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Interaction of NF-Y with the 3′-flanking DNA sequence of the CCAAT box

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Abstract NF-Y, also referred to as CCAAT-binding factor, is a major CCAAT-binding transcription factor. The present study demonstrated that the 3′-flanking region of the CCAAT box is involved in the formation of a stable NF-Y·DNA complex. An electrophoretic mobility shift assay showed that the interaction of NF-Y with DNA 15 bp downstream of the CCAAT box alters not only the affinity of NF-Y for its binding site but also the electrophoretic mobility of the NF-Y·DNA complex. This interaction is accompanied by a conformational change of NF-Y as demonstrated by a change in the reactivity of an anti-NF-YA antibody to the NF-Y·DNA complex.

Key words: Transcription factor; NF-Y; CCAAT box; DNA binding

1. Introduction

Gene expression is regulated by an assortment of transcription factors unique to specific cellular contexts. Since the original domain-swapping experiments [1], it has generally been thought that transcription factors are composed of separate, autonomous modules of DNA-binding domains (DBD), which recruit the protein to relevant promoter and transcription activation domains [2]. Recently, studies on intracellular receptor families and other classes of transcription factors have modified this view by proposing a transcription model in which DBD play a more active role [3]. That is, DNA is not only a mere binding site for transcription factors, but also an allosteric ligand whose binding can regulate the function of transcription factors. The allosteric effect is accompanied by a DNA-induced conformational change in the protein, and this structural change modifies its inherent properties including transcriptional activating potential as well as affinity for other components of the transcriptional apparatus. The DNA sequence itself can contain information that dictates a specific conformational change in the protein, resulting in a different transcriptional outcome [4–6].

Among several CCAAT-binding proteins isolated so far, the heterotrimeric transcription factor NF-Y (also referred to as CCAAT-binding factor, CBF) has been shown to be an essential transcription factor responsible for the regulation of most CCAAT box-containing promoters [7–9]. NF-Y organizes the transcriptional machinery by bending the DNA chain and interacting with other regulatory proteins including TBP, TAFs, Sp1, RFX and P/CAF [9]. All three subunits of NF-Y, namely, NF-YA, -YB and -YC, appear to participate in forming the surfaces required for DNA binding [9–11]. While NF-Y binding to the CCAAT box exhibits a strict requirement for the pentanucleotide [12], previous studies such as random binding-site selection experiments [13] and compilation of bona fide NF-Y binding sites [7] have suggested that, in addition to the pentanucleotide, short segments of adjacent flanking sequences are important for NF-Y binding. Moreover, contacts between NF-Y and the more distal flanking sequences were observed in hydroxyl radical footprinting [13]. However, it remains to be clarified to what extent these distal flanking regions of DNA are involved in NF-Y binding.

In this study, we demonstrate that the distal contact with the 3′-flanking DNA is important for NF-Y binding to DNA. Furthermore, we present evidence for a conformational change in NF-Y that is induced by the interaction between NF-Y and the 3′-flanking DNA.

2. Materials and methods

2.1. Electrophoretic mobility shift assay (EMSA)

Nuclear extract was prepared from Chinese hamster ovary (CHO) and differentiated mouse P19 embryonic carcinoma cells by the method of Dignam et al. [14]. Unless specified otherwise, EMSA and antibody supershift assay were performed as described previously [15]. The GC box sequence in the extracellular signal-regulated kinase 2 (ERK2) 28R, 31R and 33R probes used in the EMSA shown in Figs. 2–5 was altered to GttCGG to exclude Sp1/Sp3 binding [15]. Polyclonal rabbit antibodies against the NF-YA C-terminal peptide (anti-NF-YA_C7) and against NF-YB [16] were kindly provided by Dr. R. Mantovani (University of Modena, Italy). Polyclonal rabbit antibody against the NF-YA N-terminal peptide (hereafter referred to as anti-NF-YA_N) was purchased from Rockland (Gilbertsville, PA, USA). Polyclonal rabbit anti-CBF-C (NF-YC) antibody [17] was kindly provided by Dr. T. Taira (Hokkaido University, Sapporo, Japan).

