

Transcriptional Regulation of the Murine Dopamine Receptor Regulating Factor (DRRF) Gene

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The murine dopamine receptor regulating factor (DRRF) gene is transcribed from a TATA-less promoter that has several putative Sp1 binding sites. The present investigation identifies functional transcription factors that modulate the expression of this gene. In the D₂-expressing NB41A3 cells, Sp1 potently activates transcription from the DRRF promoter in pCAT-DRRF-1153/+17, but DRRF effectively inhibits it. Deletion of the 31 bp fragment between -1153 and -1122 decreased transcription down to about 60%. This fragment contains a functional AP1 binding site. In addition, deletion of the 129 bp region between -901 and -772 further decreased transcription. The latter region has a functional AP2 binding site. Using a DRRF_AP1 (bases -1153 to -1121) probe, a specific retarded band was observed, and the unlabeled AP1 consensus competitor could effectively compete away this retarded band. In addition, using a DRRF_AP2 (bases -873 to -846), a specific retarded band was observed, and the unlabeled AP2 consensus competitor could effectively compete away this retarded band. The present observations suggest that Sp1 and DRRF regulate the DRRF promoter and that both AP1 and AP2 also modulate this gene.

Key words – dopamine receptor regulating factor (DRRF), transcription factor, Sp1, AP1, AP2, gel shift assay

Transcription of genes in eukaryotes is regulated by several transcription factors that bind to distinct DNA sites. Although numerous short DNA sequences are known to be specific binding sites for transcription factors, all these factors contain one of three DNA binding motifs: zinc finger, helix-turn-helix and leucine zipper motifs[3].

The murine dopamine receptor regulating factor (DRRF) gene was cloned in the course of investigation about transcriptional regulation of the D₂ dopamine receptor gene promoter[11]. DRRF is an Sp1-like zinc finger transcription factor that has ability to regulate the D₂ as well as D_{1A} and D₃ dopamine receptor genes[5]. DRRF is alternatively designated as the basic transcription element-binding protein (BTEB)[7]. BTEB binds to the basic transcription element (BTE) and controls expression of target genes, such as the cytochrome P501A1 (CYP1A1) gene[7,8], the SM22 α gene [10], the uteroferrin gene[13], and the AP-2 α gene[6]. BTEB1 is present in most tissues, and the level of BTEB1 expression correlates inversely with that of Sp1[7]. Like Sp1, BTEB1 activates transcription from GC boxes in tandem repeats. However, in contrast to Sp1, BTEB1 could not activate against single GC boxes, and it may even repress

the activity of Sp1 at such sites[7]. DRRF is the orthologue of BTEB4. BTEB4 is a novel ubiquitously expressed member of the Sp1-like proteins family. DRRF represents a new homologue of BTEB1, originally described as a regulator of the BTE site in the CYP1A1 gene promoter[8].

Previously, we cloned and sequenced the murine DRRF genomic DNA and analyzed its regulatory 5' flanking region[9]. The promoter region of the DRRF gene lacks a TATA box and CAAT box, is rich in G+C content, and has multiple putative binding sites for the transcription factor Sp1. Transient expression assays of the DRRF gene suggested the presence of positive regulators between -1153 and -901, and between -118 and -93 while a negative regulator was found in the region between -901 and -118.

In the present investigation, we analyzed the regulatory regions of the DRRF gene in detail and examined the activity of known transcription factors on this promoter.

Materials and Methods

Construction of Plasmids

For functional analysis, the mammalian expression plasmid pcDNA-Sp1 was constructed by inserting the 3.0-kb *EcoRI-PstI* fragment of the coding region from pRmSp1 in the same sites of pcDNA3.1 (+) (Invitrogen, USA)[14]. The

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mammalian expression plasmid pcDNA- DRRF was constructed by inserting the 770-bp *EcoRI-XbaI* fragment of the coding region from pUC-DRRF in the same sites of pcDNA1.1/amp (Invitrogen, USA)[5].

Plasmid pCAT-DRRF-1153/+17 was described previously [9]. Three additional deletion mutants of the DRRF gene were made. To construct pCAT-DRRF-1122/+17, an 1140 bp fragment of the DRRF gene was generated by PCR using pCAT-DRRF-1503/+17[9] as template with sense primer DRRF-1122-F and antisense primer DRRF+17-R (Table 1). The resultant fragment was digested with *PstI* and *SalI* and inserted into the corresponding sites of pCAT-Basic. A similar strategy was employed to construct pCAT-DRRF-772/+17, or pCAT-DRRF-692/+17 with corresponding primers shown in Table 1. The integrity of all CAT constructs was verified by restriction analysis and partial sequencing.

