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Effects of the Heptasequence SPTSPTY of Rat Nuclear Factor 1-A on Interactions between the C-Terminal Regions of Mammalian Nuclear Factor 1 Proteins

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NF1 proteins are a family of DNA binding proteins which consist of two separate domains, N-terminal DNA binding domain and C-terminal transcription activation domain. The N-terminal 220 amino acids are highly conserved and are also known to mediate dimerization of NF1 proteins. The C-terminal regions of different type of NF1 proteins are heterogeneous and responsible for transcriptional activation. In this study, we tested the interaction between different domains of rat NF1-A protein by yeast two hybrid analysis and observed the interaction between C-terminal regions of NF1-A which do not contain the N-terminal dimerization domain. Our results showed that the C-terminal region of rat NF1-A between residues 231 and 509 strongly interacted not only with itself, but also with human NF1/CTF1 which is a different type of NF1. When the C-terminal region was divided into two fragments, one from residue 231 to 447 and the other from 448 to 509, the two fragments were able to interact with the C-terminal region of NF1-A significantly. This indicates that both fragments contain independent interaction domains. Analysis of the interactions with alanine substituted fragments showed that substitutions of the heptasequence, SPTSPTY of NF1-A, affected interaction between NF1 proteins. Our results strongly suggest that C-terminal regions may also be important for the formation of homo- and heterodimers in addition to the N-terminal dimerization domain. Also, the heptasequence motif may play some roles in dimer formation.

Keywords: Nuclear Factor 1, NF1-A, Protein interaction, Transcription factors

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Control of eukaryotic gene transcription is mediated by the interaction of different sets of transcription factors with specific DNA sequence elements, which are contained in promoter and enhancer regions (Mitchell and Tjian, 1989; Wolberger, 1999). Diversity of gene expression are further generated by the presence of a family of proteins which bind to a specific DNA element and by formation of homo- and heterodimers among a family of structurally related proteins (Hirai *et al.*, 1989; Zhang and Pfahl, 1993; Kruse and Sippel, 1994; Chambon, 1996). Such a family of proteins include the steroid/thyroid hormone receptor family (Zhang and Pfahl, 1993; Chambon, 1996), Fos/Jun family (Hirai *et al.*, 1989) and NF1 family (Kruse and Sippel, 1994).

Nuclear Factor 1 is a family of DNA binding proteins which was first identified as a host factor required for initiation for adenovirus DNA replication in HeLa cells (Nagata *et al.*, 1982). Later NF1 proteins were shown to play roles in transcription of many viral and cellular genes. Multiple forms of NF1 cDNAs were cloned from HeLa cells (Santoro *et al.*, 1988) and subsequently from several animal species (Gil *et al.*, 1988; Paonessa *et al.*, 1988; Rupp *et al.*, 1990; Apt *et al.*, 1994; Kruse and Sippel, 1994; Wenzelides *et al.*, 1996). Sequence homology enabled to identify at least four different genes; NF1-A, B, C, and X (Gil *et al.*, 1988; Rupp *et al.*, 1990; Kruse and Sippel, 1994). Mutiple NF1 proteins were generated in higher eukaryotes by different mechanisms, for example, existence of at least four different genes (Gil *et al.*, 1988; Rupp *et al.*, 1990; Kruse and Sippel, 1994), differential splicing (Santoro *et al.*, 1988; Rupp *et al.*, 1990; Apt *et al.*, 1994; Kruse and Sippel, 1994; Wenzelides *et al.*, 1996), and posttranslational modifications (Jackson and Tjian, 1988; Yang *et al.*, 1993).