2.2. Production of recombinant proteins

cDNAs of mouse NF-YA, -YB and -YC were amplified by PCR from 3T3-L1 cell cDNA and cloned into pGEX-6P-2 (Amersham Biosciences). To produce NF-YA protein, the pGEX-YA construct

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Abbreviations: CBF, CCAAT-binding factor; DBD, DNA-binding domains; CHO, Chinese hamster ovary; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase

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was introduced into BL21-Codon-Plus (DE3) RIL cells (Stratagene). The cells expressing the protein were lysed with buffer A (25 mM Tris–HCl (pH 7.5), 1% *n*-octylthioglucopyranoside, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2.5 μg/ml leupeptin and 2.5 μg/ml pepstatin). The inclusion bodies were collected, washed twice in 10-fold diluted buffer A, and solubilized in 8 M urea, 50 mM Tris–HCl (pH 7.0), 1 mM EDTA and 1 mM DTT. The proteins were refolded by stepwise dialysis against decreasing concentrations of urea (7, 6, 5, 4, 3, 2, 1 and 0.5 M), and then dialyzed against 50 mM Tris–HCl (pH 7.0 at 25°C), 0.15 M NaCl, 1 mM EDTA and 1 mM DTT. GST-YA was purified over glutathione Sepharose 4B (Amersham Biosciences), and YA protein was eluted by on-column cleavage with PreScission protease (Amersham Biosciences). The YB subunit was also prepared as described above for YA. The GST-YC protein was purified as a complex with the YB subunit. Three milligrams of the inclusion body fraction of GST-YC-expressing bacteria and 1.5 mg of the purified YB protein were combined in 8 M urea solution and refolded together by stepwise dialysis. After purification of the YB/GST·YC complex over glutathione Sepharose, the YB/YC moiety was eluted by on-column cleavage and further purified using a MonoQ HR 5/5 column (Amersham Biosciences).

3. Results

Our previous report characterizing NF-Y interaction with the mouse ERK2 promoter raised questions about the role of flanking sequences in NF-Y·CCAAT box binding [15]. As shown in Fig. 1 (lanes 3 and 4), NF-Y efficiently bound to the ERK2 33R probe (the number preceding 'R' in the probe nomenclature denotes the length of the 3′-flanking DNA; Table 1). In contrast, it failed to bind to either the ERK2 −93/−51 probe with a 29-bp 5′ extension (lane 5) or the 9R probe containing the CCAAT core-binding sequence (lane 2). A possible explanation for these observations is that the 9-bp

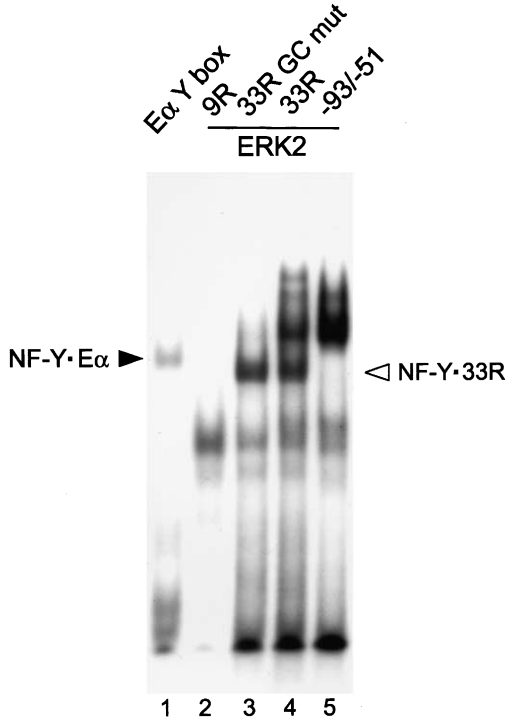

Fig. 1. Efficient binding of NF-Y to the CCAAT box requires a specific length of 3′-flanking sequence. EMSA was performed with P19 cell nuclear extract. Probes used are Eα Y box (lane 1), ERK2 9R (lane 2), ERK2 33R GC box mutant (lane 3), wild-type 33R (lane 4) and ERK2 −93/−51 (lane 5). The major bands of lower mobility in lanes 4 and 5 are derived from Sp1/Sp3 binding [15].