Cell Culture and Transient Expression Assays

The murine neuroblastoma NB41A3 cell line was obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, USA) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker) at 37°C in a humidified atmosphere containing 5% CO₂. Transfections were carried out using Lipofectin plus reagent (Invitrogen) with serum free DMEM in 60 mM dishes. Three µg of pCAT plasmid and 1.5 µg of pCMV β plasmid (Clontech, USA) were used for transfections. For competitive co-transfections, NB41A3 cells were co-transfected with 3 µg of pCAT plasmid, 1.5 µg of pCMV β plasmid (Clontech), and various amounts of Sp1 (pcDNA-Sp1) or DRRF (pcDNA-DRRF) expression plasmid. Cells were harvested 48 hr later and lysed by four cycles of freezing and thawing of the harvested cells followed by centrifugation.

Table 1. PCR primers for deletion mutants of the DRRF gene

DRRF+17-R (+17/+2)	5'- <u>GTCGACGGCAGCGGCTGCTAGAG</u> -3' <i>SalI</i>
DRRF-1122-F (-1122/-1106)	5'- <u>CTGCAGGCCTCAGTTTCCCTGTC</u> -3' <i>PstI</i>
DRRF-772-F (-772/-756)	5'- <u>CTGCAGGTCGCGCACGTGGCCCG</u> -3' <i>PstI</i>
DRRF-692-F (-692/-675)	5'- <u>CTGCAGATCCCCGACACACACAC</u> -3' <i>PstI</i>

All plasmids used in transfections were purified by the Plasmid Midi Kit (Qiagen, Inc., USA). Chloramphenicol acetyltransferase (CAT) assay was carried out using the CAT enzyme-linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals, USA). All CAT assay results were normalized to β-galactosidase activity[12].

Gel Mobility Shift Assay

Nuclear extracts from NB41A3 cells were prepared using a modification of Dignam's procedure[2,4]. DNA probes DRRF_AP1 (bases -1153 to -1121) and DRRF_AP2 (bases -873 to -846) were generated by annealing ³²P-labeled sense oligonucleotide and cold antisense oligonucleotide (Table 2). All probes were purified using Sephadex G-25 (Amersham Bioscience, USA) columns. For competition of probe binding, the unlabeled fragments corresponding to each probe, or the unlabeled AP1 or AP2 consensus oligonucleotide shown in Table 2 was added in 500-fold molar excess relative to the respective probes. DNA-protein binding reactions were carried out with 5 µg of nuclear extract from NB41A3 cells, and about 5 fmol (>50,000 cpm) of probe in the presence of 12% glycerol, 12 mM HEPES buffer (pH 7.9), 60 mM KCl, 120 µM EDTA, 50 mM NaCl and 2 µg of poly[dIdC] (Roche Molecular Bio-

Table 2. Oligonucleotide sequences of DNA probes and competitors for gel mobility shift assay

DRRF_AP1 (-1153/-1121)	
DRRF_AP1-F	5'-CCATGGAAGTTTCCAATCGCTCCATTAGTCAGC-3'
DRRF_AP1-R	5'-GCTGACTAATGGAGCGATTGAAAACCTCCATGG-3'
DRRF_AP2 (-873/-846)	
DRRF_AP2-F	5'-ATATTTTGTCCCAAGCCAAGAATGGCA-3'
DRRF_AP2-R	5'-TGCCATTCTTGGCTTGGGGACAAAATAT-3'
AP1 consensus competitor	
AP1_con-F	5'-CGCTTGATGACTCAGCCCGAA-3'
AP1_con-R	5'-TTCCGGCTGAGTCATCAAGCG-3'
AP2 consensus competitor	
AP2_con-F	5'-GATCGAACTGACCGCCCGCGCCCGT-3'
AP2_con-R	5'-ACGGGCCGCGGGCGGTGAGTTCGATC-3'

Putative or consensus binding sites for corresponding transcription factors are underlined.

chemicals, USA) in 25 μ l. Products of the binding reactions were resolved in a 1X Tris-Glycine buffer, 4% polyacrylamide (30:1) nondenaturing gel[1].

