type 2 adenovirus [7], Novikoff hepatoma cells [8] and the heterotrophic marine alga, *Cryptothecodinium cohnii* [9]. Apart from their association with DNA, there is now an increasing body of evidence that DNA-binding proteins can modify the secondary structure of polydeoxyribonucleotides and DNA-unwinding or -relaxing proteins have been reported in many eukaryotic cells [10–14]. A DNA-unwinding protein was first identified in rat prostate by Rennie *et al.* [15] and many of its properties were investigated further by Mainwaring *et al.* [16], particularly with respect to its relatively rapid induction after androgenic stimulation and the selective enhancement of DNA synthesis by the replicative 9 S form of prostate DNA polymerase in the presence of the DNA-unwinding protein. The term, DNA-unwinding protein, was originally applied to this prostate constituent because it simulated to a certain extent the denaturation or strand separation of native prostate DNA achieved by heating. The principal objective of the present study was to establish unequivocally that this prostate protein does promote restricted or localized areas of denaturation in helical DNA because this is an integral feature of DNA replication in the intact cell.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (250–270 g body wt.) were used and for certain preparations, it was necessary to take advantage of the dramatic enhancement of DNA replication evoked in the prostate by the administration of androgens to castrated animals. Bilateral orchidectomy was performed by the scrotal route under Fluothane anaesthesia and 7 days later, animals received daily injections of testosterone phenyl propionate (2.5 mg) into the flank region in arachis oil (0.25 ml). Prostate DNA-unwinding protein was isolated after 2 days of androgenic stimulation, whereas the labelling of DNA with [^{3}H]-thymidine and the isolation of the replicative 9S DNA polymerase was performed on prostate glands following 4 days of hormonal treatment. The rationale for these time periods has been reported earlier [15, 16].

Bacteria

Non-infected and T4 bacteriophage-infected *Escherichia coli* cells (strain MRE-600) were purchased from the Microbiological Research Establishment, Porton Down, Wilts., U.K. and stored at $-70°C$.

Chemicals

[Methyl-^{3}H]-thymidine (19 Ci/mmol), [8-^{3}H]-deoxyguanosine 5'-triphosphate (13 Ci/mmol) and [γ-^{32}P]-ATP (15 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks. Tris base (99.5% pure), dithioerythritol, dATP, dCTP, dGTP, dTTP, native calf thymus DNA, lysine-rich histone Hl and *E. Coli* alkaline phosphatase were supplied by the Sigma (London) Chemical Co., London, S.W.6. $(NH_4)_2SO_4$ (enzyme grade; low in heavy metals) was obtained from Fisons Chemical Co., Loughborough, Leics., U.K. and a saturated solution was titrated to pH 7.4 with 1 N NH_4OH. The antibiotic, mithramycin, was kindly donated by Pfizer, Ltd., Sandwich, Kent, U.K. in the form of the proprietary drug, Mithracin®; as parts by weight, this contains mithramycin, 1; mannitol, 40 and Na_2HPO_4-NaH_2PO_4,40. A stock solution of 200 μg/ml mithramycin was made up in 0.3 M $MgCl_2$ and stored at 4°C in a dark-glass bottle. Crystalline pancreatic DNAase†, type I, was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Thermal denaturation of native DNA was accomplished by heating of solutions in 0.1 M NaCl in a boiling water-bath for 10 min followed by rapid quenching in crushed ice. Other chemicals were of Analar grade and glass distilled water was used in making up all solutions.

