Functional Analysis of the Heptasequence SPTSPTY in the Transcriptional Activation Domain of Rat Nuclear Factor 1-A

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Nuclear Factor 1 (NF1) proteins are a family of transcriptional factors consisting of four different types: NF1-A, -B, -C, and -X. Some NF1 transcription factors contain a heptasequence motif, SPTSPSY, which is found as a repeat sequence in the carboxyterminal domain (CTD) of the largest subunit of RNA polymerase II. A similar heptasequence, SPTSPTY, is contained in rat liver NF1-A at a position between residues 469 and 475. In order to investigate the roles of the individual amino acids of the heptasequence of rat liver NF1-A in transcriptional activation, we systematically substituted single and multiple amino acid residues with alanine residue(s) and evaluated the transcriptional activities of the mutated NF1-A. Substitution of a single amino acid reduced transcriptional activity by 10 to 30%, except for the proline residue at position 473, whose substitution with alanine did not affect transcriptional activity. However, changes of all four serine and threonine residues to alanine or of the tyrosine residue along with the serine residue at position 469 to alanine reduced the activity to almost background levels. Our results indicate that multiple serine and threonine residues, rather than a single residue, may be involved in the modulation of the transcriptional activities of the factor. Involvement of the tyrosine residue is also implicated.

Keywords: Nuclear factor 1, Transcription, Transcription factors.

Introduction

Eukaryotic transcription factors possess modular structures which often consist of two functionally distinct domains such as a DNA binding domain and an activation domain. The activation domains of transcription factors have been classifed into at least four groups according to their different amino acids composition: acidic domain, glutamine-rich domain, proline-rich domain, and cysteine-containing activation domain (Mitchell and Tjian, 1989; Blau *et al.*, 1996).

Nuclear Factor 1 (NF1) proteins are a family of ubiquitous transcription factors which were originally reported to be required for the replication of adenovirus DNA (Nagata *et al.*, 1982) and also for the transcription of many eukaryotic genes including liver-specific (Colantuoni *et al.*, 1987; Jackson *et al.*, 1993), type I collagen (Ritzenthaler *et al.*, 1993), and p53 (Lee *et al.*, 1999) genes. NF1 proteins bind specifically to a sequence of dyad symmetry, $TGGCN_5GCCAA$ (Mermod *et al.*, 1989; Novak *et al.*, 1992). Sequence homology enabled researchers to identify at least four different genes, NF1-A, B, C, and X (Gil *et al.*, 1988; Rupp *et al.*, 1990). Multiple NF1 proteins have also been generated in higher eukaryotes by differential splicing (Gil *et al.*, 1988; Santoro *et al.*, 1988; Rupp *et al.*, 1990; Apt *et al.*, 1994; Kruse and Sippel, 1994; Wenzelides *et al.*, 1996).

It has been reported that the N-terminal 220 amino acids of human NF1/CTF mediate DNA-binding, dimerization, and stimulation of adenovirus DNA replication, while the C-terminal regions are capable of activating transcription in *Drosophila* and HeLa cells (Mermod *et al.*, 1989; Novak *et al.*, 1992). The N-terminal domains are highly conserved among these factors, while the C-terminal transactivation domains are heterogeneous (Gil *et al.*, 1988). Several investigators have independently reported that a transcriptional activation domain of human NF1/CTF contains a heptasequence motif, SPTSPSY (Kim and

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Roeder, 1993; Altman et al., 1994; Wendler et al., 1994; Xiao et al., 1994), which is strongly related to the heptasequence present in the carboxyterminal domain of RNA polymerase II (Cordon, 1990). The carboxyterminal regions of human NF1/CTF are able to activate transcription in yeast-like acidic activation domains (Kim and Roeder, 1993; Altmann et al., 1994; Wendler et al., 1994; Xiao et al., 1994).

Rat NF1-A was isolated from liver (Paonessa et al., 1988; Jung et al., 1993). Sequence comparisons of rat NF1-A cDNA with other NF1 cDNAs reveal that the C-terminal region of NF1 proteins is heterogeneous in contrast to the N-terminal region which is about 98% homologous among the different NF1 family members (Gil et al., 1988; Paonessa et al., 1988). Rat NF1-A also contains a similar heptasequence in the carboxyterminal region as human NF1/CTF.