Results

Activation of the DRRF promoter by Sp1 and self repression of the DRRF promoter by DRRF

We previously reported that the promoter region of the

DRRF gene has multiple putative binding sites for the transcription factor Sp1[9]. To analyze transcriptional regulation of the DRRF promoter by Sp1, NB41A3 cells were co-transfected with pcDNA-Sp1 and pCAT-DRRF-1153/+17. Co-transfection with a fixed amount of the reporter pCAT-DRRF-1153/+17, which contains 1170 bp *NcoI-NaeI* fragment of the DRRF gene and increasing amounts of the expression plasmid pcDNA-Sp1 resulted in increased CAT activity in a concentration-dependent manner (Fig. 1B).

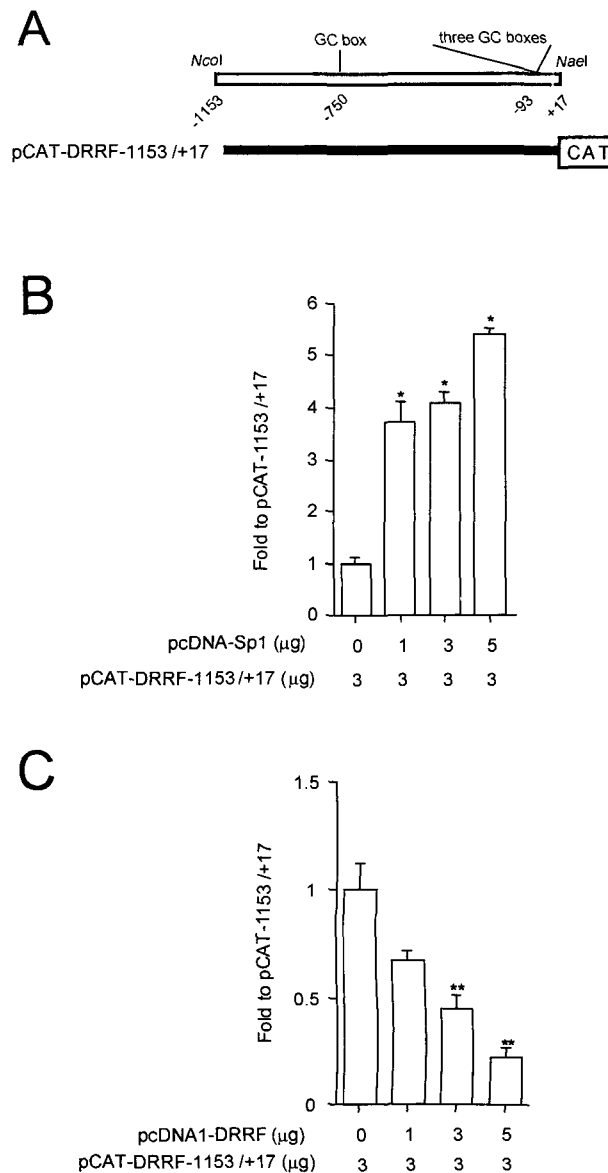


Fig. 1. Transcription activity of Sp1 or DRRF on the DRRF promoter. (A) Schematic structure of the CAT constructs. (B) Rising amounts of pcDNA-Sp1 were used to co-transfect NB41A3 cells with fixed amounts of pCAT-DRRF-1153/+17. (C) Rising amounts of pcDNA-DRRF were used to co-transfect NB41A3 cells with fixed amounts of pCAT-DRRF-1153/+17. CAT activity was measured using the CAT-ELISA kit, normalized with protein concentration and expressed as fold increase over pCAT-DRRF-1153/+17 alone. Results presented are means \pm SEM for three plates. Factorial ANOVA with Fisher's PLSD post-hoc test * $p < 0.0001$ and ** $p < 0.03$. Transfections were repeated at least twice yielding reproducible results.

DRRF is a transcription factor that contains an Sp1-like zinc finger motif and has DNA-binding activity at the same DNA sequences as Sp1[5]. To analyze transcriptional auto-regulation of the DRRF promoter by DRRF protein, NB41A3 cells were co-transfected with a fixed amount of pCAT-DRRF-1153/+17 and rising amounts of pcDNA-DRRF, and found decreased CAT activity in a concentration-dependent manner (Fig. 1C). This repressive effect of DRRF on the DRRF promoter itself in pCAT-DRRF-1153/+17 is contrary to the strong activation induced by Sp1[14].