Biochemical preparations

Native DNA, labelled with [^{3}H]-thymidine, was prepared from rat prostate and *E. coli* cells as described in detail elsewhere [15]. The specific activities of the final preparations were 1.06×10^3 and 1.52×10^5 counts/min/10 μg for the prostate and bacterial DNA, respectively; both were stored in small lots at −20°C and had sedimentation coefficients in the range 60–70 S when examined in the Spinco Model E analytical ultracentrifuge. Replicative 9 S DNA polymerase of rat prostate was isolated as described by Mainwaring *et al.* [16] and assayed by the incorporation of [^{3}H]-dGTP in the presence of complementary deoxyribonucleoside 5'-triphosphates. Polynucleotide kinase was isolated from phage-infected *E. coli* as directed by Richardson [17]. This enzyme is assayed by the incorporation of [γ-^{32}P]-ATP into the 5'-positions of calf thymus DNA that had been exposed by enzyme digestion, first with DNAase I and then alkaline phosphatase; details of this preparation may be found elsewhere [15]. A homogeneous preparation of the SI nuclease from *Aspergillus oryzae*, as originally described by Vogt [18], was a gift from Dr. Robert Kamen. Extracts containing DNA-unwinding activity were prepared as described by Mainwaring *et al.* [16], the critical feature of the isolation being the adsorption of the unwinding protein to columns of Macherey-Nagel cellulose, type 2200 ff, containing immobilized native calf thymus DNA. RNA polymerase from *E. coli* and the form B enzyme from wheat germ were purified extensively by published procedures [19, 20] and stored in 50% (v/v) glycerol at −70°C in small lots.

† DNAse, deoxyribonuclease.

Rabbit antibodies against single-stranded DNA

Our procedure was modified from the method of Erlanger and Baiser [21]. Adenosine (10 mg) was oxidised with $NaIO_4$, coupled to 25 mg BSA and the linkage of the purine derivative to the hapten stabilized by reduction with $NaBH_4$. A female New Zealand White rabbit (4 Kg body wt.) received 3 weekly injections of purine-albumin conjugate (250 μg) intramuscularly and into the foot pad in 1.0 ml of Freunds complete adjuvant. The specific antiserum was stored in small lots at −20°C; control serum was taken from non-immunized rabbits, diluted to an identical concentration of protein as the test antiserum (60 mg/ml) and stored similarly. As reported by the innovators of this procedure [21], the antibodies are directed exclusively against the purine ring of adenine rather than the pentose moiety of the antigen and may thus be used against single-stranded DNA.

Assay of DNA-unwinding activity

Two procedures have been advocated in this laboratory [15, 16]; the retention of native, [^{3}H]-labelled prostate DNA on Millipore membranes (code SX 0001300) and the enhancement of 9S DNA polymerase with native DNA as template. This replicative form of DNA polymerase has a marked requirement for denatured DNA as template, the ratio of DNA synthesis with single- as against double-stranded DNA being approx. 3.3 [16]. There is a linear relationship between response and the amount of DNA-unwinding protein in both procedures, up to a maximum input of 60 μg of protein/assay.

Analyses for single-strandedness in DNA

Medium A used in these experiments was 50 mM Tris–HCl buffer, pH 7.4, containing 0.25 mM EDTA and 1 mM dithioerythritol. Three methods were employed for the detection of restricted areas of denaturation in native DNA, but the initial step was similar to all. Native or thermally denatured DNA (1–10 μg) was incubated with up to 50 μg of various proteins in a total vol. of 100 μl of medium A for 1 min at 37°C. Depending on the nature of the assay, the DNA was non-radioactive or labelled with [^{3}H]-thymidine. (a) *Mithramycin-DNA interactions.* Stock mithramycin solution (50 μl) was added to the samples and after standing for 15 min at ambient temperature (19–21°C), 1.0 ml of medium A was finally added. The fluorescence of the samples was measured at 540 nm in quartz cuvettes using an Aminco–Bowman spectrophotofluorimeter with the excitation monochromator set at 440 nm. A slit width of 5 mm was selected on both the excitation and fluorescence light paths and an aperture of 3 mm was chosen for the photomultiplier shutter. Daily calibration of the

