Acceptor Proteins of Rat Prostate Residual Chromatin†

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ABSTRACT: The androgen acceptor sites of the rat prostate residual chromatin (2 M NaCl insoluble fraction of chromatin) have been determined by steroid exchange assay, binding of translocated androgen–receptor complex in vitro, and solubilization of the acceptor protein(s) from the residual chromatin. Binding of [^{3}H]dihydrotestosterone to the residual chromatin was saturable, displaying high affinity (K_d = 3.1 nM) and low capacity (6.3 nmol/mg of protein). The binding of [^{3}H]dihydrotestosterone by the residual chromatin was androgen specific, as shown by steroid competition experiments. Intrachromatin binding study of translocated 5α-[^{3}H]dihydrotestosterone–receptor indicated that the residual chromatin contained 31% of the total chromatin-bound androgen, thus representing one of the major chromatin–androgen binding sites. The results suggested the presence of acceptor molecules in the residual chromatin with which the androgen–receptor interacted. To ascertain this, the residual chromatin was extracted with phenol, and the phenol-solubilized protein(s) was (were) assayed for acceptor activity by interaction with [^{3}H]dihydrotestosterone–receptor complex. Comparison of phenol-solubilized residual proteins from rat prostate, spleen, and chicken erythrocyte indicated that [^{3}H]dihydrotestosterone–receptor complex bound tissue specifically to the prostate residual protein and that the interaction required the presence of DNA. The possible importance of the residual DNA was examined by reannealing with cloned cDNAs coding for the subunit components of prostatic binding protein, an androgen-regulated oligomeric protein in rat prostate. The rates of reassociation kinetics of the residual DNA with the cDNAs were faster than with total DNA, equivalent to a 3-fold enrichment in prostatic binding protein coding sequences. The high salt resistant residual chromatin acceptor(s) thus appear(s) to be preferentially associated with androgen-activated genes.

Selective retention of steroid hormones in target cell nuclei represents one of the key steps in steroid hormone action (Liao, 1975). The nuclear-retained steroid, translocated as a steroid–receptor complex, is bound to a specific chromatin component, the acceptor. Interaction of the steroid–receptor complex with nuclear acceptor presumably initiates the reactions leading to the steroid-specific transcription pattern. An understanding of the nature and function of the acceptor molecules is therefore necessary in order to comprehend the mechanism of steroid hormone action. In rat prostate, several androgen acceptors have been reported (Hiremath et al., 1980; Klysejko-Stefanowicz et al., 1976; Liao et al., 1973; Mainwaring et al., 1976; Tymoczko & Liao, 1971; Wang, 1978). The presence of an acidic protein acceptor in rat prostate nuclear extract was first demonstrated by Tymoczko & Liao (1971) and supported by the purification of a 14000-dalton acceptor protein from the 0.35 M NaCl soluble fracton of chromatin (Hiremath et al., 1980). A basic acceptor protein, isolated from the 2 M NaCl nuclear extract of rat prostate, has been reported by Mainwaring et al. (1976). Evidence indicates that the 2 M NaCl insoluble residual chromatin also interacts tissue-specifically with the androgen–receptor complex (Wang, 1976). In fact, several high salt (1–3 M NaCl) resistant preparations of rat prostate nuclei or chromatin have been shown to exhibit acceptor activity or to contain specific androgen binding sites (Barrack & Coffey, 1980; Buttyan et al., 1983; Klysejko-Stefanowicz et al., 1976; Liao et al., 1973; Rennie et al., 1983).

Since the high salt resistant chromatin contains both DNA and protein (Nyberg & Wang, 1976), the identity of the acceptor was not clear. The insolubility of the residual chromatin also impeded attempts to determine the nature of the acceptor. In this paper we report the solubilization of the acceptor proteins from rat prostate residual chromatin. Data are also presented to show that the residual chromatin DNA is enriched in androgen-specific transcribing sequences.