Transcriptional Activity of the 5' Flanking Region of the DRRF Gene

To localize the regulatory regions in the 5' flanking region of the murine DRRF gene in detail, serial deletion mutants were tested (Fig. 2). pCAT-DRRF-1122/+17, which deletes a putative binding site for the transcription factor AP1 between -1153 and -1122, showed significant decrease in activity reaching about 60% of that of pCAT-DRRF-1153/+17 (Fig. 2). pCAT-DRRF-901/+17, which deletes the region between -1122 and -901 from pCAT-DRRF-1122/+17 also revealed significant decrease in activity reaching about 30% of that of pCAT-DRRF-1122/+17 (Fig. 2). pCAT-DRRF-772/+17, which deletes a putative binding site for the transcription factor AP2 between -901 and -772 also had significantly decreased activity reaching about 40% of that of pCAT-DRRF-901/+17 (Fig. 2). On the other hand, pCAT-DRRF-692/+17, which deletes putative binding sites

for the transcription factors Sp1 and AP2 between -772 and -692 showed significant increase in activity reaching about 450% of that of pCAT-DRRF-772/+17 (Fig. 2). These results suggest that the putative AP1 binding site located between -1153 and -1122, and the putative AP2 binding sites between -901 and -772 could play roles as functional positive modulators. On the other hand, the 80 bp region between -772 and -692 of the DRRF promoter could play a role as a negative modulator.

DNA-protein interactions at the transactivation regions of the DRRF gene

We suggested that the putative binding sites for the transcription factors AP1 between -1153 and -1122 and the AP2 site between -901 and -772 could play roles as positive modulators. To investigate if these sequences could recruit corresponding transcription factors, gel mobility shift assay was performed using 5'-end labeled DNA probes shown in Table 2 with nuclear extract from NB41A3 (Fig. 3). Using DRRF_AP1 (bases -1153 to -1121) as probe, a specific retarded band was observed, and the unlabeled DNA fragment could effectively compete away this retarded band (Fig. 3A). This band could also be competed away by unlabeled AP1 consensus sequence (Table 2 and Fig. 3A). In addition, using a DRRF_AP2 (bases -873 to -846) as probe, a specific retarded band was observed, and the unlabeled DNA fragment could effectively compete away this retarded band (Fig. 3B). This band could also be competed away by the unlabeled AP2 consensus competitor

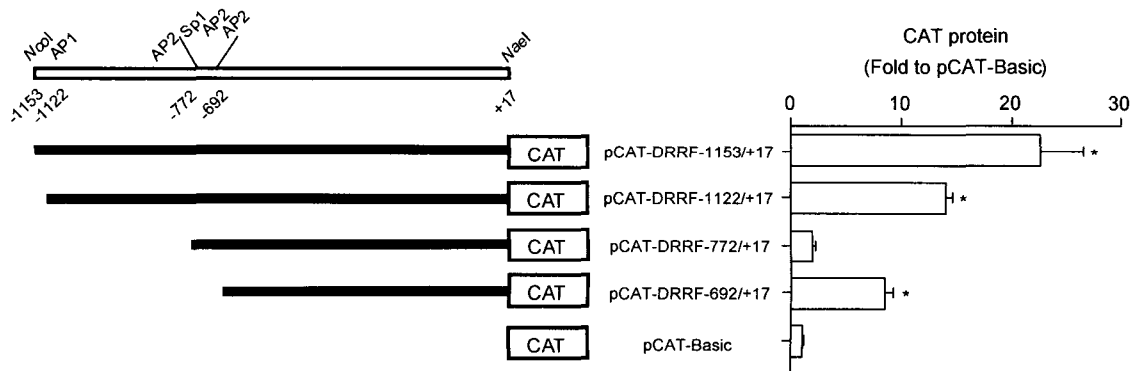


Fig. 2. Transcriptional activity of DRRF promoter deletion mutants. Schematic representation of CAT constructs containing various lengths of the DRRF 5'-flanking region is shown on the left side of upper panel. Restriction sites used for subcloning are shown. Putative transcription factor binding sites are also shown. Each CAT construct was used to co-transfect NB41A3 cells along with pCMV β as internal control. CAT activity was measured using the CAT-ELISA kit, normalized with β -galactosidase activity derived from pCMV β and expressed as fold increase over the promoterless reporter plasmid pCAT-Basic. Results presented are means \pm SEM for three to four plates. Factorial ANOVA with Fisher's PLSD post-hoc test * $p < 0.0001$ compared to pCAT-Basic. Transfections were repeated at least twice yielding reproducible results.