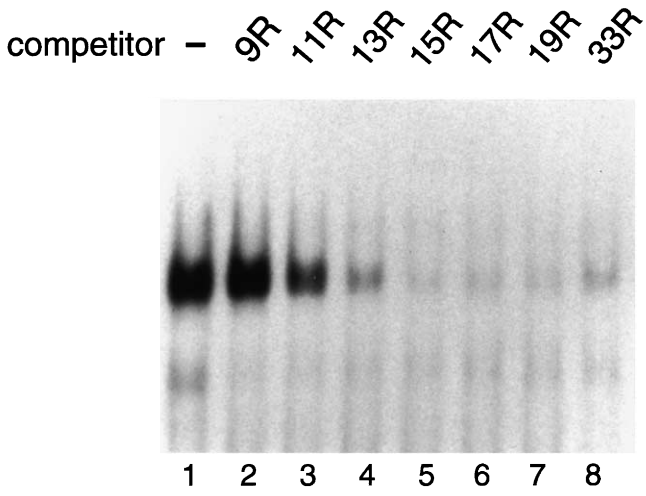

Fig. 2. Critical length of the 3′-flanking DNA for NF-Y binding to the CCAAT box probe. Competitive EMSA was performed with P19 cell nuclear extract and ERK2 33R GC box mutant probe in the absence (lane 1) or presence (lanes 2–8) of a 100-fold excess of unlabeled oligonucleotides with 3′-flanking DNA of increasing length.

3′-flanking DNA in both 9R and −93/−51 probes is inadequate for efficient NF-Y binding. We tested this possibility by a competitive EMSA using oligonucleotides consisting of 8 bp 5′-flanking DNA, the core pentanucleotide, and 3′-flanking DNA of increasing lengths. The NF-Y binding to radiolabeled 33R probe was challenged with a 100-fold excess of each unlabeled oligonucleotide (Fig. 2). As expected, 9R oligonucleotide failed to compete for NF-Y binding (lane 2). 11R and 13R oligonucleotides showed an increasing ability to compete (lanes 3 and 4). The addition of oligonucleotides having more than 15 bp of 3′-flanking DNA abolished the NF-Y binding to labeled 33R probe (lanes 5–8). This finding suggests that the efficient binding of NF-Y to the ERK2 CCAAT box requires an interaction with the 3′-flanking DNA in addition to the core pentanucleotide.

We next investigated an unexpected difference in the electrophoretic mobility between the NF-Y·DNA complexes obtained with the 33R probe (Fig. 1, lane 3, open arrowhead) and 24-bp Eα Y box probe, a classical NF-Y binding site (Fig. 1, lane 1, closed arrowhead). We compared the mobility of the NF-Y·DNA complexes formed with 17R and 28R probes derived from three different promoter sequences (ERK2, Eα and α2(I)col; Table 1) in order to examine whether the length of the 3′-flanking DNA simply affects the mobility of the NF-Y·DNA complex formed with each DNA. As shown in Fig. 3, all the NF-Y·DNA complexes obtained with the three different 17R probes exhibited almost identical mobility. On the other hand, the complexes obtained with the 28R probes all migrated faster than the complexes with the 17R probes did. This result was demonstrated with purified recombinant NF-Y subunits as well as the nuclear extracts, indicating that the result was attributed to an inherent property of NF-Y, but not to its interaction with other nuclear proteins. Thus, in all three promoters examined the length of the 3′-flanking DNA affected the electrophoretic mobility of the NF-Y·DNA complex, irrespective of the sequences surrounding the CCAT box.

