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Demonstration of DNA binding factors interacting with a fragment of the canine prostate arginine esterase gene promoter

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We have studied, by the gel mobility shift assay, the interaction of DNA binding proteins with a fragment of the proximal promoter (from nucleotides −177 to −47) of the androgen-regulated canine prostate arginine esterase gene. Several shifted bands were obtained using nuclear extracts from various tissues. In the case of the prostate, the intensity of some of the shifted bands was decreased or increased when the extracts were prepared from animals that had been castrated 12 days earlier. Several of the DNA–protein complexes could be assigned to an interaction with part or all of the sequence GGGGGTGGGGG from −124 to −114. We also obtained evidence for the presence of protein(s) interacting with an Sp1 motif present in the same fragment. These results suggest that some ubiquitous factors different from the androgen receptors could be involved in the regulation of the arginine esterase gene.

DNA binding factor; Gene regulation; Prostatic secretory protein; Androgen action

1. INTRODUCTION

Our understanding of the mechanisms by which steroids exert their actions in various target tissues is increasing rapidly (reviewed in [1]). However, in the case of androgens the picture is very fragmentary and the existence of specific androgen response elements (ARE) is still uncertain. It is known that glucocorticoid response elements, such as those present in the MMTV long terminal repeat, can confer androgen responsiveness in vitro when coupled to the CAT reporter gene [2]. Similar elements are also present in androgen responsive genes, such as in the gene encoding the C3 subunit of the rat prostatic binding protein (PBP). However, when these and other promoter elements of subunits C1 and C2 of PBP genes were cloned upstream of the CAT reporter gene and transfected into androgen receptor containing cells, the androgen inducibility was shown to be very modest [3]. Among possible explanations for these results are that the transfected cells may lack some specific regulatory factors different from the androgen receptor, or that they may contain repressors acting on negative regulatory elements of the genes [4]. Furthermore, in some systems, such as the rabbit uteroglobin gene in which the role of progesterone receptor on transcription is well demonstrated [5], it has recently become evident that the hormonal activation of gene expression requires binding of additional proteins to proximal promoter elements [6]. In the case of the rat prostate C3 gene, it has been proposed that the CCAAT element could be involved in some way in transmitting an androgen response [7]. For these reasons we have decided to study the interaction of DNA binding proteins with a fragment (from nucleotides −177 to −47) of the canine prostate arginine esterase gene containing a potential CCAAT regulatory element [8]. At the same time we have looked at the effects of castration on the patterns of these binding proteins in prostatic nuclear extracts.

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2. MATERIALS AND METHODS

2.1. *Animals and treatments*

Adult mongrel dogs weighing 15–25 kg were purchased from local suppliers through the Laboratory Animal Service of Laval University in Quebec city. Some of the dogs were used without any treatment while other dogs were castrated under general anesthesia. The castrated animals were sacrificed 12 days after surgery. Immediately after sacrifice by intravenous injection of euthanyl the tissues to be studied were removed, cut into small pieces and quickly frozen in liquid nitrogen. The frozen tissues were kept at −80°C until needed.

2.2. *Preparation of nuclei and nuclear extracts*

Nuclei were prepared from the prostate and other tissues according to the procedure previously described by Donnelly et al. [9] and the nuclear extracts according to Englander and Wilson [10]. This procedure yielded 0.4–2 mg of protein per gm of tissue. Further purification of the nuclear extracts by heparin-Sepharose was found to be useless since it resulted in major losses and did not change the DNA binding patterns.