The heptasequence in the CTD region of RNA polymerase II is phosphorylated mostly at serine and to a lesser extent at threonine and tyrosine (Trigon et al., 1998). However, it has not been demonstrated whether the heptasequence in NF1/CTF or any other NF1 protein is subject to phosphorylation. In order to test the possible roles of individual amino acids of the SPTSPTY heptasequence, of rat liver NF1-A for transcriptional activation, we analyzed the effects of each amino acid by mutagenesis. In this study, we report that multiple substitutions of serine and threonine and substitution of tyrosine along with a single serine residue resulted in marked reduction of the transcriptional activity of NF1-A, indicating that phosphorylation may be involved in the functional roles of NF1-A.

Materials and Methods

Strains and growth of cells E. coli DH5α was used for maintenance and amplification of plasmids. E.coli CJ236 was used for mutagenesis.

S. cerevisiae strain CTY1 (MATα ade⁻ 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 the expression of GAL4/NF1-A fusion plasmids. CTY1 cells were grown in YPD medium (2% bactopeptone, 1% yeast extract, 2% glucose) and the transformed CTY1 cells with pAS2-1-based plasmids were selected in Trp-synthetic dextrose medium (SD-Trp).

Plasmids pYS1 contains the complete coding sequence of rat NF1-A. pYS1r was derived from pYS1 by introducing an EcoRI site at the ATG initiation codon of NF1-A by site-directed mutagensis (Yang et al., 1996).

pAS2-1 (Clontech) was used for construction of yeast GAL4-NF1A fusion plasmids. pAS2-1 was digested with BamHI, filled-in with Klenow, and digested with SaⅡ. The 656 bp StuⅠ/SaⅡ NF1-A cDNA fragment was obtained from pYS1r, and then ligated to pAS2-1 predigested as above. The fusion plasmid was designated as pAJ448-509. Plasmid pAJ231-509 was constructed by ligation of an NcoⅠ/XhoⅠ-digested 834 bp PCR fragment to the NcoⅠ/SaⅡ DNA fragment of pAS2-1. Plasmid pAJ231-447 was constructed by ligation of the NcoⅠ/XhoⅠ-digested 648 bp PCR fragment to the NcoⅠ/SaⅡ DNA fragment of pAS2-1. pYS1 was used as a template for PCR amplification of NF1-A cDNA segments. The oligomers used for PCR amplification were oligoNF231, oligoNF447, and oligoNF509 whose sequences are listed in Table 1.

Site-directed mutagenesis Site-directed mutagenesis was performed according to a method previously described (Kunkel, 1985). The mutagenic oligomers used in this study are listed in Table 1.

β-galactosidase assay Each fusion plasmid was introduced into yeast CTY1 cells by the lithium acetate method (Ito et al., 1983) and the transformed CTY1 cells were selected in a synthetic dextrose medium containing 0.17% YNB-AA/AS, 0.5% $(NH_4)_2SO_4$, and 2% dextrose (SD-Trp).

The filter lift assay was performed using Xgal as substrate according to the supplier's protocol (Clontech #PT1030-1). β-galactosidase activity was measured using o-nitrophenyl-β-galactoside as the substrate according to a method previously

Table 1. Oligomers used in the study.

Uses	Oligomers	Sequence
PCR	NF231	5'-CATGCCATGGCAGTGTCACAAACACC-3'
	NF447	5'-CCGCTCGAGCCCTGGCCATCGGTGG-3'
	NF509	5'-GTCCTGGTACCTGGGATAAGCTCGAGGCC-3'
Mutagenesis	S469A	5'-GGTGCAGCCGCCCCCACCTCA-3'
	P470A	5'-GCACCTCCGCCACCTCACCG-3'
	T471A	5'-TCGAGTAGGTCGGTGAGGCGGGGGAGGCTGCAC-3'
	S472A	5'-CTCCCCCACCGCACCGACCTAC-3'
	P473A	5'-GTGGTGTCGAGTAGGTCGCTGAGGTGGGGGAGGC-3'
	T474A	5'-TGCTGGTGTCGAGTAGGCCGGTGAGGTGGGGG-3'
	Y475A	5'-CTCACCGACCGCCTCGACACCCAG-3'
	SP1	5'-CGGTGCAGCCGCCGCCACCTCACCGAC-3'
	SP2	5'-CTCCCCCACCGCAGCGACCTACTCGAC-3'
	SSTT	5'-CGGTGCAGCCGCCCCCGCCGCACCGGCCTACTCGACACCC-3'

described (Guarente, 1983). One unit of β-galactosidase was defined as the amount of enzyme hydrolizing 1 μmol of o-nitrophenyl-β-galactoside to o-nitrophenyl and D-galactose per min at 30°C.