The change in electrophoretic mobility of the NF-Y·DNA complex was further analyzed using ERK2 probes with the

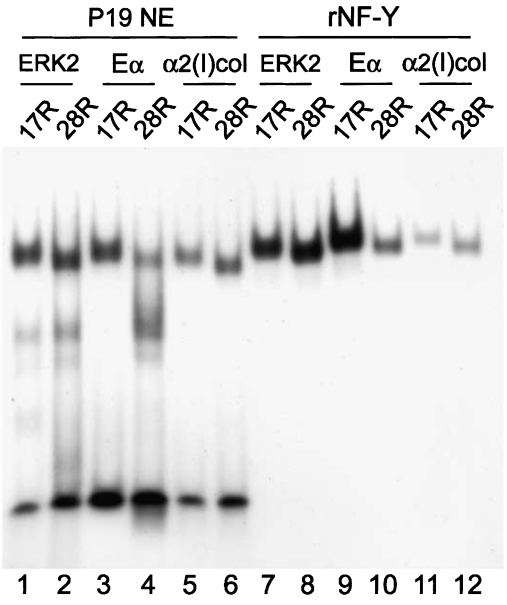

Fig. 3. Electrophoretic mobility of the NF-Y·DNA complex correlates with the length of the 3′-flanking DNA, but not with the differences in the sequences surrounding the CCAAT box. The results with P19 cell nuclear extracts (left half panel) and with recombinant NF-Y subunits are shown (right half). As αcol probes have a higher affinity for NF-Y, half as much of the protein was used for the αcol probes than for the ERK2 and Eα probes. We also observed similar results with CHO cell nuclear extracts (data not shown).

3′-flanking DNA of increasing length (Fig. 4). As expected from the results of the competitive EMSA, the affinity of NF-Y for a probe was gradually elevated as the length of the 3′-flanking DNA increased up to 22 bp (lanes 1–6). The NF-Y·DNA complexes formed with 9R, 11R and 13R probes showed the same mobility (lanes 1–3), suggesting that the mobility of the complex appeared to be insensitive to the changes in mass and charge provided by the increased length of DNA. However, the complexes formed with 17R to 28R probes showed a gradual increase in the mobility (lanes 4–8). This alteration of electrophoretic mobility, which is conferred by the interaction with the 3′-flanking DNA, suggests a conformational change in the protein, a phenomenon similar to that observed with thyroid hormone receptor, which exhibits a similar alteration of electrophoretic mobility of protein·DNA complex due to a DNA-induced conformational change in the protein moiety [18,19].

DNA binding to transcription factors can induce conformational changes which alter the immunoreactivity to their specific antibodies. This is the basis of antibody supershift assays used to detect such DNA-induced conformational changes [20–22]. We examined the conformational changes in NF-Y·DNA complexes formed with either 17R or 33R probe using antibodies against each of the three NF-Y subunits (Fig. 5). NF-Y bound to the probes was reactive to anti-NF-YA_N, anti-NF-YB and anti-NF-YC antibodies as demonstrated by the retardation of the NF-Y·DNA complexes; for unknown reasons, anti-NF-YA_C7 antibody was ineffective in the range of concentrations tested. These results confirmed that the NF-Y bound to 17R and 33R probes contains all three subunits and, therefore, excluded the possibility that the higher mobility of the NF-Y·33R complex resulted from the dissociation of any of the subunits.

NF-Y·DNA complexes formed with17R and 33R probes showed distinct reactivities to anti-NF-YA_N antibody. In contrast to a single supershifted species derived from the NF-Y·17R complex (lane 4, asterisk), reaction of anti-NF-YA_N antibody with the NF-Y·33R complex gave rise to two supershifted species (lane 10, open and closed circles). One migrated faster than the supershifted NF-Y·17R complex, and the other migrated more slowly. Of these two species, the more slowly migrating one was the main supershifted species at a low concentration of anti-NF-YA_N (lane 9, open circle). As the concentration of the antibody increased, the faster-migrating species became predominant (lanes 10 and 11, closed circle), indicating that the more slowly migrating species represents an intermediate conformation of the antibody·NF-Y·DNA complex that eventually gives rise to the faster one by reacting with a higher concentration of the antibody. Similar results with anti-NF-YA_N antibody were also observed using Eα and α2(I) collagen probes (data not shown), indicating that the observation was not unique to the ERK2 sequence. Taken together, these observations with the anti-NF-YA_N antibody corroborated the structural change in NF-Y induced by the 3′-flanking DNA.