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2.3. *Preparation of the promoter fragment*

A 1.3 kb *Sph*I–*Kpn*I genomic DNA fragment of the arginine esterase gene was obtained from a recombinant subclone containing a 2.8 kb insert derived from λDAgE1 [8]. The 1.3 kb fragment was digested with *Sau*3A1 and the resulting digestion products were subcloned into the *Bam*HI site of the polylinker of pTZ18R. A subclone containing a 279 bp fragment was selected and its insert was sequenced. It corresponded to nucleotides −177 to +101 of the arginine esterase gene. An *Apa*I restriction site was present in the fragment at a position corresponding to position −47 of the gene. For further studies, the recombinant bearing the 279 bp fragment was digested with *Hin*dIII in the polylinker and was labeled at its 5'-end on the coding strand using [^{32}P]γ-ATP (5,000 Ci/mmol) (Amersham) and T-4 polynucleotide kinase (Gibco-BRL). After the labeling reaction a second digestion was performed with *Apa*I resulting in a fragment containing 131 bp of genomic sequences and 30 bp derived from the polylinker. The genomic sequences harboured a potential CCAAT element and a potential Sp1 binding site. That fragment was purified by polyacrylamide gel and Elutip-D filtration.

2.4. *Gel shift assays and methylation interference footprinting*

Binding reactions contained 2–3 ng of end-labeled DNA fragment (25,000 cpm), 5 μg of crude nuclear extract, 2 μg of poly dIdC (Pharmacia-LKB, Canada) in buffer (20 mM HEPES-NaOH pH 7.9, 50 mM KCl, 16 μg of BSA/ml, 12% glycerol and 0.1 mM dithiothreitol) and were conducted at 25°C for 15 min. Reaction mixtures were loaded on either a 6 or 8% polyacrylamide gel and run at 125 V for 6 h in Tris-glycine buffer (50 mM Tris, pH 8.5, 380 mM glycine and 2 mM EDTA). The gels were dried and the DNA–protein complexes detected by autoradiography. For competition experiments we used double-stranded oligonucleotides containing either the NF1-L binding site from the human retinol binding protein gene or the Sp1 high-affinity binding site from the chicken H5 histone promoter as previously described [4]. We also used 2 oligonucleotides derived from the sequence of the arginine esterase gene promoter [8]: the first one is G5TG5 = 5'-CCAATAGCCCTGGGGGTGGGGGCTGTCCAC-AC-3'; the second one (5'-CCCTCCAATAGCCC-3') contained only the potential CCAAT element in the absence of the G-rich sequence.

The methylation interference footprinting on specific bands eluted from gels of gel shift experiments was done as described previously [4].

3. RESULTS

We first studied the effect of incubating nuclear extracts prepared from different tissues of an adult male dog with the labeled fragment containing 131 bp from the arginine esterase gene promoter. Several DNA–protein complexes were observed with all the nuclear extracts examined (Fig. 1). All the shifted bands were abolished by an excess of cold fragments (results not shown). Some similarities and differences were apparent between the prostate and the other tissues. Very few (if any) major bands appeared to be prostate-specific. We next tested whether castration of the animals would have any effect on the band shift pattern generated by prostatic nuclear extracts (Fig. 2). Several changes could be noted. At least one major band (band 2) was absent in castrated animals while another (band 4) was present only in the 12 days-castrated animals. Two other bands (bands 1 and 3) were increased after castration. During that same period there were no major modifications in the proportion of stromal and epithelial cells although the prostatic acini presented evident signs of atrophy at histology (results not shown). Furthermore, the band shifts patterns with kidney and liver nuclear extracts were not changed after castration (results not shown).

