Molecular Cloning and Functional Characterization of the Transcription Factor YY2*

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YY1 is a ubiquitous zinc finger transcription factor that binds to and regulates promoters and enhancers of many cellular and viral genes. Here we report the isolation of a human cDNA encoding a DNA sequence-specific binding protein with significant homology to the transcription factor YY1. A sequence analysis of this novel protein, YY2, revealed an overall 65% identity in the DNA sequence and a 56% identity in protein sequence compared with human YY1. The most pronounced similarity between YY1 and YY2 exists within the zinc finger regions of the two proteins, and consistent with this observation, YY2 can bind to and regulate some promoters known to be controlled by YY1. Similar to YY1, YY2 contains both transcriptional activation and repression functions. The finding of a protein with structure and function similar to YY1p.m. provides a new opportunity to explore additional mechanisms by which YY1-responsive genes can be regulated and suggests that gene regulation by YY1 is far more complicated than previously assumed.

YY1 is a ubiquitously expressed zinc finger protein that binds to many different cellular and viral promoters in a sequence-specific manner to regulate transcription (1–5). The activities of YY1 are regulated by acetylation (6), phosphorylation (7, 8), poly-ADP-ribosylation (9), and O-GlcNAc-ylation (10). YY1 has been identified and cloned in Xenopus and Drosophila in addition to humans and mice (11–15).

Early investigations firmly established that YY1 activates or represses the transcription of many different genes, and recent studies are beginning to uncover the biological functions and the underlying mechanisms of YY1 action. For example, we now know that the targeted disruption of YY1 in mice results in peri-implantation lethality, thus arguing that YY1 is essential for mouse embryo development (16). In vivo studies in Drosophila show that YY1 functions as a polycistronic group protein that maintains transcriptional repression patterns during embryogenesis (15). YY1 also participates in checkpoint functions that regulate cell cycle transitions in differentiated cells (17). YY1 is overexpressed in failing human hearts and in a transgenic mouse model of hypertrophic cardiomyopathy (18). Additionally, YY1 is a component of a repressor complex that binds to a chromosomal repeat that is deleted in facioscapulohumeral muscular dystrophy (19).

A chief mechanism by which YY1 activates and represses transcription is the interaction with cellular transcription factors including TBP, TAFs, TFIIB, and Sp1 (7, 20–23). Alternatively, although not mutually exclusively, YY1 recruits histone modification enzymes including p300, HDACs, and PRMT1 to regulate transcription (24–26). It is estimated that >7% of all vertebrate gene promoters contain at least one YY1 consensus-binding site (27). Thus, YY1 potentially controls the expression of a vast array of genes ranging from those that are important in basic cellular processes such as DNA replication, transcription, and cell cycle control to genes that are directly linked to the immune response, cancer, viral infections, and development (28, 29).

Proteins that occupy a critical role in the cell often occur in multiple homologous yet distinct forms. Many transcription factors, once thought to exist as a single member, are now known to belong to a family of proteins. For example, several highly conserved proteins homologous to one of the first and best characterized DNA-binding transcription factors, Sp1, exist in humans (30–33). Reciprocally, DNA-control elements such as AP1- and cAMP-response element-binding sites located in the promoters and enhancers of many eukaryotic genes bind families of multiple transcription factors (34, 35).

Here we report that, similar to Sp1 and many other human DNA sequence-specific binding transcription factors, YY1 also exists in a family of more than one protein. Using DNA and amino acid sequence data base analysis, we discovered that at least one protein with a structure and functions similar to those of YY1 (hereafter designated YY2) is present in humans. As determined by Northern blot analysis, YY2 is derived from multiple mRNAs with a predominant transcript of ~7.3 kb. YY2 migrates as a protein of ~58 kDa on SDS-polyacrylamide gels and is expressed in multiple tissues. In gel shift assays, YY2 binds specifically to a YY1-consensus sequence and to some, but not all, promoter sequences previously shown to interact with YY1. Deletion analysis of a Gal4-YY2 fusion protein indicates that YY2 contains both activation and repression domains. Finally, using overexpression and siRNA technology, we show that YY2 activates a number of promoters previously demonstrated to be responsive to YY1. Our results suggest that YY2 is intimately involved in the regulation of genes previously known to be controlled by YY1.
Identification and cDNA Cloning of YY2—The NCBI BLASTP program was used to search for proteins with a similarity to YY1. Several human clones that potentially encode proteins with significant homology to YY1 were found, and primers were designed to amplify one of the human clones that potentially encode proteins with significant homology to YY1. Several degenerate oligonucleotide primers were designed, and these were used in PCR assays to amplify the full-length YY2 fragment from HeLa cells followed by resuspension in 10 mM Tris-Cl, pH 7.5, and 10% glycerol.