Experimental Procedures

Animals and Materials. Male Sprague-Dawley rats weighing about 300 g were bought from Harlan Sprague-Dawley, Indianapolis, IN. The animals were castrated by the scrotal route under ether anesthesia and used 2 days after the operation. 1,2-[^{3}H]Dihydrotestosterone (60 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Nonradioactive steroids were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were of analytical grade.

Preparation and Solubilization of Residual Chromatin. Nuclei were isolatead from rat ventral prostates by the procedure of Blobel & Potter (1966). The homogenization medium and all subsequent solutions were supplemented with 1 mM phenylmethanesulfonyl fluoride. The nuclei were used either for the nuclear binding experiments or for the isolation of chromatin as described elsewhere (Wang, 1978). For preparation of the residual chromatin, the prostate chromatin was extracted with 1000 volumes of 2 M NaCl/20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] pH 8.0, for 6–8 h, and the suspension was centrifuged for 90 min at 45000 rpm in a Spinco 50Ti rotor. This extraction was repeated once. The resulting pellet was washed 4 times with 2 M NaCl/Tris buffer and twice with 20 mM Tris-HCl, pH 8.0, by suspension and centrifugation; the resulting pellet was the residual chromatin.

The residual chromatin was solubilized by phenol according to the procedure of Teng et al. (1971). Briefly, the residual chromatin was extracted with cold 0.2 N HCl, and the acid-

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[1] Abbreviations: DHT, dihydrotestosterone (17β-hydroxy-5α-androstan-3-one); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TEM, 20 mM Tris-HCl (pH 7.5)/10 mM EDTA/1 mM β-mercaptoethanol; TKMM, 20 mM Tris-HCl (pH 7.5)/25 mM KCl/1 mM $MgCl_2$/1 mM β-mercaptoethanol; NHCP, non-histone chromosomal protein; PBP, prostatic binding protein; RNP, ribonucleoprotein; NP, nucleoprotein.

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insoluble residue, suspended in 0.1 M Tris-HCl, pH 8.4/10 mM EDTA/0.14 M β-mercaptoethanol, was extracted with redistilled phenol. The phenol phase was collected and dialyzed successively against the following solutions: (i) 0.5 M acetic acid/8 M urea/0.14 M β-mercaptoethanol; (ii) 0.01 M Tris-HCl, pH 8.4/0.01 M EDTA/8 M urea/0.14 M β-mercaptoethanol; (iii) 0.1 M NaCl/0.01 M Tris-HCl, pH 8.4/0.01 M EDTA/0.14 M β-mercaptoethanol; (iv) 20 mM Tris-HCl, pH 7.5, with three changes of the last buffer.

Determination of Androgen Binding by [³H]DHT Exchange Assay. Androgen binding by the insoluble residual chromatin was determined by the exchange assay of Anderson et al. (1972). The reaction mixture for measurement of total androgen binding, in a total volume of 100 μL, contained 50 μg of residual chromatin protein, various concentrations of [³H]DHT (0–18 nM), 10 mM Tris-HCl, pH 7.6, and 1.5 mM EDTA. For nonspecific binding, the above reaction mixture contained, in addition, 1000-fold molar excess of nonradioactive DHT. The reaction was carried out at 2 °C for 18 h, after which the mixture was centrifuged to pellet the residual chromatin. The pellet was washed as described by Barrack & Coffey (1980). In steroid competition experiments, the binding assays were done in the presence and absence of nonradioactive competing steroids in 1–1000-fold excess concentrations as indicated in Figure 3.

Preparation of Androgen–Receptor Complex. For acceptor assay of the solubilized residual protein, a 40% saturated ammonium sulfate precipitate fraction of cytosol from castrated rat prostate was used as the receptor preparation. The receptor was incubated with 2.5 nmol of [³H]DHT in 0.1 M NaCl containing 0.02 M Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM β-mercaptoethanol according to Fang & Liao (1971).