NF1 family proteins bind as dimers (de Vries *et al.*, 1987; Mermod *et al.*, 1989; Gounari *et al.*, 1990) to the DNA sites that are homologous to the palindromic consensus sequence 5'-YTGGCA(N)$_3$TGCCAR-3' (de Vries *et al.*, 1987; Meisterernst *et al.*, 1988; Meisterernst *et al.*, 1989; Goyal *et al.*, 1990). All forms of NF1 proteins have a highly conserved

N-terminal domain (Paonessa *et al.*, 1988; Santoro *et al.*, 1988; Gil *et al.*, 1988; Rupp *et al.*, 1990; Apt *et al.*, 1994), and the N-terminal 220 amino acids of human NF1/CTF1 and rat NF1-A have been shown to be sufficient for DNA-binding, dimerization and stimulation of adenovirus DNA replication (Mermod *et al.*, 1989; Gounari *et al.*, 1990; Kruse and Sippel, 1994). It has been demonstrated that a family of NF1 proteins bind to DNA as homodimers and also as heterodimers (Gounari *et al.*, 1990; Kruse and Sippel, 1994).

The C-terminal regions are capable of activating transcription in yeast (Kim and Roeder, 1993; Altmann *et al.*, 1994; Kim and Roeder, 1994; Wendler *et al.*, 1994; Xiao *et al.*, 1994) as well as in HeLa cells (Mermod *et al.*, 1989; Novak *et al.*, 1992; Nebl *et al.*, 1995). Several investigators have independently reported that a transcriptional activation domain of human NF1/CTF1 contains a heptasequence motif, SPTSPSY (Kim and Roeder, 1993; Altmann *et al.*, 1994; Kim and Roeder, 1994; Wendler *et al.*, 1994; Xiao *et al.*, 1994), which is strongly related to the heptapeptide sequence present in the carboxyterminal domain of RNA polymerase II (Corden, 1990). Rat NF1-A also contains a related heptapeptide sequence, SPTSPTY and has been shown to be important for transcriptional activation (Yang *et al.*, 1996; Hwang *et al.*, 1999).

In this paper, we showed that the C-terminal fragments from residue 231 to 509 of rat NF1-A interacted with the C-terminal fragment itself and with human NF1/CTF1 using a yeast two-hybrid system. This demonstrates that there are interaction domains in the C-terminal region of NF1 in addition to the N-terminal dimerization domain. Multiple alanine substitutions of serine and threonine residues of the heptasequence of NF1-A affected the interaction of the C-terminal regions of the proteins. This suggests that the heptasequence of the proteins may be important for interaction of NF1 proteins.

Materials and Methods

Growth of yeast cells *S. cerevisiae* strain CTY1 (*MAT ade2-101 ura3-52 his3-200 leu2-3 leu2-112 lys2-801 trp1-901 gal4 gal80 URA3::GAL1-lacZ LYS2::GAL1-HIS3)* was used as host cells for yeast two hybrid plasmids. CTY1 cells were grown in a YPD medium (2% bactopeptone, 1% yeast extract, 2% glucose).

Plasmid construction pAS2-1 (Clontech, Palo Alto, USA) was used for construction of yeast GAL4 fusion plasmids with NF1. Fusion plasmids of yeast GAL4 DNA binding domain with various segments of rat NF1-A were designated as pAJ, and followed by numbers which indicate the positions of the amino acid residues. pAJ231-509, pAJ231-447, and pAJ448-509 were described previously (Hwang *et al.*, 1999). Plasmid pAJ1-230 was constructed by ligation of EcoRI/XhoI-digested 690bp PCR fragment to EcoRI/SalI-digested pAS2-1.

Fusion plasmids of yeast GAL4 activation domain with various segments of rat NF1-A were designated as pAH, and followed by numbers which indicate the positions of the amino acid residues.

Table 1. Oligomers used in this study

Oligomers	Sequence
NF1	5'-CCGGAATTCCGCTATTCTCCGCTCTGTCTG-3'
NF230	3'-CTGACTCGATCATTCTCGGCGAGCTCGCC-5'
NF231	5'-CATGCCATGGCAGTGTCACAAACACC-3'
NF447	3'-GGTGGCTACCGGTCCCGAGCTCGCC-5'
NF509	3'-CCTTAGCTCGAATAGGGTCCATGGTCCTG-5'

pAH448-509 was constructed by ligation of 677bp NcoI/SalI fragment of pAJ448-509 to NcoI/XhoI-digested pACT2 (Clontech). pAH1-230 was constructed by ligation of EcoRI/XhoI-digested 690bp PCR fragment to EcoRI/SalI-digested pACT2. pAH231-447 was constructed by ligation of the NcoI/XhoI-digested 648bp PCR fragment to NcoI/XhoI-digested pACT2. pAH231-509 was constructed by ligation of the NcoI/XhoI-digested 834bp PCR fragment to NcoI/XhoI-digested pACT2. pAHCTF1 was constructed by ligation of the NcoI/EcoRI digested fragment of the pBS plasmid (Santoro *et al.*, 1988) to NcoI/EcoRI-digested pACT2.