instrument was carried out with 25 ng of quinine sulphate, diluted from a 1 mg/ml stock solution just prior to use with 0.1 N H_2SO_4. (b) *Digestions with S1 nuclease.* The samples of [^{3}H]-labelled DNA were diluted with 200 μl of 0.1 M sodium acetate–acetic acid buffer, pH 4.6, containing 0.1 M NaCl and 4.5 mM $ZnSO_4$, placed in a water bath at 37°C for 5 min to attain temperature equilibration and 1 μl (1.2 μg) of Sl nuclease was added. At timed intervals, 25 or 50 μl aliquots were removed and pipetted onto DEAE-cellulose discs (Whatman DE 81; 2.5 cm. dia.). (c) *Immunological precipitation of DNA* [^{3}H]-labelled DNA was used in this assay and both the control and specific antisera were diluted with an equal vol. of medium A just prior to use. Diluted serum (100 μl) was added to the samples and after gentle but thorough mixing by tapping with the finger, the samples were allowed to stand undisturbed at ambient temperature for 15 min. The tubes were placed in an ice-bath and 400 μl of 75% saturated $(NH_4)_2SO_4$ in medium A were added dropwise. After standing for 15 min at 0°C, the samples were sedimented at 10,000 $g_{(av)}$ for 10 min in a Christ type UJ 15 centrifuge and duplicate samples (100 μl) of clear supernatant were counted to assess the amount of non-sedimented [^{3}H]-DNA. A visible precipitate was rapidly formed when thermally denatured DNA was mixed with the specific antibody for single stranded DNA; this was not evident with native DNA or on admixture of denatured DNA and the control serum. From this, it follows that direct centrifugation would give a measure of the selective precipitation of denatured [^{3}H]-DNA, but two reasons prompted our adoption of $(NH_4)_2SO_4$ precipitation of protein-bound [^{3}H]-DNA. First, even the precipitation of single-stranded [^{3}H]-DNA was more complete; second, owing to the elimination of non-specific adsorption of serum proteins to native [^{3}H]-DNA under conditions of high ionic strength, the distinctive sedimentation of denatured [^{3}H]-DNA was made even more marked.

Other procedures

Protein was determined by the Folin–Ciocalteau reagent [22] with BSA as the standard. DNA was measured by the diphenylamine-acetaldehyde reaction [23] with calf thymus DNA as reference. In all radioactive tracer work, radioactive DNA was collected on Whatman DE 81 discs which were washed extensively, first with 5% (w/v) Na_2HPO_4 and second, 50% (v/v) ethanol [15]. Millipore membranes and DE 81 discs were dried under an I.R. lamp and counted in a non-aqueous phosphor containing 5 g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (or BPD)/litre of toluene, where the efficiency for ^{3}H counting was 50%. Aqueous samples (up to 0.5 ml) were counted directly in 10 ml of a water-miscible phosphor (15 g of BPD dissolved in 1770 ml of toluene, 110 ml of water and 625 ml of Triton X-100), where the efficiency for ^{3}H counting was 40%. T.l.c. of ATP, ADP, AMP and H_3PO_4 was performed on Macherey–Nagel sheets (Polygram; type 300 PE I) with 1 M LiCl as the developing medium [24].

RESULTS

Rat spleen contains very significant amounts of DNA-unwinding activity, as judged by our methods of assay [15, 16] and is seemingly identical to the activity in the prostate except that it is not subject to androgenic regulation. Spleen DNA-unwinding activity was used in all preliminary trials and the final procedures were then repeated with prostate preparations derived from castrated animals, subsequently given 2 days of androgenic stimulation *in vivo*. Similar considerations also applied to the source of [^{3}H]-labelled DNA. The high specific activity of [^{3}H]-DNA from *E. coli* expedited initial studies which were then corroborated with [^{3}H]-prostate DNA.

Enzyme contamination of extracts containing DNA-unwinding activity

The interpretation of the present results would be placed in doubt if the preparations of prostate DNA-unwinding activity were contaminated with certain enzymes. The absence of non-replicative (4S) and replicative (9S) DNA polymerases has already been reported [16]. Phosphatase activity of both acidic and alkaline types can also be discounted as 20 μg of ATP, supplemented with 2 μCi of [γ-^{32}P]-ATP, may be incubated with 200 μg of DNA-unwinding extracts at either pH 5 or pH 10 for 1 h at 37°C with less than 2% release of the terminal phosphate group, as established by t.l.c. DNAase contamination was not detected in our earlier report [16] and this important result is substantiated in the more sensitive assay, depicted in Fig. 1. Furthermore, the random cleavage of the phosphodiester bonds in native DNA by DNA-unwinding extracts seems unlikely from the evidence presented in Table 1. Native prostate DNA was first incubated with DNA-unwinding protein and then incubated with alkaline phosphatase, with intervening extractions with phenol. The final product served as a very poor substrate for polynucleotide kinase in the presence of [γ-^{32}P]-ATP, whereas intentionally nicked DNA was heavily labelled in the 5′-positions under similar experimental conditions.