Binding of Translocated [³H]DHT–Receptor Complex to Chromatin Fractions. Cell nuclei isolated from castrated rat prostate were suspended in 0.32 M sucrose/TKMM and incubated with an equal volume of the [³H]DHT–receptor complex for 30 min at 20 °C. After incubation, the mixture was chilled on ice, diluted with an equal volume of 0.32 M sucrose/TKMM, and centrifuged at 1000*g* for 10 min to recover the nuclei. The nuclear pellet was washed 3 times by centrifugation with 0.32 M sucrose/TKMM. Chromatin was isolatead from the washed nuclear pellet and extracted with 0.35 M NaCl, followed by extraction with 2 M NaCl/0.02 M Tris-HCl, pH 8.0. The 2 M NaCl extract was dialyzed against 13 volumes of distilled water containing 1 mM phenylmethanesulfonyl fluoride to precipitate the DNA–histone. The supernatant contained the NHCP. The fractionation procedure yielded four fractions: the 0.35 M NaCl soluble NHCP, the 2 M NaCl soluble NHCP, the DNA–histone, and the 2 M NaCl insoluble residual chromatin. The radioactivity of each fraction was extracted with ethanol and counted in dioxane cocktail in a liquid scintillation spectrometer.

Interaction of [³H]DHT–Receptor Complex with Solubilized Residual Protein. The acceptor activity of the phenol-solubilized residual protein was assayed by the procedure of Tymoczko & Liao (1971). The solubilized residual protein, in amounts as indicated in Figure 4, was incubated at 4 °C for 30 min with 0.6 mg of protein of the [³H]DHT–receptor preparation and 200 μg of rat liver DNA in a total volume of 1.5 mL of 0.1 M NaCl/TEM. After incubation, the resulting complex was collected by filtration on a Millipore filter (RA 1.2 μm), and the filter was washed 5 times with 3 mL of 0.2 M NaCl/TEM. A control reaction mixture without the residual protein was run in parallel to provide the background counts.

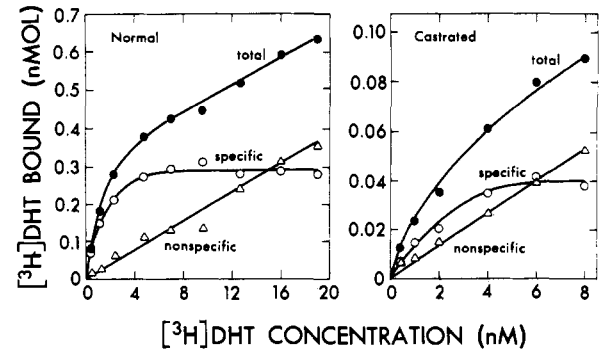

FIGURE 1: Binding of [³H]DHT to prostate residual chromatin of normal (left) and castrated (right) rats. Binding assays without (total) and with (nonspecific) 1000-fold excess of nonradioactive DHT were carried out as described under Experimental Procedures. Specific binding was calculated as the difference between total and nonspecific bindings. The ordinate scale indicates nanomoles of [³H]DHT bound per assay containing 50 μg of protein of the residual chromatin.

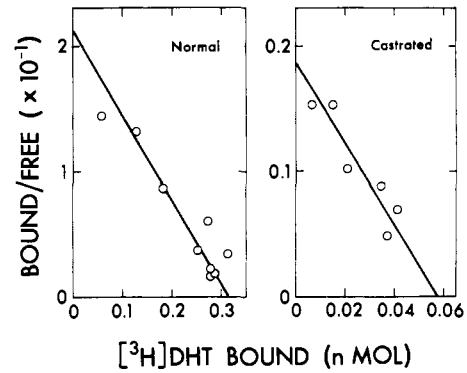

FIGURE 2: Scatchard analysis of androgen binding to the residual chromatin. The plots were constructed from the data in Figure 1.