pYS1 (Yang *et al.*, 1996) was used as a template for PCR amplification of NF1-A cDNA segments. The oligomers used for PCR amplification are oligoNF1, oligoNF230, oligoNF231, oligoNF447, and oligoNF509, of which sequences are listed in Table 1. The numbers of the oligomers represent the positions of oligomers corresponding to the amino acid residues.

Site-directed mutagenesis Site-directed mutagenesis of the heptasequence motif of rat NF1-A was reported previously (Hwang *et al.*, 1999). The alanine substitution mutant of rat NF1-A was obtained in this study, and the mutation was confirmed by nucleotide sequencing.

Yeast transformation and β-galactosidase assay Yeast two-hybrid plasmids were introduced into yeast CTY1 cells by the lithium acetate method. Transformed CTY1 cells with two hybrid plasmids were then selected on synthetic dextrose medium without tryptophan and leucine (SD-Trp-Leu). Yeast cells containing two hybrid plasmids were grown in SD-Trp-Leu medium to an OD_{600nm} of 0.7-1.5, and the activities of β-galactosidase of yeast crude extracts were determined by the method previously described (Hwang *et al.*, 1999). β-galactosidase activities were measured in triplicate samples and at least four differnet experiments were carried for individual samples.

Results and Discussion

Interaction between the C-terminal regions of NF1-A Homo- and heterodimer formations among NF1 proterins have been previously demonstrated *in vitro* by the methods which require DNA binding domains (Gounari *et al.*, 1990; Kruse and Sippel, 1994). The DNA binding domain of NF1 proteins is located in the N-terminal region from residue 1 to 220 which is also known to mediate dimerization of NF1 proteins (Mermod *et al.*, 1989; Gounari *et al.*, 1990; Kruse

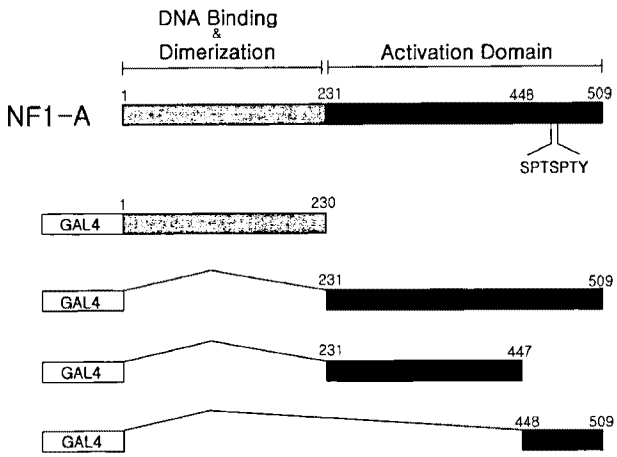

Fig. 1. Schematic diagram of rat NF1-A domains fused with GAL4 DNA binding domain and activation domain. The cDNA fragments coding different domains of rat NF1-A were cloned into two yeast hybrid plasmids, pAS2-1 and pACT2, as described in the "Materials and Methods". The numbers above the boxes indicate the positions of the amino acid residues of fused segments.