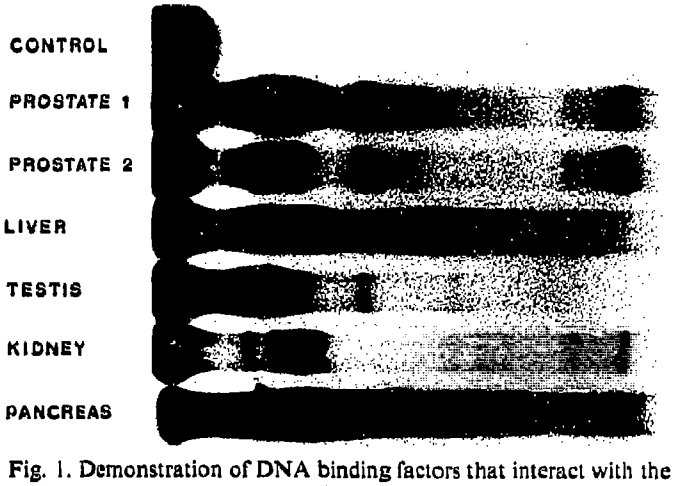

Fig. 1. Demonstration of DNA binding factors that interact with the arginine esterase gene promoter fragment −177/−47 by the gel mobility shift assay. The amount of protein used in each incubation was 5 μg with the exception of the control lane which contained only the radioactive fragment.

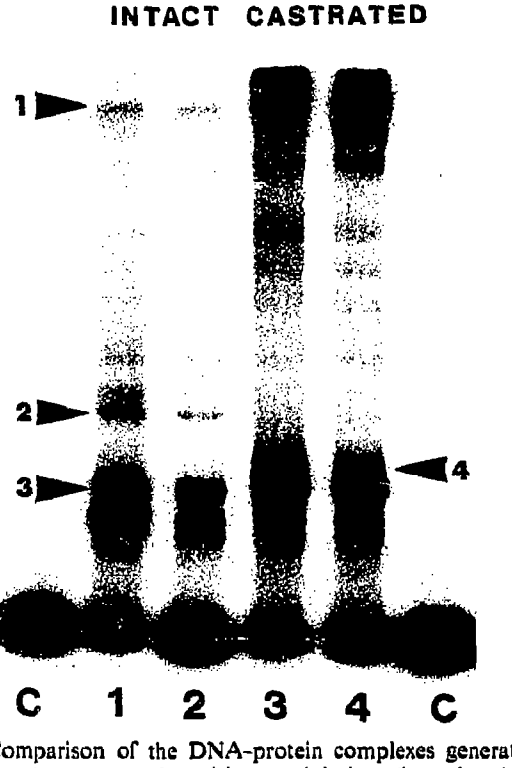

Fig. 2. Comparison of the DNA-protein complexes generated by prostatic nuclear extracts from 2 intact adult dogs (lanes 1 and 2) and from 2 dogs that had been castrated 12 days before sacrifice (lanes 3 and 4). The arrowheads (numbered 1 to 4) indicate some major complexes whose concentrations were consistently modified by castration.