DNA–DNA Reassociation. Reannealing of [³H]cDNA with residual DNA and total prostate DNA was carried out as described (Hiremath & Wang, 1981). Single-stranded cloned [³H]cDNAs coding for the three subunit components, C1, C2, and C3, of PBP were prepared as described elsewhere (Mpanias et al., 1983; Xu et al., 1983). Driver DNA was prepared from the residual chromatin and unfractionated prostate chromatin according to Norman & Bekhor (1981).

Results

Androgen Binding to the Residual Chromatin. Figure 1 shows binding of [³H]DHT to the residual chromatin as determined by the steroid exchange assay. Both normal and castrated preparations showed binding which was calculated as the difference betwen total and nonspecific bindings. Castration for 2 days reduced the specific binding to about 15% of the normal level. Scatchard plots (Figure 2) of the data yielded apparent K_d values of 1.5 and 3.1 nM, and specific DHT binding sites (n) of 6.3 and 1.1 nmol/mg of protein for normal and castrated residual chromatins, respectively. The specific binding of [³H]DHT to prostate residual chromatin was also evident by competition with nonradioactive DHT as compared to competitions with progesterone, estradiol, and cortisone (Figure 3). Table I illustrates the distribution of translocataed androgen bound to the four chromatin fractions prepared as described under Experimental Procedures. The data indicate that the residual chromatin is one of the major androgen binding sites, presumably the acceptor sites, of prostate chromatin.

Interaction of [³H]DHT–Receptor Complex with Solubilized Residual Protein. Since the residual chromatin contains

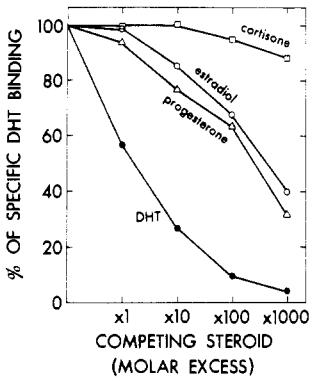

FIGURE 3: Inhibition of [^{3}H]DHT binding to rat prostate residual chromatin by competing steroids. The residual chromatin (50 μg of protein) was incubated with [^{3}H]DHT (10 nM) and nonradioactive DHT, progesterone, estradiol, and cortisone in various concentrations (10–10000 nM) as shown. Control androgen binding without addition of competitor steroid is set at 100%.

Table I: Distribution of [^{3}H]DHT in Chromatin Fractions of Rat Ventral Prostate[a]

fraction	rel concn[b]	[^{3}H]DHT bound cpm (×10^{-3})	[^{3}H]DHT bound %
0.35 M NaCl/solubilized NHCP	0.26	61.5	47.7
2 M NaCl/solubilized NHCP	0.37	22.3	17.3
DNA–histone	1.25	4.6	3.6
residual chromatin	0.10	40.4	31.4

[a] Translocation of [^{3}H]DHT–receptor to castrated rat prostate nuclei, isolation of the radioactive DHT–receptor-bound chromatin, and fractionation of the chromatin were done as described under Experimental Procedures. Radioactivity of the translocated [^{3}H]DHT = 230 × 10^3 cpm. [b] Relative concentration with respect to DNA where [DNA] = 1.

DNA as well as protein and since the androgen–receptor complex also binds to DNA, whether the acceptor is protein or DNA is not clear. To resolve this problem, the prostate residual chromatin was extracted with phenol as described under Experimental Procedures, yielding a phenol-soluble protein preparation free of nucleic acid. Initial experiments determining the acceptor activity of the solubilized residual protein by [^{3}H]DHT–receptor binding failed to demonstrate any interaction between the two. Addition of rat liver DNA to the acceptor assay system, however, resulted in increasing and saturable binding of [^{3}H]DHT–receptor complex in response to increasing amounts of the solubilized residual protein and in addition to the control counts due to binding of [^{3}H]DHT–receptor to DNA (Figure 4). In the presence of rat DNA, residual proteins similarly solubilized from chicken erythrocytes interacted negligibly with the [^{3}H]DHT–receptor complex, whereas rat liver residual protein showed low binding activity (about 20% of prostate binding activity). These and the previous data support that, in addition to the soluble acceptor (Tymoczko & Liao, 1971; Hiremath et al., 1980), the residual protein is a major acceptor fraction in the rat prostate.