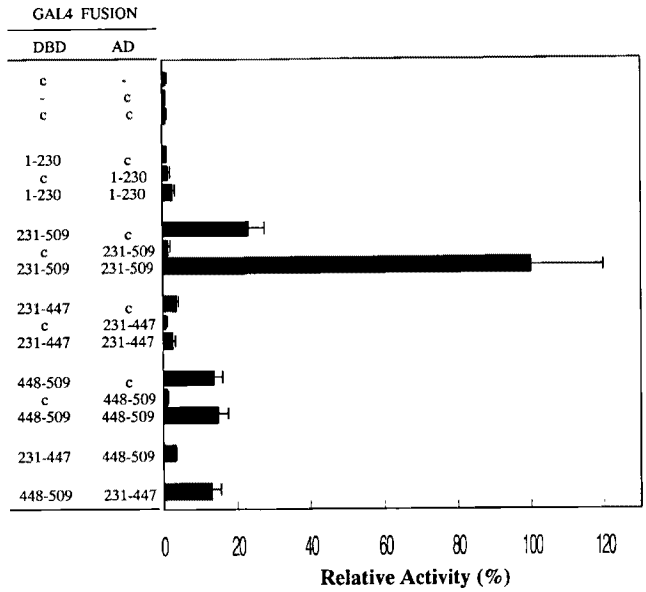

Fig. 2. Interaction between the C-terminal regions of NF1-A from residue 231 to 509. The numbers indicate the positions of the amino acid residues of rat NF1-A which were fused with either GAL4 DNA binding domain (DBD) or activation domain (AD). The letter "c" represent control plasmids, pAS2-1 or pACT2, for the left panel and the right panel, respectively. β-galactosidase activities were measured in triplicate samples and at least four different experiements were carried out for individual samples.

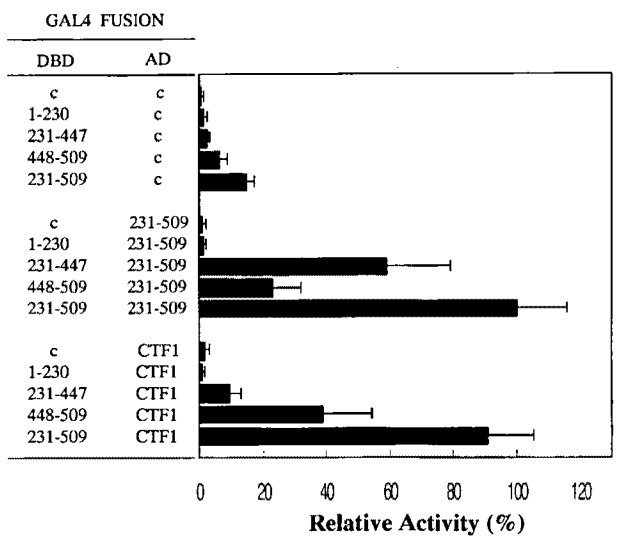

Fig. 3. Interaction of different C-terminal segments of NF1-A with the C-terminal region of NF1-A and with NF1/CTF1. The symbols are the same as described in Figure 2. CTF1 represent human NF1/CTF1 from residue 1 to 499 fused with GAL4 activation domain.

and Sippel, 1994). We attempted to identify additional domains in the C-terminal region of NF1-A which may contribute to the interactions of NF1 proteins. The C-terminal fragments of rat NF1-A activate transcription in yeast (Hwang *et al.*, 1999) as do those of human NF1/CTF1 (Kim and Roeder, 1993; Altmann *et al.*, 1994; Kim and Roeder, 1994; Wendler *et al.*, 1994; Xiao *et al.*, 1994).

In order to test interaction between different domains of NF1-A, we constructed yeast two-hybrid plasmids containing different segments of NF1-A as shown in Figure 1. When the plasmids containing the C-terminal region of NF1-A from residue 231 to 509 fused with the GAL4 activation domain and the GAL4 DNA binding domain, they were introduced together into yeast cells. The activation of transcription was increased by about four fold, indicating that interaction between the C-terminal regions of NF1-A occurred in the absence of the N-terminal dimerization domain (Figure 2). In contrast, the N-terminal regions between residue 1 and 220 unexpectedly didn't interact with each other in two-hybrid system probably due to (1) the DNA binding property of the fragments, (2) improper folding of the N-terminal domain for interaction, and/or (3) some other unknown reason. The expression of GAL4/NF1-A fusion proteins was detected by Western blot analysis (Data not shown).