Western blot analysis Yeast-transformed cells containing appropriate plasmids were grown in minimal liquid medium (SD-Trp) and harvested at a cell density of OD_{600} 0.5–1.0. Cell pellets were resuspended in alkaline lysis buffer (50 mM NaOH, 2mM EDTA, 2% SDS, 10% glycine, 5% β-mercaptoethanol, 160 μg/ml Benzamidine, 10 μg/ml PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin), boiled, and neutralized with HCl. After centrifugation, proteins in the supernatant were separated on an SDS-polyacrylamide gel. Proteins were transferred onto ECL-nitrocellulose membrane (Amersham) and incubated with a primary mouse monoclonal antibody against the GAL4 DNA-binding domain (Santa Cruz Biotechnology). Goat anti-mouse IgG-HRP antibody (Biorad) was used as secondary antibody. The complex was detected by ECL detection system (Amersham Braunschweig, Germany) according to a method described previously (Hwang, 1998).

Results and Discussion

The proline-rich activation domain of mammalian NF1 transcription factors is transcriptionally active, similar to an acidic transcription activation domain, in both yeast and mammalian cells (Kim and Roeder, 1993; Altmann *et al.*, 1994; Wendler *et al.*, 1994; Xiao *et al.*, 1994; Yang *et al.*, 1996). In order to measure the transcriptional activity of rat liver NF1-A, we fused C-terminal segments of NF1-A with the yeast GAL4 binding domain (1–147) contained in the pAS2-1 plasmid, which was then introduced into yeast cells. As shown in Fig. 1A, all GAL4/NF1-A fusion proteins containing three different segments of C-terminal domains activated transcription in yeast. The C-terminal domain from amino acid residues 448 to 509 of rat NF1-A, containing the sequence motif SPTSPTY, had about 60% activity, while the segment between residues 231 and 447 had about 30% activity of that of the entire activation region between amino acid residues 231 and 509 (Fig. 2). This result indicates that there are two transcriptional activation domains in rat NF1-A which can act independently of each other. We eliminated the 1–230 portion because the N-terminal portion of NF1-A contains a DNA-binding domain and the GAL4 fusion protein containing the whole NF1-A had little transcriptional activity (Yang *et al.*, 1996).

The region of rat NF1-A from residues 469 to 475 contains a sequence, SPTSPTY, which is similar to the heptapeptide sequence of the largest subunit of eukaryotic RNA polymererase II, with only one amino acid substitution from S to T at the sixth residue. The functional roles of each amino acid residue of the heptasequence motif was not yet demonstrated thoroughly, although deletions of the sequence motif of human NF1/CTF abolished the transcription activity of human NF1/CTF
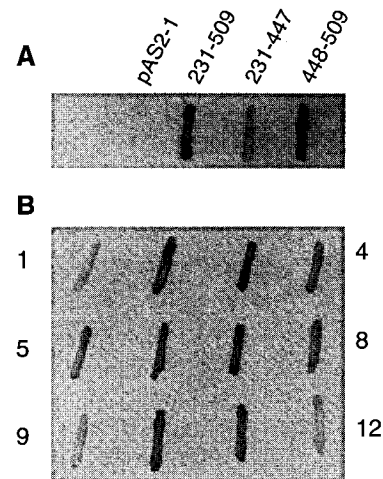

Fig. 1. Measurement of the transcriptional activity of GAL4/ NF1-A fusion proteins by the filter lift assay. Assays were carried out as described in the Materials and Method section. **A.** Transcriptional activation by the GAL4 DNA-binding domain fused with different C-terminal segments of rat NF1-A proteins. **B.** Transcriptional activation by the GAL4 DNA-binding domain fused with the NF1-A C-terminal segment from amino acid residues 448 to 509. The fusion plasmids are as follows: 1, pAS2-1; 2, pAJ448-509; 3, pAJS469A; 4, pAJP470A; 5, pAJT471A; 6, pAJS472A; 7, pAJP473A; 8, pAJT474A; 9, pAJSY; 10, pAJSP1; 11, pAJSP2; 12, pAJSSTT.
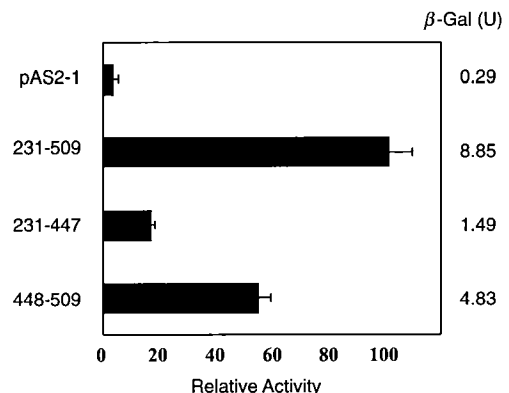