DNA of the Residual Chromatin. The association of the residual protein with DNA and the DNA dependence of the solubilized residual acceptor in its interaction with the androgen–receptor complex suggest that the DNA may play a role in the acceptor's function. As one approach to evaluate this problem, we have examined the sequence specificity of the DNA in the prostate residual chromatin. Since PBP mRNAs represent the major androgen-regulatead mRNAs in rat prostate (Hiremath & Wang, 1981; Parker et al., 1980; Peeters et al., 1980), we have used cloned cDNAs coding for the three subunit components of PBP (Mpanias et al., 1983) as probes to reanneal with the prostate residual DNA and

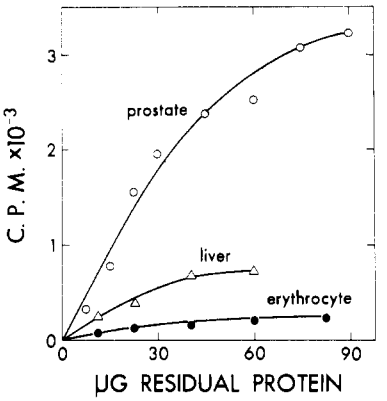

FIGURE 4: Interaction of solubilized residual protein with androgen–receptor complex. The phenol-solubilized residual protein from castrated rat prostate, in amounts as shown on the abscissa, was incubated with 0.6 mg of protein of a [^{3}H]DHT–receptor preparation (32000 cpm) and 200 μg of rat liver DNA, in 0.01 M NaCl/TEM. Incubation was at 4 °C for 30 min. Millipore filtration of the resulting complex and washing with 0.2 M NaCl/TEM were done as described under Experimental Procedures. For comparison, phenol-solubilized residual protein from rat liver and chicken erythrocyte were run in parallel in the presence of 200 μg of rat liver DNA. Control samples of the same 0.6 mg of [^{3}H]DHT–receptor complex and 200 μg of rat liver DNA without the acceptor gave 1127 cpm, and this value was subtracted from the experimental cpm.

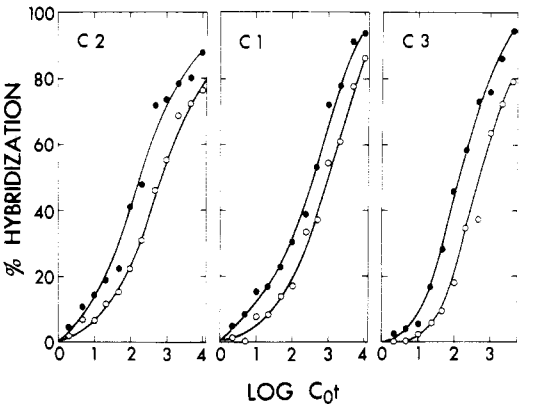

FIGURE 5: Reassociation kinetics of [^{3}H]cDNA with prostate residual DNA. Single-stranded cloned cDNAs coding for C1, C2, and C3 subunit components of PBP were annealed to prostate residual DNA (●) and total prostate DNA (○) as described elsewhere (Hiremath & Wang, 1981).

compared it with total prostate DNA. The hybridization data in Figure 5 show that the rates of reassociation of the cDNAs with the residual DNA were faster than those with total DNA by 0.4–0.6 log C_0t unit, equivalent to about a 3–4-fold increase in PBP gene sequences. The residual acceptor(s) thus appear(s) to be associated with androgen-regulated transcribing DNA sequences.