In order to define the interaction domains, we divided the C-terminal region into two fragments, one from residue 231 to 447 and the other from 448 to 509. We then tested the interaction of the fragments with the C-terminal region of NF1-A. As shown in Figure 3, both of the fragments were able to interact with the C-terminal region from residue 231 to 509, although neither of NF1-A (231-447) nor NF1-A (448-509) interacted with each other in any combinations. Failure of interaction may be due to conformational instabilities of the domains when expressed as separate fragments. The C-terminal region between residue 231 and 509 appears to form

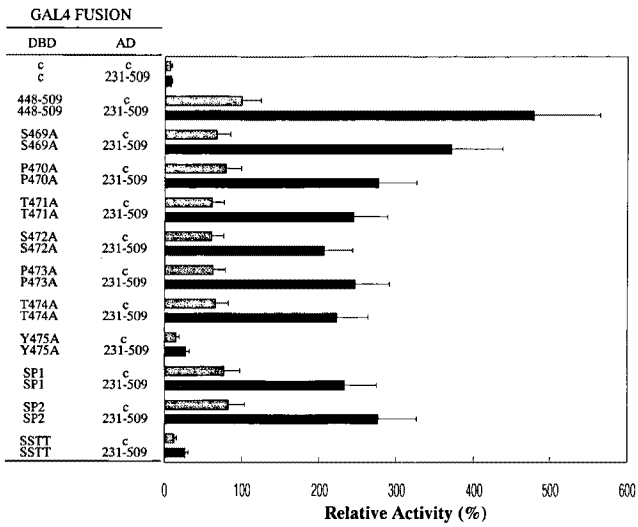

Fig. 4. Effects of the heptasequence, SPTSPTY, of rat NF1-A on the interaction of the C-terminal region of NF1 proteins. The positions of multiple alanine substitutions are indicated in Table 2. The symbols are same as described in Figure 2.

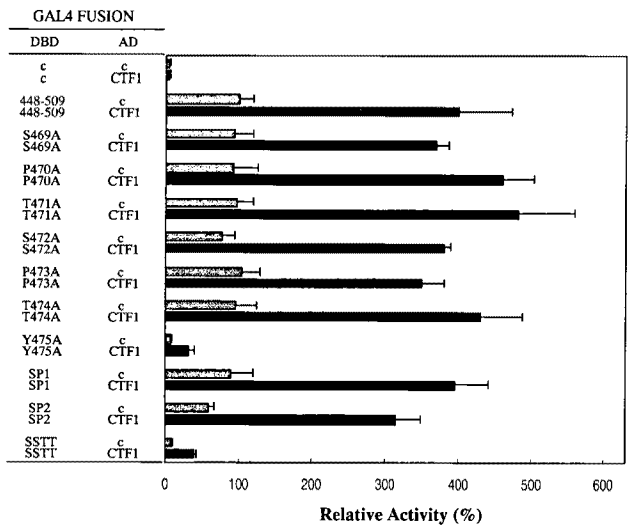

Fig. 5. Effects of the heptasequence, SPTSPTY, of rat NF1-A on interaction between NF1-A and NF1/CTF1 protein, isoform of NF1 family. The positions of multiple alanine substitutions and the symbols are same as those of Figure 4.

Table 2. Amino acid changes of the multiple substitutions in NF1-A

Plasmid	Amino acid change
pSP1	Ser469→Ala Pro470→Ala
pSP2	Ser472→Ala Pro473→Ala
pSSTT	Ser469→Ala Thr471→Ala Ser472→Ala Thr474→Ala

stable conformation. It interacts independently with the separate fragments formed in right conformation at any instance and thus derives the formation of right conformation of the fragments. Our results showed that both fragments contain interaction domains, which can interact with the entire C-termianl region between 231 and 509. This indicating that the interaction between the C-terminal regions of the rat NF1 may be mediated through two separate domains located between 231-447 and between 448 and 509.

