

## IDENTIFICATION OF AN ANDROGEN-INDUCED C3-P4 DNA BINDING PROTEIN IN THE CYTOSOL OF RAT VENTRAL PROSTATE

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**Summary.** A DNA binding protein C3-P4 was detected in the rat ventral prostate cytosol by gel retardation assay using a <sup>32</sup>P-labeled 31 base pair synthetic oligomers (sequence deduced from the rat prostatic steroid binding protein C3-1 gene promoter -149 to -119) as a probe. The DNA binding activity of C3-P4 DNA binding protein is sequence specific, with preference for single strand and coding strand exclusive. This protein can be detected in many androgen target tissues and controlled well by androgen in the rat ventral prostate. We speculate that this DNA binding protein may function as an accessory factor to androgen receptor (AR) for the regulation of the C3-1 gene expression. © 1993 Academic Press, Inc.

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C3 is one of the androgen target genes and represents about 30% of total proteins in the rat ventral prostate (1). Structural analysis of C3-1 gene indicated that the 5' flanking region of C3-1 gene contains the TATA and CAAT box (2,3). Recent experimental evidences revealed that androgen response element (ARE) was located in the first intron of C3-1 genes (4). The mechanism by which androgen receptor (AR) interacts with the general transcriptional factors to enhance the synthesis of C3-1 protein is still unclear. In addition to ARE, the requirement of extra DNA sequence for the specific gene expression has been demonstrated in the synthesis of sex limited protein (5). It also has been proposed that the presence of other factors which can bind to the specific DNA sequence to cooperate with AR may contribute to the expression of androgen target genes (5). During the search for AR accessory factors in the regulation of C3-1 gene expression, we were able to use gel retardation assay to observe a prostatic cytosol protein which can bind to a specific <sup>32</sup>P-labeled 31 bp DNA fragment in the C3-1 gene promoter (-149 to -119). The binding characterization of this protein, named as C3-P4, was followed and the potential significance of this finding was discussed.

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**Abbreviations:** AR, androgen receptor; ARE, androgen response element; HRE, hormone response element; GRE, glucocorticoid response element; PRE, progesterone response element; MRE, mineralocorticoid response element; PSBP, prostatic steroid binding protein; MMTV, mouse mammary tumor virus; ERE, estrogen response element; DRE, vitamin D response element; TRE, thyroid hormone response element.

## MATERIALS AND METHODS

**Animals:** Male retired Sprague-Dawley rats were from the King Animal Laboratories (Oregon). Castration was operated by excision of both lobes of the testis. Androgen administration was made by injecting subcutaneously testosterone at a dose of 5 µg/g body weight/day.

**DNA oligomers & probes:** The oligonucleotide used for protein binding assay was 31 base pair DNA, derived from the -149 to -119 DNA fragment of the C3-1 gene (referred to as C3-P4). The oligonucleotides used for competitors in the competition assay were hormone response elements (HRE) of mouse mammary tumor virus (MMTV) (6), glucocorticoid response element (GRE) of rat tyrosine amino transferase gene (7), estrogen response element (ERE) of vitellogenin gene (8,9), vitamin D response element (DRE) of osteocalcin gene (10), thyroid hormone response element (TRE) of rat growth hormone gene (11), TATA of rat PSBP C3-2 gene (1), CCAAT of rat PSBP C3-1 gene (2), and C3-P4 (2,3). All oligonucleotides were synthesized by DNA synthesizer. The sequence of each oligonucleotide was shown in Table 1. All the synthetic oligomers were purified on the native polyacrylamide gel electrophoresis (PAGE) and annealed with their corresponding strand. Single strand oligomers were <sup>32</sup>P-5'-end labeled by  $\gamma^{32}$ P-ATP with T4-nucleotide kinase (Pharmacia) resulting in a single strand probe with a specific activity around 10,000 cpm/ 50 pg. The <sup>32</sup>P-labeled single strand probes were then annealed with hot or cold opposite strand resulting in double strand probes with both 5'-end labeled or only one strand 5'-end labeled.