4. Discussion

Contacts between NF-Y and the 3′-flanking DNA have been shown by hydroxyl radical footprinting [13]. In addition to the CCAAT box, distal flanking regions located on each side of the core sequence were protected from hydroxyl radical cleavage. The reported distal boundary of the 3′ protected region, which is 14–16 bp away from the pentanucleotide, agrees well with the boundary of the 3′-flanking DNA determined in this study as the minimal length required for efficient NF-Y binding. Similar distal contacts as well as central se-

Table 1
Oligonucleotide probes used in this study

Eα Y box	5′-CAACTTTTAACCAATCAGAAAAAT-3′
ERK2 9R	5′-AAGAGAAGCCAATCAACACGCA-3′
ERK2 17R	5′-AAGAGAAGCCAATCAACACGCACGCCCTTG-3′
Eα 17R	5′ ACTTTTAACCAATCAGAAAAATGTTTCAGA 3′
α2(I)col 17R	5′-CGTCTCCACCAATGGGAGGGCTAGGGCTAG-3′
ERK2 28R	5′-AAGAGAAGCCAATCAACACGCACGCCCTTGACTCCGCCCTA-3′
Eα 28R	5′-ACTTTTAACCAATCAGAAAAATGTTTCAGACTGACACATCT-3′
α2(I)col 28R	5′-CGTCTCCACCAATGGGAGGGCTAGGGCTAGGGGCCCGGGAC-3′
ERK2 33R	5′-AAGAGAAGCCAATCAACACGCACGCCCTTGACTCCGCCCTAGCGCC-3′
ERK2 −93/−51	5′-CGAGACTCCGCCCTCTCTACCAAGAGAAGCCAATCAACACGCA-3′

The sequences were taken from: Eα, mouse MHC class II Eα gene (GenBank accession number M17389); ERK2, mouse ERK2 gene (D87264); α2(I)col, mouse α2(I) collagen gene (M13124). The CCAAT box and GC box are underlined.

quence-specific ones have also been used in several DNA-binding proteins to form a stable DNA·protein complex [23–25]. Recently, a crystal structure of the NF-YB/NF-YC heterodimer has been reported [26]. The structure of the NF-YB/NF-YC dimer is highly similar to that of the histone dimer H2A/H2B [27], as well as NC2α/2β [28], a heterodimeric complex that modulates TATA-dependent transcription. A comparison of the structure of NF-YB/NF-YC to that of the H2A/H2B·DNA complex in the nucleosome core particle suggests that the NF-YB/NF-YC dimer may also interact with DNA at two different sites, α1α1 and L1L2 [26]. The interactions made by the two loops that comprise the L1L2 site, located at the extremity of the dimer, are thought to occur in the distal CCAAT box-flanking region identified in this report. It has also been reported that the L1L2 site makes contacts with the DNA backbone, which could explain the lack of an apparent sequence preference in this region of NF-Y binding sites.

It was previously indicated that a stable NF-YB/NF-YC heterodimer initially forms an unstable complex with NF-YA by weak interaction, and then binding of DNA to the trimeric complex stabilizes the interaction between NF-YA and the heterodimer, resulting in a stable NF-Y·DNA complex [10]. For this stabilization, a conformational change in NF-Y is likely to be important since the increase in the amount of the NF-Y·DNA complex formed correlated with that in the electrophoretic mobility when the length of the 3′ DNA sequence of the probe increased (Fig. 4, lanes 1–7).

Thermodynamic studies have shown that DNA binding drives the structural changes in DNA-binding proteins [29]. In a nuclear magnetic resonance study of a HoxB1·Pbx1·DNA complex, it was also demonstrated that a DNA-induced conformational change in Pbx1 promotes the formation of a ternary complex by facilitating HoxB1 binding to a reorganized α-helix of Pbx1 [30]. Such examples suggest that a conformational change in NF-Y could affect the transcriptional apparatus by altering the interaction between NF-Y and other regulatory components. For example, a structural change in NF-Y might account for the cooperative binding of NF-Y and RFX to the X-Y promoter elements even though NF-Y and RFX do not interact in solution in the absence of DNA [31]. The findings obtained in this report will facilitate future studies investigating the coordinate effects of the DNA-binding, structural change, and concomitant functional modulation of NF-Y.