Protein specificity in the unwinding of native DNA

From the evidence presented in Table 2, the unwinding of native DNA is a specific process. Proteins such as casein and γ-globulin, which have no functional requirement for interacting with DNA, are ideal controls for our assay procedures. The change in the secondary structure of DNA is not simply attributable to the association of the relatively basic unwinding protein to the negatively charged phosphate groups of polydeoxyribonucleotides, as the process is countered by thermal denaturation of the protein.

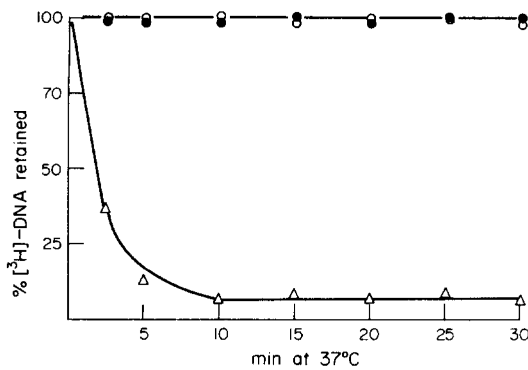

Fig. 1. The absence of DNAase activity in prostate extracts containign DNA-unwinding activity. [^{3}H]-DNA from *E. coli* (10 μg; 3 × 10^5 disintegrations/min) was incubated at 37°C with 200 μg of prostate proteins isolated by DNA-cellulose chromatography in either 0.3 ml of 0.1 M acetic acid-sodium acetate buffer, pH 5.0 or 0.1 M Tris–HCl buffer, pH 8.5, in the presence or absence of 1 mM $MgCl_2$. One incubation mixture at pH 8.5 with $MgCl_2$ contained 0.25 μg of pancreatic DNAase I. At times intervals, 25 μl samples were pipetted onto DE 81 discs and radioactivity in a high mol. wt. form was determined. Controls, incubated with medium A alone, 0; prostate extracts at pH 5.0 or 8.5, ± $MgCl_2$, ●; pancreatic DNAase I and pH 8.5 in the presence of MgCl, △.

Consistent with the findings of others [25, 26], the binding of bacterial and eukaryotic RNA polymerases provokes discrete changes in the structure of helical DNA, a property not ostensibly shared by DNA polymerase. In accord with the observations of Vogel and Singer [27], the lysine-rich histone Hl markedly changes the physicochemical properties of native DNA. However, three lines of argument suggest that the effects mediated by histone Hl are less subtle than those achieved by other proteins and are essentially explained by strong ionic interactions. First, a visible precipitate forms immediately on mixing DNA and histone Hl; this is not the case with DNA-unwinding protein or RNA polymerase. Second, histone-DNA interactions are only marginally reduced by heating, in contrast to the deleterious effects of heating on the activity of the DNA-unwinding protein. Third, the presence of histone Hl powerfully inhibits DNA synthesis; this is directly opposed to the enhancement promoted by the DNA-unwinding protein.

Evidence that the DNA-unwinding protein partially denatures native DNA

(a) *Studies with SI nuclease.* The SI nuclease discovered by Vogt [18] is a particularly useful enzyme for probing the structure of DNA and its unique preference for single-stranded regions has played an invaluable part in the development of nucleic acid hybridisation and other procedures. Our own evidence, presented in Fig. 2a, confirms that SI nuclease degrades denatured [^{3}H]-prostate DNA remarkably rapidly but was without detectable effect on native [^{3}H]-prostate DNA. As shown in Fig. 2b, prior treatment of native DNA with the unwinding protein changed the structure in a subtle manner such that the SI nuclease could release approx. 23% of the radioactivity into a soluble form. In an important control, this sensitivity to digestion by SI nuclease was not evident if inactivated DNA-unwinding protein was used. By contrast, denatured DNA was degraded equally rapidly by the nuclease, even in the presence of functional DNA-unwinding activity. In additional experiments, not included in Fig. 2, the sensitivity of native [^{3}H]-DNA to SI nuclease was not significantly altered by γ-globulin or *E. coli* RNA polymerase, even though the latter protein binds to DNA (see Table 2).