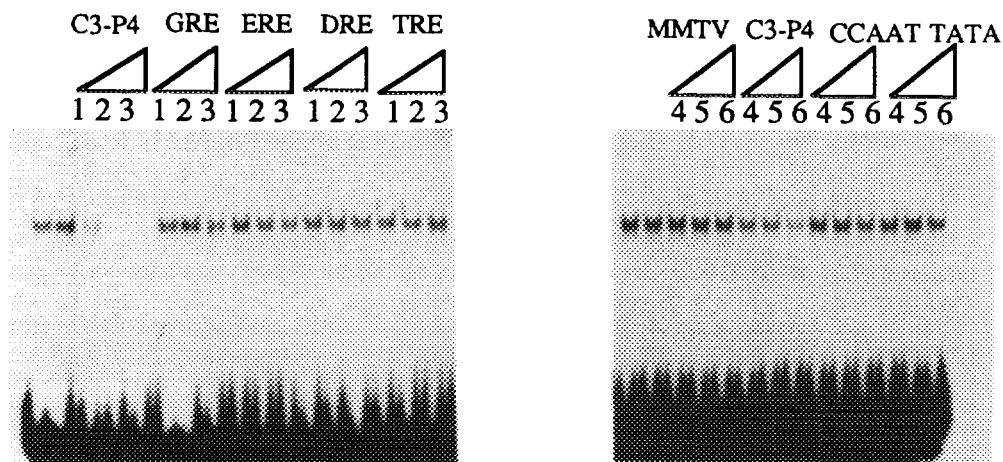
**Cytosol preparation:** Various fresh tissues were collected from sacrificed animals. Human normal and cancer prostate samples were from prostate sample bank in University of Wisconsin-Madison. Washed tissue was minced, homogenized in the sodium phosphate buffer (25 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.2, 1.5 mM EDTA, 2 mM DTT, 10% Glycerol, 10 mM NaF, 10 mM Na<sub>2</sub>MoO<sub>4</sub>, 1 mM PMSF, 0.1 mM Bacitracin (Sigma), 1 µg/ml aprotinin (Sigma)) by polytron. After centrifugation at 105,000g for 30 min at 4° C, the supernatant regarded as cytosol was aliquoted and stored at -70° C. The protein concentration of the cytosol was determined by the Bradford assay with bovine serum albumin as a standard (12).

**Nuclear extract preparation:** Preparation of the nuclear extracts from rat tissue was performed by using the method of Gorski et. al. (13) and as described previously (3).

**Gel retardation assay:** 10 µg-100 µg proteins of cytosol or 20 µg proteins of nuclear extracts were incubated with 1- 10 µg of polydI/dC (pharmacia) as non-specific DNA competitor and 10,000 cpm/50 µg of probe in a total volume of 20 µl binding reaction buffer (25 mM Hepes pH 7.9, 1 mM EDTA, 5 mM DTT, 10% Glycerol, 150 mM NaCl) at room temperature for 30 min. The binding mixture was loaded on 6% PAGE, separated in 0.5x Tris-boric acid-EDTA buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) at 10 V/ cm for 2 h. Then, the gel was fixed in 10% methanol, 10% glacial acetic acid, dried in vacuum and exposed to x-ray film with intensified screen overnight at -70° C.

## RESULTS AND DISCUSSION

**A DNA sequence-specific binding protein in the rat ventral prostate:** A DNA-protein complex was detected (Fig. 1) by native PAGE when a cytosol from rat ventral prostate was incubated with a <sup>32</sup>P-labeled synthetic 31 bp oligonucleotides (C3-P4). As shown in Fig.1, while cold C3-P4 oligomer can compete well with this DNA-protein complex, other DNA oligomers from different hormone response elements (HRE) and other oligonucleotides corresponding to different regions of the C3 gene promoter (shown in the Table 1) used as DNA competitors were unable to compete the binding. These results indicated that the binding reaction was highly sequence-specific. In addition to the sequence-specific, C3-P4 DNA-binding protein showed stronger binding affinity to the single strand DNA than to the double strand DNA. When double strand C3-P4 probes were denatured by