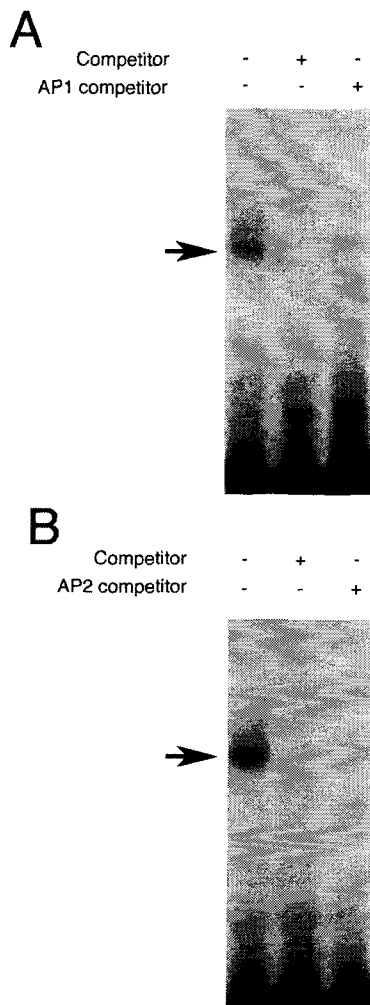


Fig. 3. Interaction of AP1 and AP2 with their respective binding sites in the DRRF promoter. Gel mobility shift assay was done with DNA probes DRRF_AP1 (A) and DRRF_AP2 (B) and nuclear extracts prepared from NB41A3 cells. Competitors indicate the corresponding unlabeled DNA fragments. AP1 and AP2 competitors indicate the corresponding unlabeled consensus competitors shown in table 2, respectively. The arrow indicates a specific shifted band.

(Table 2 and Fig. 3B).

Discussion

In the present report, transcriptional regulation of the DRRF gene was analyzed. This promoter has multiple putative binding sites for the transcription factor Sp1[9], and DRRF protein itself has DNA-binding activity on the same DNA sequences as Sp1[5]. We found that Sp1 indeed activates the DRRF gene, but DRRF has the ability to repress transcription of the gene.

The present study also exhibit to involve in transcriptional activity to regions in the DRRF promoter that have functional binding sites for AP1 and AP2. Deletion of an AP1 binding site located between -1153 and -1122 of the DRRF gene resulted in significant decrease in transcriptional activity, and deletion of an AP2 binding site between bases -1122 and -772 resulted in further decrease in activity. Gel mobility shift assays also substantiated the binding of corresponding nuclear factors to these activation regions. Interestingly, it was reported that BTEB-1 is a strong activator of AP2 promoter activity[6]. AP2 proteins interact with the palindromic consensus recognition site detected in promoters of numerous gene promoters, including genes that are expressed specifically in neural, glial, urogenital, and epidermal cells. On the other hand, a significant increase in activity was observed by deleting the 80 bp region between -772 and -692 (Fig. 2). This region possesses consensus sequences for one Sp1 and two AP2 putative binding sites. Whether the latter sites are functional remain to be investigated.

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초록 : 생쥐 도파민 수용체 조절인자 (DRRF) 유전자의 전사조절

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생쥐의 도파민 수용체 조절인자 (DRRF) 유전자는 몇몇 Sp1 결합부위를 가지고 TATA가 없는 프로모터로부터 전사된다. 본 연구에서는 이 유전자의 발현을 조절하는 기능성 조절인자들을 밝힌다. D₂ 도파민 수용체를 발현하는 NB41A3 세포에서 Sp1은 pCAT-DRRF-1153/+17에 포함된 DRRF 프로모터부터의 전사를 촉진시키지만 DRRF는 전사를 억제시켰다. -1153과 -1122 사이의 31 bp 단편의 결손에 의해 전사활성은 약 60% 정도 감소하였다. 이 단편은 기능성 AP1 결합부위를 포함하고 있다. 게다가, -901과 -772 사이의 129 bp 영역의 결손에 의해 전사활성이 더욱 더 감소하였다. 이 영역은 기능성 AP2 결합부위를 가진다. DRRF_AP1 (bases -1153 to -1121) 탐침을 이용한 gel shift 실험에서 특정 밴드가 관찰되었고, 이 밴드는 AP1 상보성 경쟁자에 의해 효과적으로 사라졌다. 더욱이, DRRF_AP2 (bases -873 to -846) 탐침을 이용한 gel shift 실험에서도 특정 밴드가 관찰되었고, 이 밴드도 AP2 상보성 경쟁자에 의해 효과적으로 사라졌다. 본 연구결과로, Sp1과 DRRF가 DRRF 프로모터를 효과적으로 조절한다는 사실과, AP1과 AP2 역시 이 유전자를 조절한다는 사실을 알 수 있었다.