(b) *Fluorescence analysis with mithramycin.* In the presence of Mg^{2+} ions, the intensity of fluorescence of mithramycin is considerably enhanced by the presence of very low amounts of DNA [28, 29]. However, this fluorescence increment is markedly dependent upon the ordered secondary structure of the DNA, being reduced on separation of the strands and totally negated by chemical or enzymic hydrolysis [29]. These observations were confirmed with prostate DNA, as indicated in Fig. 3. A more extensive investigation of mithramycin-DNA interactions is presented in Table 3. Provided that the DNA-unwinding protein is in a functional or active state, it reduces the fluorescence of mithramycin in the presence of native prostate DNA by approximately 15%. This small but

Table 1. Evidence that extracts containing DNA-unwinding activity do not extensively cleave the phosphodiester bonds of native DNA. Native prostate DNA (500 μg) was incubated with 50 μg of DNA-unwinding protein and then extracted with phenol. After precipitation with ethanol, the DNA was incubated with 10 μg of alkaline phosphatase and again extracted with phenol. Samples (50 μg) of the final DNA product were incubated in triplicate with polynucleotide kinase in the presence of 2.5 μCi of [γ-^{32}P]-ATP. Nicked calf thymus DNA was prepared by incubation with pancreatic DNAase I, followed by alkaline phosphatase

Treatment of DNA	Incorporation of [γ-^{32}P]-ATP (Disintegrations/min/50 μg of DNA)
None	3100 ± 200
DNA-unwinding protein alone	5300 ± 100
Alkaline phosphatase alone	5900 ± 300
Unwinding protein and then phosphatase	6300 ± 200
Nicked DNA	220,000 ± 8000

Table 2. Protein specificity in the unwinding of native prostate DNA. The ability of various proteins (25 μg each) to promote the unwinding of DNA was assessed by two procedures; the retention of [^{3}H]-DNA (10 μg; 2.0×10^4 d.p.m.) on Millipore filters and the stimulation of DNA synthesis by replicative 9S DNA polymerase (60 μg) with 25 μg of DNA as template. In certain experiments, proteins were heated at 100°C for 1 min prior to assay. Assays were performed in duplicate; experimental error ± 10%

Protein added	I. Filtration assay [^{3}H]-DNA retained (d.p.m./assay)	II. Enzyme assay [^{3}H]-dGTP incorporated (d.p.m./assay)
None: controls	610	3100
Unwinding protein	12,300	10,700
Unwinding protein (heated)	730	3300
Histone Hl	18,400	500
Histone Hl (heated)	14,700	540
γ-Globulin	580	3300
Casein	620	not determined
DNA polymerase (9S)	900	not determined
RNA polymerase (*E. coli*)	13,700	not determined
RNA polymerase (wheat germ)	11,400	not determined

reproducible reduction in fluorescence could not be evoked by γ-globulin, casein or denatured DNA-unwinding protein. The low fluorescence in the presence of denatured prostate DNA was not reduced further by the presence of the unwinding protein.

(c) *Immunological studies.* As originally suggested by Erlanger and Boise [21], it is possible to raise antibodies in rabbits that are highly efficient reagents for the selective precipitation of single-stranded DNA. The specificity of our own antiserum against [^{3}H]-prostate DNA is illustrated in Fig. 4. Under these conditions of assay, [^{3}H]-DNA alone is not sedimented in 50% saturated $(NH_4)_2SO_4$. The secondary structure of the [^{3}H]-DNA was the determining factor for its precipitation by the specific antiserum, because denatured or single-stranded DNA was almost completely precipitated. This effect could not be mimicked by the control serum and native or double-stranded DNA remained soluble in 50% saturated $(NH_4)_2SO_4$, even in the presence of the specific antiserum. Presumably the hydrogen bonds responsible for the maintenance of the structure of helical DNA prevent the access of the antibody to the immunological determinants, the purine ring of adenine bases.

