Identification of a nuclear protein interacting with a novel site on rat androgen receptor promoter after transcription factor NFkB is displaced from adjacent site

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Received 2 August 2002; accepted 19 November 2002

Key words: androgen receptor gene, DNA-protein interaction, FRN, gene expression, NFkB

Abstract
Sequence-specific DNA-protein interactions mediate the regulation of rat androgen receptor (rAR) gene expression. Previously, DNase I footprinting revealed that nuclear factor kappa B (NFkB) binds to region -574 to -554 on rAR promoter and represses its expression. In this study, we demonstrate that when NFkB protein is removed from its site by competitor DNA in DNase I footprinting reaction, a new DNase I protected region is formed overlapping adjacently (-594 to -561). This indicates that another nuclear protein (named here as FRN, factor repressed by NFkB) binds to rAR promoter only after NFkB protein is displaced. By competitive electrophoretic mobility shift assay and mutation analysis, we confirmed the formation of FRN-DNA complex. FRN interacts with a novel sequence on rAR promoter and may play a role in regulation of rAR gene expression in concert with NFkB.

Abbreviations: EMSA – electrophoretic mobility shift assay; FRN – factor repressed by NFkB; NFkB – nuclear factor kappa B; rAR – rat androgen receptor.

Introduction
The androgen receptor (AR) is a ligand-activated transcription factor belonging to the steroid/thyroid hormone receptor superfamily [1, 2]. AR plays a central role in the coordination of the male specific sexual phenotype and in the development of the male reproductive organs [3]. It also plays role in prostatic hyperplasia and carcinogenesis [4, 5]. Hepatic androgen responsiveness declines during aging in rat and it is correlated with decrease of AR expression by almost 100-fold from adult to old [6, 7]. NFkB is a cytokine-inducible transcription factor that plays a key role in the expression of variety of genes involved in inflammatory responses and cell survival [8, 9]. NFkB is composed of homo- and heterodimeric complexes of members of Rel family of proteins, consisting of p65 (Rel A), c-Rel, Rel B, p50 and p52 and most abundant of these complexes is the p50/p65 heterodimer. Mostly NFkB exists in an inactive form in the cytoplasm, bound to an inhibitory protein, IκB. Phosphorylation of the inhibitor results in ubiquitination and degradation of the inhibitor and translocation of active p50/p65 to the nucleus, followed by a specific regulation of gene expression [10].

Earlier, Supakar et al. have reported that NFkB binds to rAR promoter at region spanning -574 to -554 and functions as negative regulator of rAR gene expression [11]. Contribution of NFkB to the age-dependent down-regulation of the rAR gene expression is indicated by the observation that a 10-fold increase of NFkB DNA binding activity occurs in old rats [11]. In this report, we demonstrate by DNase I footprinting and electrophoretic mobility shift assay (EMSA) that NFkB binds to -574 to -554 on rAR promoter and when NFkB is removed from its site by using excess of NFkB competitor DNA, a new adjacent protected footprint appears in the region -594 to -561. This indicates that in the absence
Table 1. Formation of FRN and NFκB complexes with wild type, deletion and site-directed mutant oligonucleotide duplexes by electrophoretic mobility shift assay

<table>
<thead>
<tr>
<th>Oligonucleotide duplexes used in electrophoretic mobility shift assay</th>
<th>Formation of NFκB complex</th>
<th>Formation of FRN complex</th>
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<tbody>
<tr>
<td>FRN</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>FRN-ΔNFκB</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>ΔFRN-NFκB</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>FRN-mNFκB</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>mFRN-NFκB</td>
<td>+</td>
<td>−</td>
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Materials and methods

Preparation of nuclear extract

Fischer 344 rats were obtained from National Institute of Nutrition, Hyderabad, India. All animal care was approved by institutional animal ethics committee. Liver nuclear extracts were prepared from 4–6 rats (20–22 months old) using the procedure of Hattori et al. [12]. Briefly, liver homogenate was centrifuged through a 2.2 M sucrose cushion to obtain the purified nuclei. Nuclear pellets were resuspended and lysed in a buffer containing 10 mM Hepes (pH 7.6), 10% glycerol, 100 mM KCl, 3 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT and the nuclear proteins were extracted with addition of 0.4 M ammonium sulfate. Extracts were resuspended and precipitated proteins were extensively dialyzed in 20 mM Hepes (pH 7.6), 0.1 M KCl, 0.2 mM EDTA, 2 mM DTT, 20% glycerol and 1 mM NaMoO₄. The dialysate was centrifuged and flash frozen in liquid nitrogen. All buffers contained 2 µg/ml each of protease inhibitors (aprotinin, leupeptin, bestatin) and 0.1 mM phenylmethylsulfonyl fluoride which were added just prior to use. All manipulations were carried out at 4 °C. Protein concentrations were determined by the Bio-Rad protein reagent [13].

DNase I footprinting

A radiolabeled 262 bp DNA fragment was generated by PCR using template DNA and two primers (³²P-labeled 5′ primer at -736 and unlabeled 3′ primer at -475) and was gel purified. Either rat liver nuclear extract (50 µg) or recombinant NFκB subunit p50 (4 U, Promega, USA) were preincubated in binding reaction mixture containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol and 0.2–2 µg poly(dI-dC). After 5 min at room temperature, 2 ng (50,000 cpm) of radiolabeled DNA fragment was added and incubated for 30 min further. Subsequent to the binding reaction, 7.5 mM MgCl₂ and 5 mM CaCl₂ were added, and DNA digestion was carried out with DNase I (0.025 U for control and p50 reactions, and 0.25 U for reactions with nuclear extract). Nuclease digestion was stopped by the addition of an equal volume of 10% SDS, 20 mM EDTA, 200 mM NaCl, 100 µg/ml yeast tRNA and 200 µg/ml proteinase K. Samples were incubated at 45 °C for 60 min and extracted twice with phenol/ chloroform, precipitated with ethanol. Digestion pattern was analyzed