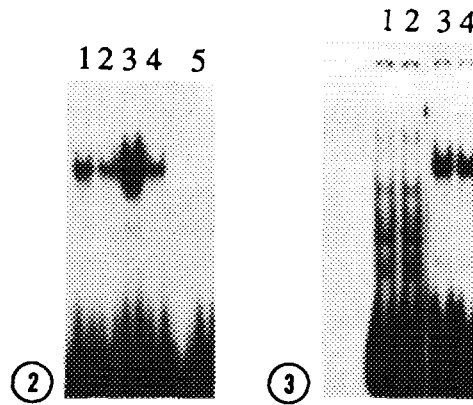


**Fig. 1.** Gel retardation analysis of binding proteins on the C3-P4 DNA probe and the competition of the binding with different HREs and oligomers from other regions of the C3 gene promoter. Each lane contained 10  $\mu$ g of cytosol of rat ventral prostate, 20,000 cpm/25  $\mu$ g of  $^{32}$ P-C3-P4 DNA probe, 1  $\mu$ g of poly dl/dC, with and without different cold DNA competitors as indicated. The wedge indicated an increasing amount (1, 2 and 3 represented 50x, 500x and 1,000x folds molar excess of each HREs and 4, 5 and 6 represented 25x, 50x and 100x folds molar excess of each DNA for HRE of MMTV, C3-P4, CCAAT and TATA, respectively).

heat at 94°C for 3 mins and treated either by cooling down immediately in the ice or reannealing in the gradually cooling condition, the former (Fig. 2, lane 1) showed stronger DNA binding activity than the latter (Fig. 2, lane 2) in the gel retardation assay. To further examine if C3-P4 DNA binding protein has preference in the binding to the sense or antisense DNA of C3-P4, 5'-end labeled single sense strand and antisense strand of C3-P4 were used

Table 1. Sequences of the synthetic oligonucleotides used in the competition assay

HRE OF MMTV	5'-TATGGTTACAACTGTTCTTAAAACGA-3'
GRE	5'-CTGTACAGGATGTTCTAGCTAGC-3'
ERE	5'-GGTCACAGTGACC-3'
DRE	5'-GGTGACTCACCGGGTGAACG-3'
TRE	5'-TCAGGTCATGACCTGA-3'
TATA	5'-TAAGGTGATTGCCTGAGCAATAAATAGAGGAA CACTGAGGT-3'
CCAAT	5'-GACTTGGGTTCTTATTTATTGGGCAAGACT-3'
C3-P4	5'-TGAAACCAGTGTCTTTGGCTCTTCTTCGCC-3'

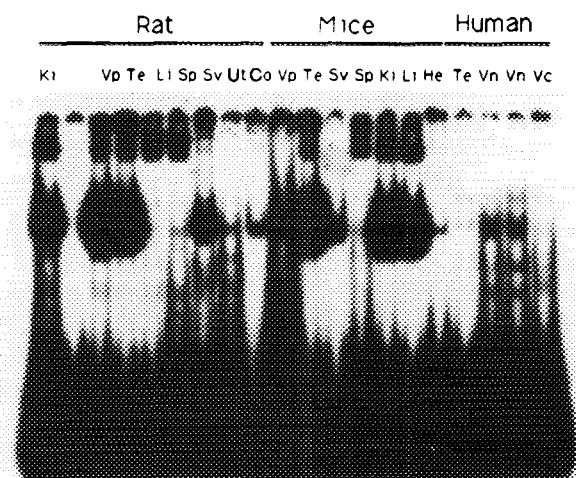


**Fig. 2.** The single strand preference and coding strand exclusive phenomenon in the DNA binding reaction. Each lane contained 10  $\mu$ g of total cell lysates of rat ventral prostate, 1  $\mu$ g polydI/dC and 10,000 cpm/12.5  $\mu$ g of C3-P4 DNA probes in different features. Lane 1 and lane 2 represented double strand DNA labeled by  $^{32}$ P on both 5'-end but heat denatured for lane 1. Lane 3 and lane 4 represented single strand and double strand probe, respectively, and both strands were labeled by  $^{32}$ P on 5'-end of coding strand only. Lane 5 represented single non-coding strand probe end-labeled by  $^{32}$ P on the 5'-end.