Fig. 2. Transcriptional activities of GAL4/NF1-A fusion proteins. β-galactosidase activities of CTY1 cells harboring the GAL4/NF1-A fusion plasmids were measured in triplicate samples. The segments of NF1-A are indicated at the left side of the figure.

(Wendler *et al.*, 1994). Thus, we further investigated the roles of the sequence motif by systematic mutations of the heptasequence. Amino acid substitutions made in this study are summarized in Table 2. The nucleotide sequences of the mutated regions were confirmed by nucleotide sequencing (data not shown). The mutation of S469A and Y475A was fortuitously obtained using oligoY475A, and was probably due to error during synthesis by T7 DNA

Table 2. Nucleotide and amino acid changes in the mutant forms of NF1-A.

Plasmid[a]	Nucleotide position[b]	Nuceotide change	Amino acid change
S469A	1405	TCC→GCC	Ser469→Ala
P470A	1408	CCC→GCC	Pro470→Ala
T471A	1411	ACC→GCC	Thr471→Ala
S472A	1414	TCA→GCA	Ser472→Ala
P473A	1417	CCG→GCG	Pro473→Ala
T474A	1420	ACC→GCC	Thr474→Ala
SY	1405	TCC→GCC	Ser469→Ala
	1423, 1424	TAC→GCC	Tyr475→Ala
SP1	1405	TCC→GCC	Ser469→Ala
	1408	CCC→GCC	Pro470→Ala
SP2	1414	TCA→GCA	Ser472→Ala
	1417	CCG→GCG	Pro473→Ala
SSTT	1405	TCC→GCC	Ser469→Ala
	1411	ACC→GCC	Thr471→Ala
	1414	TCA→GCA	Ser472→Ala
	1420	ACC→GCC0	Thr474→Ala

[a] The mutated cDNA segments coding NF1-A from residues 448 to 509 were cloned into pAS2-1 plasmid.

[b] The nucleotide position (+1) corresponds to A of the initiation codon, ATG.

polymerase. The amino acid residues from 469 to 474 were changed to alanine, and double and quadruple changes were also made in order to change two SP motifs and all serine and threonine residues to alanine. Mutated NF1-A segments from residues 448 to 509 were fused with the GAL4 DNA binding domain of pAS2-1 plasmids and introduced into yeast CTY1 cells. Expression of the fused mutant proteins was detected by Western blot analysis, as shown in Fig. 3. The fused mutant proteins were expressed in yeast cells to almost the same extent. Measurement of β-galactosidase activities showed that single amino acid substitutions of the heptasequence did not significantly affect transcriptional activities (Figs. 1B and 4). The transcriptional activity of S472A was reduced by about
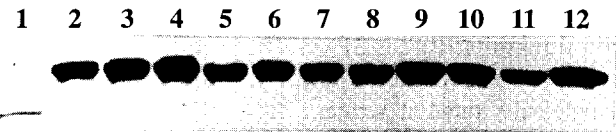