More extensive use of the specific antiserum as a probe for areas of denaturation or single-strandedness in DNA is presented in Table 4. Treatment of native [^{3}H]-prostate DNA with the unwinding protein made the [^{3}H]-DNA more susceptible to precipitation by

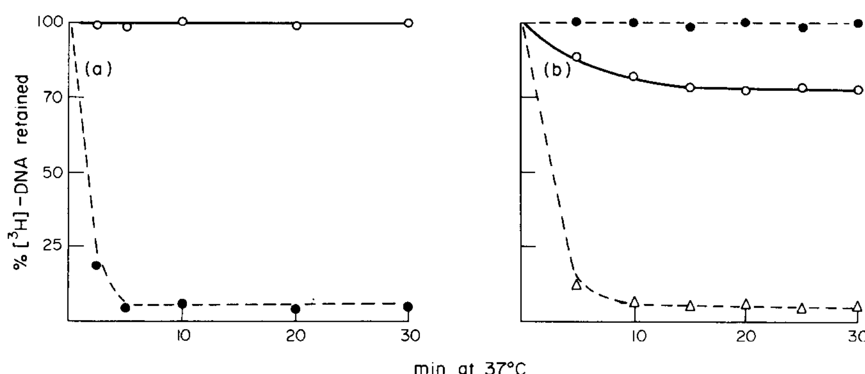

Fig. 2. The digestion of [^{3}H]-prostate DNA by Sl nuclease. [^{3}H]-Prostate DNA (10 μg; 2.0×10^4 d.p.m.) was incubated directly at pH 4.6 with 1.2 μg of Sl nuclease in the presence of Zn^{2+} ions or with DNA-unwinding protein (25 μg) in medium A, after which the medium for Sl nuclease digestions and the enzyme were added. After timed intervals at 37°C, 50 μl samples were pipetted directly onto DE 81 to assess the radioactivity released into a soluble form. Where indicated, DNA-unwinding protein was inactivated by heating at 100°C for 1 min prior to mixing with the [^{3}H]-DNA. (a) [^{3}H]-DNA without unwinding protein. Native DNA, ○; denatured DNA, ● (b) [^{3}H]-DNA with unwinding protein. Native DNA and inactive unwinding protein, ●; native DNA and functional unwinding protein, ○; denatured DNA and inactive or functional unwinding protein (the curves were superimposable), △.

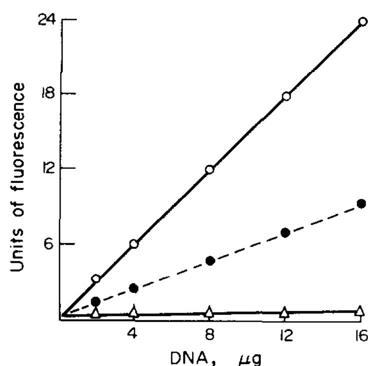

Fig. 3. The fluorescence of mithramycin in the presence of prostate DNA. The fluorescence of mithramycin was measured after mixing with DNA in the presence of Mg^{2+} ions. All values are corrected for the intrinsic fluorescence of mithramycin in the absence of DNA. Fluorescence units were calculated from the light transmission and meter sensitivity settings. Native DNA, ○; denatured DNA, ●; DNA digested with DNAase I (1 μg), △.

the specific antiserum, an effect which could not be simulated by γ-globulin, casein or heat-inactivated unwinding protein. By contrast, the DNA-unwinding protein had little effect on the immunological precipitation of denatured DNA.

DISCUSSION

Taking the present evidence overall, it would now seem that our original nomenclature for a prostate protein as a "DNA-unwinding" factor was essentially correct. Using three independent means of analysis, the prostate DNA-unwinding protein seems to be capable of introducing limited or localized areas of strand separation in native DNA. In all the methods of analysis employed, the changes evoked in the secondary structure of helical DNA were far less extensive than those achieved by thermal denaturation of DNA. Nevertheless, from the biological standpoint, these subtle changes are important because they provide the replicative 9S form of DNA polymerase with its favoured form of DNA template. Earlier speculations [15, 16] on the implications of the DNA-unwinding protein in the androgenic control of DNA replication now appear to be vindicated.