Ribonuclease Protection Assays—Total RNA was purified using TRIzol reagent (Molecular Research Center Inc.) and injected into New Zealand White rabbits. Polyclonal anti-YY2 antibodies were affinity-purified using GST-YY2 protein cross-linked to Affi-Gel 15 (Bio-Rad). Anti-Gal4 antibody was purchased from Santa Cruz Biotechnology. For immunoblotting, cell lysates were resolved on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat milk for 1 h and then incubated with the primary antibody followed by a horseradish peroxidase-conjugated secondary antibody. The proteins were visualized using Supersignal West Penta (Pierce).

Electrophoretic Mobility Shift Assays (EMSA)—Single-stranded oligonucleotides were end-labeled individually using [γ-32P]ATP and T4 polynucleotide kinase, heated together at 90 °C, and allowed to anneal by slowly cooling to room temperature. Binding reactions were performed in a 20-μl reaction volume containing 12 μM HEPES (pH 7.9), 10% glycerol, 1 mM MgCl2, 60 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, 50 μg/ml bovine serum albumin, 0.05% Nonidet P-40, 0.1 μg of poly(dI-dC), 100 ng of purified YY1 or YY2 protein, and 5 fmol of radiolabeled DNA. Reactions were incubated for 10 min at room temperature and separated on 5% non-denaturing polyacrylamide gels. The gels were then blotted and exposed to film.

Assays of Endogenous c-myc and b-actin Expression—Human osteosarcoma U2OS cells grown on (5-cm dishes, 10 6 μg of PBS/U6 or PBS/U6-YY2 plasmid using LipofectAMINE™ 2000 (Invitrogen). After 72 h, total RNA was isolated with TRIzol reagent according to the manufacturer’s suggested protocol. Reporter and effector plasmids were cotransfected into HeLa cells grown in 6-well plates at 50–70% confluence (seeded at 5 × 105 cells/well a day earlier). 48 h post-transfection, cells were harvested and luciferase activities were measured using a Berthold Lumat model LB 9501 lumimeter. Each experiment was repeated at least three times, and all of the transfections were performed in duplicates to ensure reproducibility.

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Ribonuclease Protection Assays—Total RNA was purified using TRIzol reagent (Invitrogen). After separation on formaldehyde-agarose gels, RNA was transferred onto Biobond nylon membranes (Sigma) and cross-linked to the membranes in a Stratalinker. An 870-bp EcoRI/HindIII cDNA fragment encoding the N-terminal portion of YY2 was labeled with [γ-32P]ATP (PerkinElmer Life Sciences) using a DECA prime II kit (Ambion). Hybridizations with the radiolabeled probes were performed overnight in PerfectHyb Plus Solution (Sigma) at 65 °C. Blots were washed twice at room temperature with 2× SSC, 0.1% SDS before exposure to x-ray film and subsequent visualization by autoradiography.

Ribonuclease Protection Assay—pG4A-YY2-1(–102) served as a template in a PCR reaction to generate a YY2 probe with primers 5'-GGATTAGGGCGACCATC-3' and 5'-TTAATAGGGCGACCATC-3'. The PCR product was gel-purified and labeled with [α-32P]UTP using the MAXIScript™ in vitro transcription kit (Ambion). Ribonuclease protection assays were performed according to the protocol described in the RPA III kit (Ambion). The labeled probe was precipitated with the indicated amount of total RNAs isolated from HeLa cells followed by resuspension in 10 μl of hybridization buffer. After overnight incubation at 42 °C, the RNA sample mixture was digested for 30 min at 37 °C using the RNase A/RNase T1 mixture at 1:300 dilutions. The reaction was terminated, and the sample was precipitated and subsequently resuspended in 10 μl of gel loading buffer and resolved on a 6% denaturing polyacrylamide gel. The gel was transferred to 3 μl of blotting paper, dried, and exposed to x-ray film overnight at −80 °C.