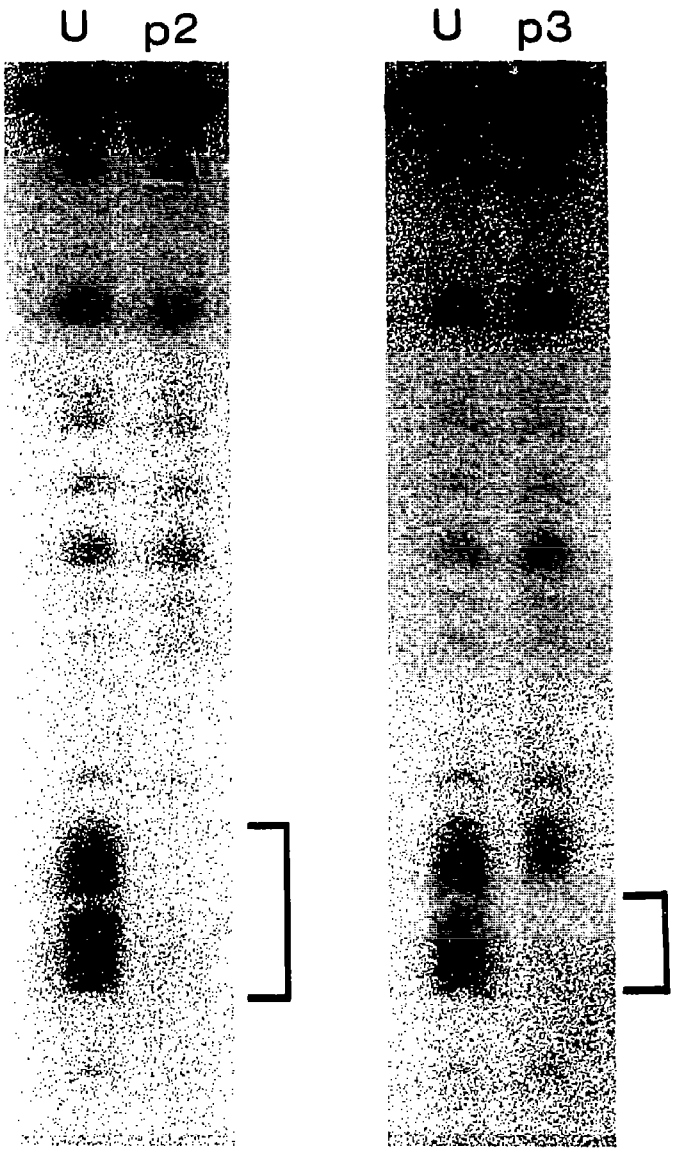

Fig. 3. Methylation interference footprinting of prostatic complexes 2 and 3. The double-stranded promoter fragment was methylated using dimethylsulfate and used for the gel shift procedure. The specifically retarded complexes 2 and 3 were eluted, treated with piperidine and loaded on an 8% polyacrylamide sequencing gel. U, unbound probe; p2, prostatic complex 2; p3, prostatic complex 3. The brackets indicate the position of the protected G's along the labeled fragment.

In the next experiment we used DMS-methylation interference footprinting to determine the precise DNA binding site for the prostatic complexes 2 and 3 which responded to castration in an opposite fashion. Complex 2 produced clear footprints at the sequence GGGGGTGGGGG from −124 to −114, while complex 3 reacted with only part of the same sequence: GGGGGTG (Fig. 3). Because of these results we then performed competition experiments in order to determine how many of the shifted bands were due to this sequence. Surprisingly, the oligonucleotide designated as G5TG5 abolished almost all of the DNA–protein complexes generated with prostatic nuclear extracts both from intact and castrated animals (Fig. 4). One of the slow migrating bands (complex 1) may be due to the interaction of transcription factor Sp1 since an oligonucleotide containing an Sp1-binding site preferentially abolished this band. Two other synthetic oligonucleotides containing either a CCAATT or an NF1-L sequence were found not to compete for binding at 100-fold excess (results not shown).

4. DISCUSSION

The results of our study suggest that there are probably several DNA binding proteins that interact with the −177/−47 promoter region for the arginine esterase gene. Most of these proteins appear to interact with G-rich sequences either of the classical Sp1 type or of G_nTG_n type. At present the exact DNA sequence with which these proteins interact has not been determined for all the DNA–protein complexes detected in gel shift assays. Furthermore, it is not known whether the different bands are really due to distinct proteins or just to different oligomers or isoforms of a few proteins which have been post-translationally modified. Contrary to the results obtained with the rat prostate [7], we have found no evidence for the presence of CCAAT binding proteins interacting with the canine arginine esterase gene promoter, despite the presence of such a CCAAT sequence. While it is possible that these specific factors have been lost during the preparation of our nuclear extracts these results could also be caused by their much lower abundance in the canine prostate.

The presence of DNA binding proteins interacting specifically with proximal promoter sequences is generally associated with regulatory function in gene transcription. More definitive conclusions on this matter must await the development of a system for transfection studies or for in vitro transcription studies because our experiments have been conducted with nuclei prepared from whole tissues and thus from mixed cell populations (though predominantly epithelial) [11]. However, the demonstration of castration-induced changes in the patterns of DNA–proteins interacting with the arginine esterase gene promoter suggests that these interactions are physiologically significant. Most interestingly, the potential androgenic regulation of Sp1 and/or Sp1-like factors has not been described previously. A role for these factors would not exclude the possibility of a direct intervention of androgen receptors in gene regulation, since a putative ARE has been found further upstream of the fragment used in this study [8].