Discussion

In rat prostate, four high salt resistant nuclear or chromatin fractions which manifest acceptor activity have been described. These are the nuclear RNP particles (Liao et al., 1973), the nuclear matrix (Barrack & Coffey, 1980), the tightly bound chromosomal nucleoprotein (NP) fractions (Klyzsejko-Stefanowicz et al., 1976), and the residual chromatin (Wang, 1978). Comparison of these acceptor fractions suggests that they may not be as diversified as they appear. The nuclear RNP, for example, is derived from 1 M NaCl extracted nuclei which are then treated with DNase I and nonionic detergent, whereas in the preparation of the nuclear matrix, DNase I digestion of nuclei procedes the salt extraction. In the rat

prostate nuclear matrix, androgen binding to the internal RNP network accounts for 70–85% of the specific DHT binding sites (Barrack & Coffey, 1980). The acceptor activity of the RNP and the high-affinity androgen binding of the nuclear matrix are both strongly dependent on their RNA. The chromatin NP fraction, a DNA–protein complex, is the high salt resistant chromatin fraction solubilized by 2.5 M NaCl/5 M urea/0.05 M Tris-HCl, pH 8.0 (Chiu et al., 1975), and is likely a part of the residual chromatin. These characteristics suggest the possibility of two kinds of acceptors in the high salt resistant fraction of prostate nuclei, one predominantly localized in the RNP and the other in the tightly bound chromatin DNA–protein complex.

By definition, the acceptors must be tissue specific. Indeed, the liver nuclear matrix of diethylstilbesterol-treated chicks binds estradiol and not DHT, progesterone, or cortisol, while only prostate nuclear matrix contains specific androgen binding sites (Barrack & Coffey, 1980). The bound steroids apparently involve the steroid–receptor complexes (Buttyan et al., 1983; Rennie et al., 1983). Similarly, only prostate (and testis) NP fraction (Klyzsejko-Stefanowicz et al., 1976) and nuclear RNP (Liao et al., 1973) bind the [^{3}H]DHT–receptor complex. Another high salt resistant steroid acceptor, the progesterone AP3 fraction, which is obtained by solubilization of chromatin with 3 M NaCl/7.5 M urea/0.05 M Tris-HCl, pH 8.5, is oviduct specific (Spelsberg et al., 1972). The preferential binding of [^{3}H]DHT–receptor complex to prostate, but not to liver or chicken erythrocyte residual protein, as shown in the present work, is consistent with the concept of acceptor.

Unlike the saline-soluble acceptor whose activity is stimulated by, but not dependent on, DNA (Tymoczko & Liao, 1971; Hiremath et al., 1980), the interaction of the residual protein with androgen–receptor complex is DNA dependent. Why and how DNA is involved in this interaction are unknown. One possibility is that binding of residual protein to DNA may confer a certain conformation on the protein for interaction with the androgen–receptor complex. Since the androgen–receptor contains steroid as well as DNA binding sites (Mainwaring & Irving, 1973; Hu et al., 1978) and binds specifically to nuclear matrix DNA from rat prostate (Buttyan et al., 1983), it is also possible that both DNA and protein are required to provide binding sites for stable and specific interaction with the receptor complex.

The enrichment of active transcribing and cell-specific gene sequences in tightly protein-bound DNA has led Norman & Bekhor (1981) to suggest that the proteins of the high salt resistant NHCPs preferentially interact with active gene sequences and function as regulatory molecules in transcription. Kuo (1982), who observed a 3-fold enrichment of ovalbumin gene sequences in 2 M NaCl resistant protein-bound DNA in chicken oviduct, suggests that the tightly DNA-bound protein complex may represent transcription complexes containing factors engaged in transcription. In either case, the association of the residual acceptor with androgen-specific gene sequences suggests a possible function in transcription for the high salt resistant androgen acceptor.

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