Because of the prevalent interest in the modification of the secondary structure of DNA, it is worthwhile to highlight the distinctive properties of the prostate DNA-unwinding protein. First, it is the only DNA-unwinding or -relaxing factor to be subject to hormonal regulation and the induction of this activity in castrated animals by androgens is consistent with its involvement in the onset of DNA replication [16]. Second, other DNA-unwinding proteins are isolated by chromatography on denatured DNA-cellulose columns, whereas the prostate protein is isolated by adsorption to immobilized native DNA. Third, with the exception of recent work on calf thymus gland [30], it is the only DNA-unwinding protein to enhance the replication of a native DNA template

Table 3. Further studies on the fluorescence of mithramycin in the presence of prostate DNA. Samples of DNA (16 μg) were mixed with proteins (25 μg) in medium A and after the addition of mithramycin, the fluorescence was measured at 540 nm. Where indicated, DNA-unwinding protein was incubated at 100°C for 1 min prior to mixing with DNA. Assays were conducted in triplicate

Protein	Arbitrary fluorescence units I. Absence of DNA	II. Native DNA	III. Denatured DNA
None	15.0 ± 1.0	40.0 ± 1.5	24.0 ± 1.0
γ-Globulin	15.0 ± 0.5	40 ± 1.0	23.5 ± 0.5
Casein	15.0 ± 1.0	40 ± 0.5	—
Unwinding protein	14.5 ± 0.5	35.0 ± 0.5	24.0 ± 1.0
Unwinding protein (heated)	15.0 ± 0.5	40.0 ± 1.0	—

Table 4. Further investigations on the precipitation of [^{3}H] prostate DNA by the specific antibody. [^{3}H]-Prostate DNA (10 μg; 2.0 × 10 d.p.m.) was incubated with various proteins (25 μg) in medium A. Specific antiserum against single-stranded DNA was then added and proteins were precipitated at an overall saturation of 50% with respect to $(NH_4)_2SO_4$. The [^{3}H]-DNA remaining in the supernatant after centrifugation was determined by scintillation spectrometry. Where indicated, DNA-unwinding protein was heated at 100°C for 1 min prior to mixing with the [^{3}H]-DNA. Assays were conducted in triplicate

Protein	[^{3}H]-DNA remaining in a soluble form (d.p.m.) I. Native DNA	II. Denatured DNA
None	19,000 ± 500	1800 ± 100
γ-Globulin	19,300 ± 100	2000 ± 100
Unwinding protein	14,100 ± 100	1400 ± 200
Unwinding protein (heated)	19,500 ± 100	1700 ± 100

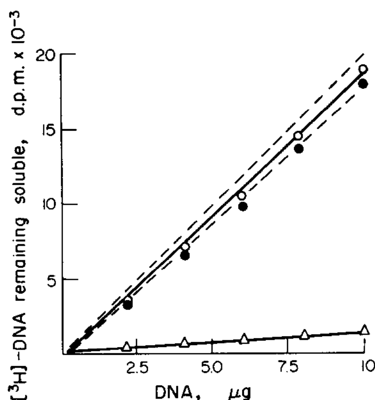

Fig. 4. The specificity of the rabbit antiserum for single-stranded DNA [^{3}H]-Prostate DNA (2–10 μg) was incubated with either the specific antiserum raised against the purine-hapten conjugate or control serum in a total vol. of 200 μl in medium A. Then 400 μl of 75% saturated $(NH_4)_2SO_4$ in medium A was added to precipitate the immunoglobulins, including DNA-antibody complexes, and after centrifugation at 10000 g, the [^{3}H]-DNA remaining in solution was determined by scintillation spectrometry. Denatured DNA and control serum, ○; native DNA and specific antiserum, ●; denatured DNA and specific antiserum, △; controls to assess the input of radioactivity, where denatured DNA was treated with $(NH_4)_2SO_4$ in the absence of serum proteins, ---.