Antibodies and Western Blots—Histidine-tagged YY2(1–167) fusion protein was expressed in bacteria using pET-YY2(1–167), purified in a nickel column (Novagen) as described previously (2), and injected into New Zealand White rabbits. Polyclonal anti-YY2 antibodies were affinity-purified using GST-YY2 protein cross-linked to Affi-Gel 15 (Bio-Rad). Anti-Gal4 antibody was purchased from Santa Cruz Biotechnology. For immunoblotting, cell lysates were resolved on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat milk for 1 h and then incubated with the primary antibody followed by a horseradish peroxidase-conjugated secondary antibody. The proteins were visualized using the SuperSignal West Penta (Pierce).

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RESULTS

Identification and Cloning of Human YY2 cDNA—The human YY1 amino acid sequence (2) was used to screen the NCBI data base for proteins with homology to YY1. Our search identified a cDNA clone (AK091850) that potentially encodes a protein with a similarity to YY1. This clone was submitted previously to the NCBI data base by the NEDO human cDNA sequencing project group. We refer to this clone as YY2. The YY2 DNA sequence also was found in a genomic clone (U73479) derived from a chromosome X-specific cosmid library (41). Another clone (BC012905) possessed an identity to YY2, but it may have retained exons and introns from the S2P (site 2 protease) gene.

YY2 and YY1 are 56.2% identical in an overall amino acid sequence and 86.4% identical in the zinc fingers region (Figs. 1, A and B). The YY2 protein has a theoretical molecular mass of 41.4 kDa and an isoelectric point of 5.98. As determined by a human genome BLAST analysis using the NCBI Map Viewer program, the YY2 gene is located on Xp22.1–22.2 of the human X chromosome. An analysis of YY2 DNA sequence reveals a poly(A) addition signal (AATAAA) 54 nucleotides downstream of the YY2 stop codon as well as an in-frame stop codon (TAA) 9 nucleotides upstream of the presumed start codon. YY2 cDNA isolated from HeLa cells by PCR migrates as a single band of 1.1 kb in an agarose gel (Fig. 1C). There are two nucleotide differences between our PCR product and the cDNA clone (AK091850), which was deposited in GenBank™. Nucleotides 26 and 431 are C and G, respectively, in our PCR product and T and A, respectively, in the AK091850 clone. Thus, the YY2 cDNA we have cloned contains a threonine at residue 9 and an arginine at residue 144 rather than an isoleucine and a lysine, respectively.

Analyses of YY2 Transcripts in HeLa Cells—To demonstrate the existence of YY2 RNA, a Northern blot was performed using total RNA isolated from HeLa cells and a unique 5′ region of the YY2 cDNA as the probe. As shown in Fig. 2A, the YY2 transcript of 11.9 and 10.9 kb was observed. Two faint minor bands of ~11.9 and 10.9 kb and a band of ~6.3 kb with intermediate intensity were also detected. To further confirm the existence of YY2 RNA, we performed a ribonuclease protection assay. As shown in Fig. 2B, antisense YY2 RNA probes were protected from ribonuclease digestion when hybridized with total RNA isolated from HeLa cells (lanes 3–5). This finding demonstrates the presence of YY2 mRNA in HeLa cells.

Expression of YY2 Protein—To examine the expression of YY2 protein, a polyclonal anti-YY2 antibody was raised in rabbits using recombinant YY2 (residues 1–167) fused to six histidines. The anti-YY2 antibody recognized recombinant GST-YY2 (Fig. 3A, lane 7) but not GST (lane 6) or GST-YY1 (lane 8) in Western blots. It also recognized a protein of ~58 kDa in whole cell lysates of HeLa cells (Fig. 3B, left panel). Extracts from various normal human tissues were tested for the expression of YY2. As shown in Fig. 3B, right panel, YY2 is expressed in kidney, liver, spleen, and testicle but not in the colon (compare lane 1 with lanes 2–5).