Interaction of C-terminal region of NF1-A with CTF1, an isoform of NF1-C proteins The first 220 amino acids of the N-terminal region of rat NF1-A are highly conserved among different types of the NF1 protein family. However, the remaining C-terminal portion shows an heterogeneity, both among NF1 proteins derived from different genes as well as among differentially spliced forms (Gil *et al.*, 1988; Paonessa *et al.*, 1988; Santoro *et al.*, 1988; Rupp *et al.*, 1990; Apt *et al.*, 1994). The C-terminal region of rat NF1-A shows an identity of about 50% with human NF1/CTF1 (Paonessa *et al.*, 1988; Santoro *et al.*, 1988). We tested whether or not interactions can occur through the heterogenous C-terminal regions of NF1-A and NF1/CTF1. Figure 3 shows that C-terminal domains of rat NF1-A interact with human NF1/CTF1 to the comparable levels as that with rat NF1-A. The two interaction domains of NF1-A appear to have different affinities to NF1-A and NF1/CTF1. One interaction domain located between residue 231 and 447 seems to have a higher affinity to NF1-A itself, while the other domain between residue 448 and 509 seems to have slightly higher affinity to NF1/CTF1. Our results suggest that the C-terminal region of NF1 proteins may contribute to formation of homo- and heterodimers among different forms of the NF1 protein family in addition to the N-terminal dimerization domains.

Effect of heptqsequence of CTD region on the interaction of NF1 proteins Rat NF1-A contains a sequence, SPTSPTY, in the position from residue 469 to 475. It is similar to the heptapeptide sequence of the largest subunit of the eukaryotic RNA polymererase II where one amino acid has a substitution from S to T at the sixth residue. Single substitution of amino acid residue from 469 to 474 did not affect the transcription activation ability of NF1-A (448-509) in yeast cells. However, multiple changes of serine and threonine residues, or changes at both 469 and 475 residues, reduced the transcription activity markedly in yeast cells (Hwang *et al.*, 1999).

In order to test whether or not the heptasequence motif also affects interactions between NF1 proteins, we analyzed the interaction of alanine substituted NF1-A in the heptasequence motif NF1-A and with NF1/CTF1. The amino acid residues from 469 to 475 were changed to alanine, and double and quadruple changes were also made in order to change two SP motifs (SP1, SP2), which were predicted to form β-turn

structure, and all serine and threonins residues to alanine (SSTT). As shown Figures 4 and 5, alanine substitution of tyrosine at the 475th residue and all four substitutions of serine and threonine resulted in marked reduction of the interaction with NF1-A itself (Figure 4), and also with NF1/CTF1 (Figure 5). However, single amino acid substitutions from the amino acid residues 469 to 474 affected the interactions only slightly. Our results suggest that the heptasequence motif plays some roles in the interaction between the C-terminal regions of NF1 proteins.

The C-terminal domain (CTD) of the largest subunit of eukaryotic RNA polymerases contain multiple repeats of the heptasequence SPTSPSY, which occurs 52 times in the largest subunit of human RNA Pol II (Cordon, 1990). To date, a number of kinases capable of phosphorylating the CTD of eukaryotic RNA pol II *in vitro* have been identified in addition to TFIIH (Watanabe *et al.*, 2000). Although there is no evidence of phosphorylation of the heptasequence of NF1 proteins, it may be a good candidate for phosphorylation, which may result in the modulation of transcriptional activity by altering the interaction with other accessory proteins or affecting dimer formation. Interactions between the C-terminal regions of NF1 proteins may be affected by the phosphorylation state of the proteins, although we cannot exclude the possibility that changes in the amino acid sequence *per se* may affect interaction between NF1-A(448-509) and NF1-A(231-509). Further studies, such as GST pull-down assays and co-immunoprecipitation experiments, are needed to demonstrate the direct interaction between the C-terminal regions of NF1 and also between the heptasequence motif mutant NF1 proteins and wild type NF1. Direct interactions between the C-terminal regions of NF1 proteins are currently under investigation *in vitro*. Futhermore, proteins, and any protein kinase with which NF1 factors interact, remain to be further elucidated.

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