Fig. 3. Western blot analysis of GAL4/NF1-A fusion protein containing NF1-A segments with substituted amino acid(s) between residues 448 and 509. Total cell lysates of CTY1 cells harboring fusion plasmids were separated on 15% SDS-PAGE, transferred onto an ECL-nitrocellulose membrane and probed with monoclonal antibody against GAL4DBD. Lanes 1, pAS2-1; 2, pAJ448-509; 3, pAJS469A; 4, pAJP470A; 5, pAJT471A; 6, pAJS472A; 7, pAJP473A; 8, pAJT474A; 9, pAJSY; 10, pAJSP1; 11, pAJSP2; 12, pAJSSTT.
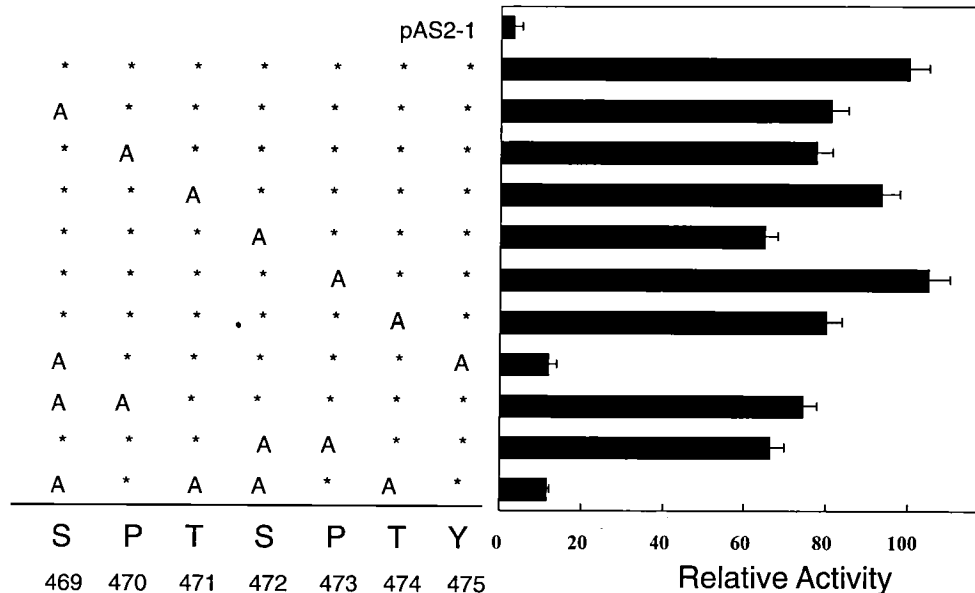

Fig. 4. Transcriptional activities of GAL4/NF1-A fusion proteins containing NF1-A segments with substituted amino acid(s) between residues 448 and 509. The heptasequence, SPTSPTY, between residues 469 and 475 is indicated in the left panel of the figure. Above the heptasequence are indicated amino acid substitutions with wild-type amino acids symbolized as asterisk marks (*). β-galactosidase activities of all extracts were measured in triplicate samples.

40%, the lowest among the singly-substituted fused proteins. Double mutation of S469A and Y475A unexpectedly affected the transcriptional activity markedly, with activity reduced to about 10% of that of wild-type protein. This result indicates that the tyrosine residue at position 475 may be important in the transcriptional activity or that substitution of the two residues may have resulted in some structural changes of the domain which may in turn have affected transcriptional activity. Substitutions of two serine residues and two threonine residues resulted in marked reduction of transcriptional activity, indicating that phosphorylation of multiple serine and threonine residues may be involved in the transcriptional activity of the factor.

The SP motif in the heptasequence has been predicted to form a β-turn structure (Kim *et al.*, 1994). In order to test the possible role of the β-turn structure in the activation, we made alanine substitutions at the two SP motifs. The alanine-substituted SP motifs retained significant transcription activities at levels slightly lower than factors single-substituted at serine.

NF1 transcription factors may activate transcription by binding to specific binding sites as a dimer. It has been demonstrated that CTF/NF1 was bound to DNA as homodimers (Mermod *et al.*, 1989) and that different combinations of heterodimers could also be formed (Kruse and Sippel, 1994). NF1 transcription factors may interact with components of the basal transcription initiation machinery and facilitate the assembly of functional transcriptional initiation complexes. Tanese *et al.* (1991) reported that the TFIID fraction stimulated transcription by proline-rich activators *in vitro*, while purified TBP did not, indicating that certain TAFs of TFIID may interact with proline-rich transcription factors. NF1 proteins promote transcription of the mouse mammary tumor virus LTR promoter by synergistic cooperation with steroid receptors, which may be involved in the chromatin-regulated transcriptional control (Kusk *et al.*, 1996). Recently, it has been reported that NF1 transcription factors mediate expression of the collagen gene stimulated by TGF-β (Ritzenthaler *et al.*, 1993). NF1 and any other proteins interacting with NF1 may serve as potential targets for TGF-β signaling pathways.

Although there is no evidence of phosphorylation of the heptasequence of NF1, it may be a good candidate for phosphorylation, resulting in the modulation of transcriptional activity by altering its interaction with other accessory proteins or affecting dimer formation. Proteins and protein kinases with which NF1 factors might interact remain to be further elucidated.

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