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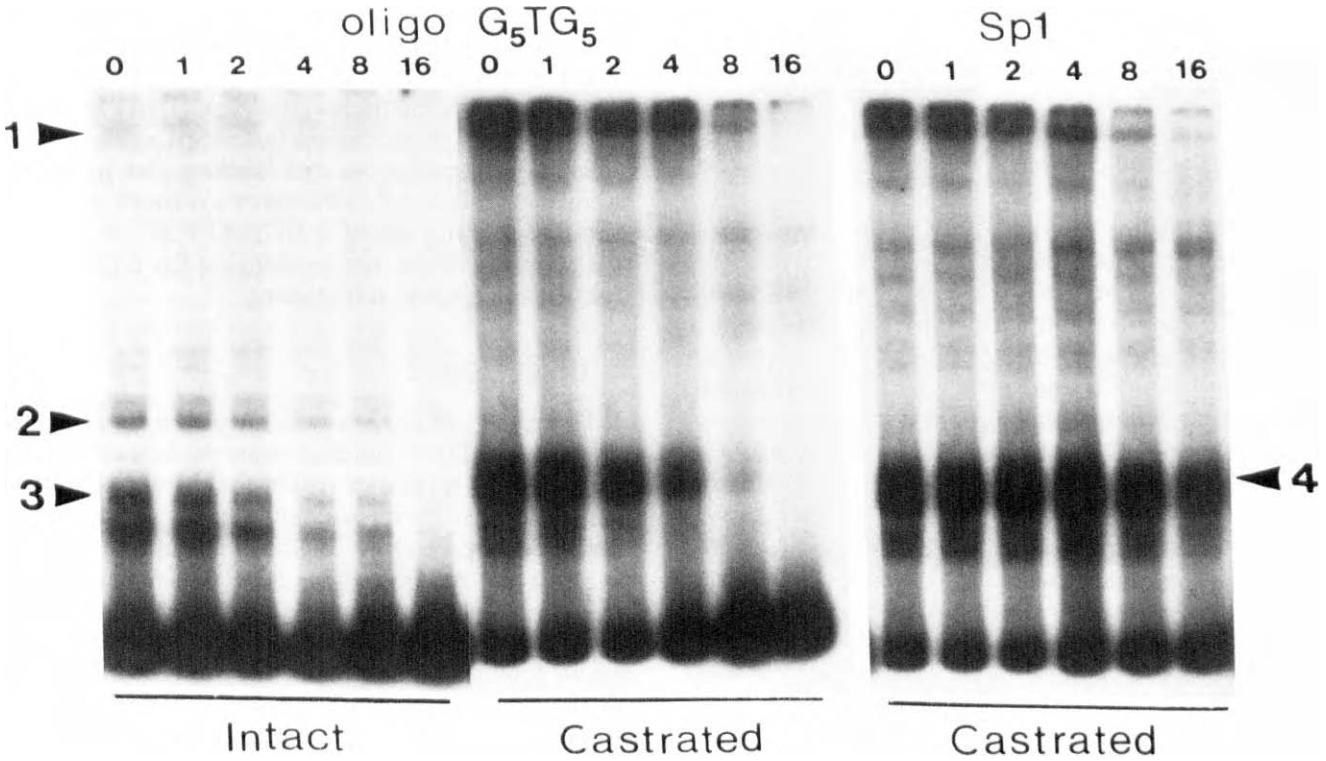

Fig. 4. Competition of DNA–protein complexes with double-stranded synthetic oligonucleotide containing either the G-rich sequence (G5TG5) or the histone H5 high-affinity Sp1 site. The cold competitor oligonucleotides were added in the binding reaction at 0 to 16-fold molar excess. Prostatic nuclear extracts were prepared from an intact dog and from a dog castrated 12 days before.

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