DNA Binding Properties of YY2—The presence of highly conserved Kruppel-like zinc fingers in YY2 suggests that YY2 is a sequence-specific DNA-binding protein. The 86% identity between the zinc finger regions of YY2 and YY1 predicts that YY2 will interact with a consensus YY1 DNA-binding site. As shown by EMSA, this is indeed the case. In these assays, purified His-YY2 was incubated with a 32P-labeled double-stranded oligodeoxynucleotide probe containing a YY1 consensus site (Table 1). Two protein-DNA complexes were detected by non-denaturing gel electrophoresis (Fig. 4, lane 2). The formation of these two YY2-DNA complexes are specific, be-
are present in a number of different promoters (Table I and Fig. 5A).

As demonstrated by EMSA, YY2 interacted with oligodeoxynucleotides containing YY1-binding sites of promoters for granulocyte macrophage-colony-stimulating factor, p53, c-Myc, c-Fos, and CXCR4, albeit with different affinities (Fig. 5A, lanes 2, 4, 8, 9, and 11).

The binding of YY2 to these promoter sequences is specific because it could be competed by excess unlabeled specific oligodeoxynucleotides but not by oligodeoxynucleotides containing an AP-1-binding site (Fig. 5B, lanes 2–8). Under identical conditions, YY2 did not interact with the YY1 sites of promoters for interferon γ, ENOS, Spi2.1, LDLR, or H4 (Fig. 5A, lanes 3, 5–7, and 10).

Activation and Repression Domains of YY2—Full-length YY1 represses the transcription of promoters containing Gal4-binding sites when transiently expressed in cells as a Gal4 fusion protein (2, 25, 42–45). However, deletion analysis of YY1 demonstrates that YY1 contains the multiple activation and repression domains. To determine whether YY2 mimics YY1 in this respect, we cotransfected HeLa cells with an expression plasmid encoding Gal4-YY2 and a target plasmid, pGal4tk-Luc, that contains five Gal4-binding sites. As shown in Fig. 6A, Gal4-YY2 maximally repressed the activity of pGal4tk-Luc 10-fold. As determined by a detailed deletion analysis, YY2 contains a potent activation domain in the N terminus (residues 32–102) and a repression domain in the C terminus (residues 237–372) (Fig. 6B). Additional repression domains may be present in the region between residues 102 and 237. As assessed by Western blots using an anti-Gal4 antibody, all of the Gal4 fusion proteins were expressed in HeLa cells or human tissues and the anti-YY2 polyclonal antibody. The blot displayed on the right panel was stripped and re-probed with an anti-β-actin antibody to ensure equal loading and transfer of proteins.
functions of YY2 are closely analogous to those of YY1.

Regulation of Promoter Activities by YY2—As shown in Fig. 5, YY2 interacts with some but not all of the promoters containing YY1-binding sites. To determine whether the binding of YY2 to these promoters affects their activities, we cotransfected HeLa cells with a YY2 expression plasmid (pcDNA3-YY2) and a luciferase reporter linked to a promoter that interacted with YY2 in our EMSA experiments. Similar to YY1 (46), YY2 activated the p53 promoter (Fig. 7, top left panel). However, in contrast to YY1, which represses the activity of c-Fos (47–49), YY2 increased the activity of the c-Fos promoter (bottom left panel). The activation of the c-Fos promoter was more pronounced at lower concentrations of YY2 than at higher concentrations. Previous studies have shown that YY1 activates the c-Myc promoter (50) but reduces the activity of the CXCR4 promoter (51). Intriguingly, YY2 activated both promoters at low concentrations and repressed them at high concentrations (right panels). Therefore, the activities of four promoters that bind YY2 are regulated by YY2 but in different manners.

To demonstrate the need for YY2 in activating the promoters examined in Fig. 7, we depleted YY2 in HeLa cells by RNA interference. YY2 siRNA synthesized from the BS/U6 template efficiently inhibited transiently expressed (Fig. 8A) and endogenous (Fig. 8B) YY2 but did not affect the expression of YY1 (Fig. 8A) or the control protein β-actin (Fig. 8B). In agreement with the observation that overexpression of YY2 increases the activity of p53, c-Fos, c-Myc, and CXCR4, the depletion of YY2 significantly reduced the expression of these promoters as assessed by luciferase reporter assays (Fig. 8C).
FIG. 6. Transcription activation and repression by the cloned YY2. A, transient transfection followed by luciferase assays show that a Gal4-YY2 fusion represses transcription when targeted to a promoter containing Gal4-binding sites. Gal4-YY1 was used for comparison. Amount of DNA transfected is as follows: 1.5 μg (Gal4); 1.5, 3, and 4.5 μg (Gal4-YY2); 1.5 μg (Gal4-YY1); and 0.75 μg (reporter). B, names and schematic drawing of plasmids used in transient transfections. All of the transfections were normalized to equal amounts of DNA with parental expression vectors. The results are the mean ± S.D. from at least three separate transfections. Bottom panel, a Western blot was performed on extracts prepared from transfected cells using an anti-Gal4 antibody to show approximately equal expression of each fusion protein.