**Fig. 3.** Gel retardation assay of the  $^{32}$ P-C3-P4 probe bound with proteins in cytosol and nuclear extracts of the rat ventral prostate. Each lane contained 20,000 cpm/25  $\mu$ g of  $^{32}$ P-C3-P4 probe, 5  $\mu$ g of polydI/dC and 20  $\mu$ g of nuclear extracts for lanes 1 and 2 as duplicate or 20  $\mu$ g of cytosol for lane 3 and lane 4 as duplicate.

as probe separately in the gel retardation assay and the high DNA binding activities were shown in the sense strand (Fig. 2, lane 3) but not the antisense strand (Fig. 2, lane 5). When 5'-end labeled sense strand was then annealed with cold antisense strand to form double strand, the binding activity was lower than the single sense strand only (Fig. 2, lane 4) after normalization in the same specific activity as the above assay. These results showed convincingly that the binding has single strand preference and also sense strand exclusive.

**The distribution of C3-P4 DNA binding protein:** Generally, most of the DNA binding proteins are in the nuclei functioning as trans-acting factors. However, we could only identify C3-P4 DNA binding protein from the cytosol and failed to find this DNA binding protein in the nuclear extracts from rat ventral prostate using double strand as probe (Fig. 3). Interestingly, the distribution of C3-P4 DNA binding protein was confined to the most of androgen target tissues (Fig. 4). The amount varied with different tissues, being abundant in the ventral prostate and testis, lower in kidney, seminal vesicle, coagulating gland, and very little in liver, spleen and uterus. To ask the species specificity of C3-P4 DNA binding protein, different tissues from mice and human have been tested. The results showed that there was no species difference in C3-P4 DNA binding protein distribution with only slight variations in the amount detected in different species, for example, abundant in the mouse liver and less in the human prostate. It is noted that while C3-P4 DNA binding protein can be found in normal human prostate, it was undetectable in sample from human prostate cancer (Fig. 4) and cell



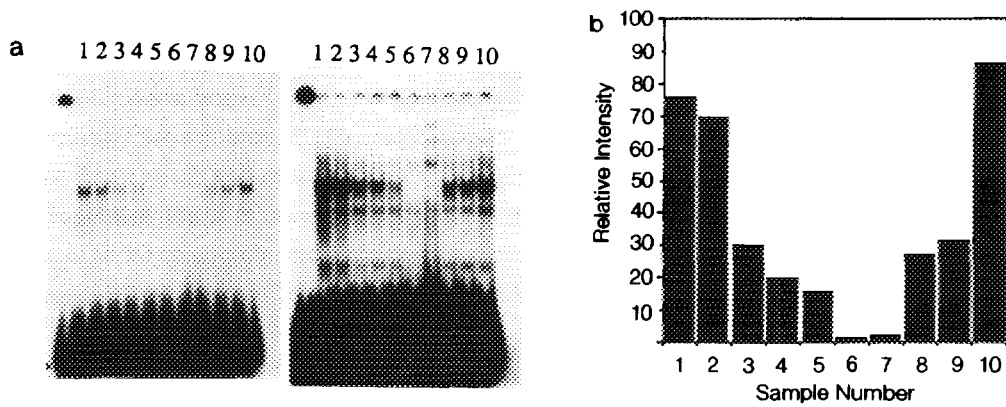
**Fig. 4.** The distribution of the protein bound on the  $^{32}\text{P}$ -C3-P4 probe in different tissues of rat, mice and human. Each lane contained 10,000 cpm/ 12.5  $\mu\text{g}$  of probe (single coding strand), 5  $\mu\text{g}$  polydI/ dC and 20  $\mu\text{g}$  of cytosol from different sources as indicated. (Ki=Kidney, Vp= Ventral prostate, Te=Testis, Li=Liver, Sp=Spleen, Sv=Seminal vesical, Ut=Uterus, Co=Coagulating gland, He=Heart, Vn=Human normal prostate, Vc=Human prostate cancer.)

lines from human prostate cancers (Data not shown). We are in the process to further confirm the possibility of differential expression of C3-P4 DNA binding protein in the different stages of prostate cancers.