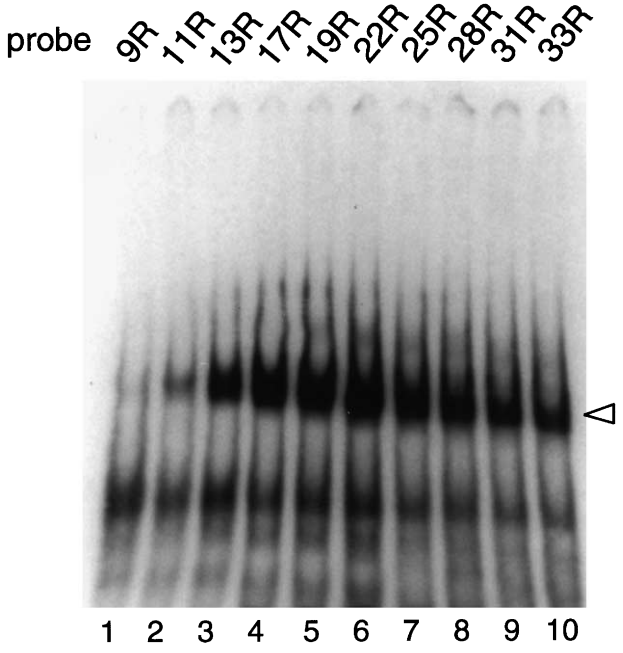

Fig. 4. A 3′-flanking DNA-induced increase in electrophoretic mobility of the NF-Y·DNA complex. EMSA was performed with P19 cell nuclear extracts and oligonucleotide probes with 3′ DNA of increasing length. The NF-Y·DNA complexes were resolved on a 3% acrylamide gel to improve separation. The probes were radiolabeled to a comparable specific activity.

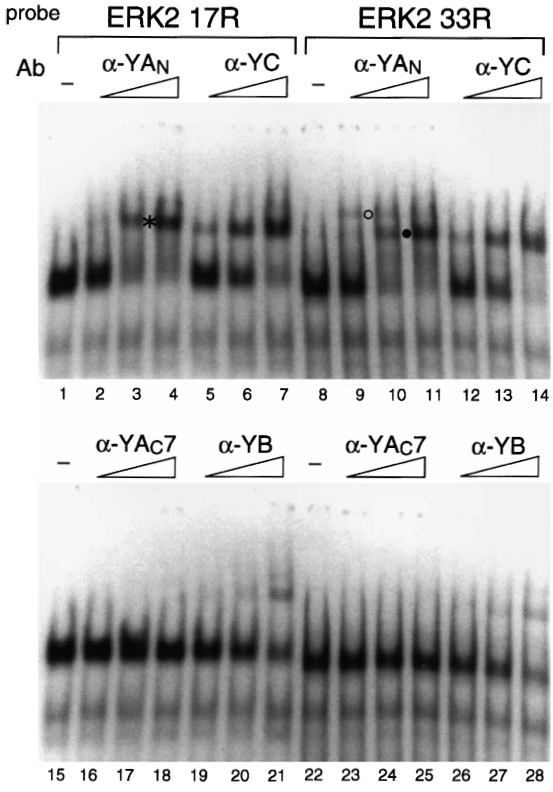

Fig. 5. An antibody supershift assay analysis demonstrates that the 3′-flanking sequence alters the conformation of DNA-bound NF-Y. P19 cell nuclear extracts were incubated in the absence (−) or presence of increasing amounts of the indicated anti-NF-Y antibody prior to the addition of the probe: ERK2 17R (left half panel) or ERK2 33R (GC box mutant: right half panel) probe. The amounts of antibody added are: anti-NF-YA_N and anti-NF-YC, 50, 100 and 200 ng; anti-NF-YA_C7 and anti-NF-YB, 200, 500 and 1000 ng (from left to right).

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