of eukaryotic origin using a homologous DNA polymerase. For example, Yeh *et al.* [13] assay their eukaryotic DNA-unwinding activity with the DNA polymerase from *E. coli*; in our experience, this Kornberg or type I enzyme is singularly unsuitable for the assay of prostate DNA-unwinding protein [16]. Fourth, our studies are unique in that we have tried to monitor the changes in the secondary structure of the natural DNA, here prostate DNA. In most other studies [5, 7, 10–13, 31], supercoiled (closed-circular) DNA derived from viruses and particularly the marine bacteriophage, PM2, serve as model substrates for DNA-unwinding proteins; assays include electrophoresis in dilute agarose gels [11] or changes in the fluorescence of ethidium bromide [10, 12, 32] for demonstrating the relaxation of these DNA structures. Such model DNA's, however, may be far removed from the authentic biology of the DNA-unwinding proteins in many mammalian cells. Finally, many proteins nick and then close the phosphodiester bonds of DNA [6, 10–12, 32] and are aptly described as DNA-relaxing proteins. On current evidence, this mode of action seems fundamentally different from the prostate DNA-unwinding protein for this appears to introduce local areas of single-strandedness into helical DNA.

Acknowledgement—The authors gratefully acknowledge the assistance of Mrs. Margaret Barker in the preparation of the manuscript.

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DISCUSSION

Rousseau. Did you consider or check the possibility that this unwinding protein could also increase the template activity of chromatin, that is RNA transcription from DNA?

Mainwaring. We have done this experiment in fact, as I indicated on one of the slides. The DNA unwinding activity with respect to the DNA polymerase is exceedingly specific because you cannot show this change with RNA polymerase. Furthermore it seems to be very much less active when one uses chromatin as template rather than DNA. In other words, it seems to be specific to the replicative 9*S* form of DNA polymerase.

Spelsberg. Just a point about unwinding proteins for polymerase. In bacteria, I believe the unwinding protein really doesn't unwind the DNA but really alters or perturbates the DNA helix. It's a little different from the unwinding per DNA replication. A couple of small questions. How tightly bound is your protein to your DNA? Do you have any evidence for specificity of sites? Is the source of the material obtained from nuclei or chromatin?

Mainwaring. It may be done either from nuclei or from whole tissue but it's all in our last paper.

Spelsberg. So you conclude that it is nuclear localized.

Mainwaring. We believe so, but we haven't definite evidence for that. On your point about specificity, I cannot answer your questions. One of the very unfortunate aspects of this protein which is hampering our progress is that the amount in the prostate gland is exceedingly small and to do the sort of experiments you're envisaging, that is the interaction of the protein with DNA, we wanted to make the protein radioactive with iodine^{125}. Unfortunately, irrespective of the method that we have employed thus far, we introduced a large amount of iodine^{125} into the protein but we inactivated it. We are trying to do the experiments raised by your question, but I have nothing specific to add at this time.

Spelsberg. How many per cell did you add.

Mainwaring. We think there are of the order of 2 or 3,000, if our molecular weight determination is correct. This is quite a lot.

Liao. Does the winding protein act on RNA–RNA or RNA–DNA duplex? Also, have you tried to isolate the protein from other mammalian tissues?

Mainwaring. We have not tried experiments relevant to your first question. We've tried to isolate it from the prostates of normal animals. The one other source, which is very active in fact, is the spleen. All the enzymes that Rennie and others have looked are very high in the spleen and so is the DNA unwinding activity but the distinctive property of the spleen unwinding protein is that it is not subject to androgenic regulation.

Siiteri. Ian, perhaps I missed it, is there any interaction of this protein with the steroids.

Mainwaring. We do not know.

Crabbé. Would it be so, Dr. Mainwaring, that from a cell biology viewpoint the larger the number of mitosis in a tissue the larger the amount of unwinding protein.

Mainwaring. This is a strong possibility Dr. Crabbé, but I have not actually looked at that. As I showed on the introductory slide, we have based all of our work on replication in prostate gland.