**Androgen regulation of C3-P4 DNA binding protein synthesis in the rat ventral prostate:** Since C3-P4 DNA binding protein was found in most of androgen target tissues, the effects of androgen on this protein synthesis was investigated by castration and administration of exogenous testosterone to the rats after castration. The results revealed that the amounts of C3-P4 DNA binding protein in the rat ventral prostate was decreased gradually after castration and became undetectable at 7 days after castration (Fig. 5). However, it could be restored by a single injection of testosterone at dose of 5  $\mu\text{g}/\text{g}$  body weight/day and reached to the normal level at 6 days after injection (Fig. 5).

Like other known AREs, the C3-ARE found in the first intron of C3 gene (4) can also be competed by GRE/PRE of rat tyrosine amino transferase (14) and share the same consensus sequence with the GRE and PRE (4,15,16). This raises a very interesting question to ask why androgen, but not glucocorticoid and progesterone, can specifically induce the C3 gene *in vivo* ?

Four possible mechanisms may explain the specificity of hormone action : (i) A given target cell might express only one of the closely related receptors; (ii) a given target cell might inactivate one of the hormonal ligands; (iii) sequences distinct from the common DNA element might exist and this sequence determines the transcriptional specificity ; (iv) the receptors



**Fig. 5.** Effects of testosterone on the protein bound on the C3-P4 in the rat ventral prostate. **a.** Each lane contained 20,000 cpm / 55  $\mu$ g of C3-P4 single coding strand probe, 1  $\mu$ g of polydI/dC and 10  $\mu$ g of cytosol from rat ventral prostate in different hormone status (lanes 1 and 2 from normal ventral prostate, lanes 3, 4 and 5 from castrated 1 day, 2 day and 3 day, respectively, lanes 6 and 7 from castrated 7 days, lanes 8, 9 and 10 from castrated 7 day rat administrated testosterone 1 day, 2 days and 6 days, respectively.) Left Fig. for short exposure. Right Fig. for long exposure. **b.** Relative intensity measured by densitometry scanning.

might interact differentially with other factors (17). The first two can not exclude out one argument that only one receptor being activated can turn on all the genes having common response element in the promoter region. In support of the third explanation, one case has demonstrated that the presence of another cis-element in addition to ARE is essential for androgen specific action (5). This result further suggests that another trans-factors may bind to this cis-element to cooperate with AR for determining precise gene activation. As described in the fourth hypothesis, it means that a complex formed by the interaction between DNA-protein or protein-protein may determine the androgen specificity.

As we known, the binding to the specific HRE by steroid receptors may come to nanomolar affinity with a specificity of less than 1000 fold to nonspecific DNA (18; Wang, *et al.*, unpublished data). Compared to the some procaryotic DNA binding protein, such as lambda repressor binding to specific operator sequences with affinity as high as  $10^{-13}$  M and binding to specific DNA 500,000-fold better than to nonspecific DNA (19), AR show only to be a modest degree of specificity and affinity in their interaction with specific DNA. However, the overall specificity of transcription complex may be much higher with the cooperative and multiple protein-DNA and protein-protein interactions (20). The search for such proteins may be an important step to further study the specificity of androgen action.

Based on (a) C3-P4 DNA binding protein can bind tightly to the DNA sequence in the C3 gene promoter region, (b) expression of this protein is tightly controlled by the androgen and (c) C3-P4 DNA binding protein was found in most of androgen target tissues but not in human prostate tumor cells whose growth is not under androgen control. It is possible that

this DNA-protein binding effect may involve in the transactivation of C3 gene expression by cooperation with AR. From our gel retardation results, the DNA binding activity of C3-P4 DNA binding protein is sequence specific, with preference for single strand and coding strand exclusive. It is likely that the binding to the sense strand of C3 promoter can stabilize the DNA unwinding condition which is induced by general transcription factors and AR to enhance the transcription activation. Further cloning by southwestern screen technique and characterization of C3-P4 DNA binding protein may provide more information for us to know if the binding of this protein to DNA may enhance the specificity of androgen on the induction of C3-1 and other androgen target genes.

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