FIG. 7. YY2 regulates transcription from promoters with YY1-binding sites. Luciferase reporter assays showing that overexpression of YY2 can alter transcription of promoters containing YY1-binding sites. In each experiment, 0.05, 0.1, 0.5, 1, 2, or 4 μg of effector plasmid pcDNA3-YY2 and 0.5 μg of reporters were used.

Whether more than one form of YY2 protein exists in humans is in progress.

An inspection of the predicted YY2 protein sequence revealed two notable features. First, a serine-rich region exists between residues 122 and 163 of YY2. Second, four C2H2-type zinc finger motifs with a striking similarity (86% identity) to the zinc fingers of YY1 are present between residues 256 and 365. Proteins containing Ser-rich regions are common in nature, and the functions of such domains vary widely. Of particular interest is the fact that some Ser/Thr-rich domain proteins can activate transcription (e.g. Refs. 52–54). In our current study, we found that the YY2 transcriptional activation domain (residues 32–102) lies outside the Ser-rich region, suggesting that the Ser-rich motif in YY2 possesses functions unrelated to transcription activation.

C2H2 zinc fingers are one of the most common DNA binding motifs in eukaryotic transcription factors. YY1 contains four C2H2 zinc finger motifs with a striking similarity (86% identity) to the zinc fingers of YY1 are present between residues 256 and 365. Proteins containing Ser-rich regions are common in nature, and the functions of such domains vary widely. Of particular interest is the fact that some Ser/Thr-rich domain proteins can activate transcription (e.g. Refs. 52–54). In our current study, we found that the YY2 transcriptional activation domain (residues 32–102) lies outside the Ser-rich region, suggesting that the Ser-rich motif in YY2 possesses functions unrelated to transcription activation.

C2H2 zinc fingers are one of the most common DNA binding motifs in eukaryotic transcription factors. YY1 contains four C2H2 zinc fingers that are related to those of the GLI-Krüppel family of proteins and to those of the REX-1 protein in particular (2, 55). The YY1 zinc fingers are extremely conserved among different species with a 95% identity between human and mouse (4, 5), 95% identity between human and Xenopus (11), and 91% identity between human and Drosophila (14). In YY1, the zinc finger motifs together serve as a DNA sequence-specific binding domain and also contribute to the ability of YY1 to repress transcription (reviewed in Refs. 28 and 29). We show that YY2 contains a transcriptional repression domain.
that overlaps with its zinc fingers domain and that YY2 interacts with DNA sequences that recognize YY1. These findings attest to the remarkable similarity of the zinc finger regions of YY1 and YY2. In the future, it would be interesting to determine whether the zinc finger motifs of YY2 are highly conserved among various species.

Unlike the similarity between REX-1 and YY1, which is restricted to the zinc finger motifs, the homology between YY1 and YY2 extends beyond the zinc fingers. The spacer region of YY1 (residues 200–297) is 62% identical to residues 164–255 of YY2. The significance of this homology is not yet known, because no function has been ascribed to this region of YY1 or YY2. Intriguingly, between human and Xenopus YY1, the spacer is the next most homologous region following the zinc fingers. Furthermore, a small region of the spacer is present in the Drosophila YY1 protein, and no other similarity exists for Drosophila and human YY1 outside of the zinc fingers and spacer regions. Based on these observations, it is reasonable to speculate that the spacer regions of YY1 and YY2 possess important functions yet to be discovered.

In our Northern blot analysis, a nonconserved portion of YY2 cDNA hybridized to several mRNA species of different sizes present in total RNA prepared from HeLa cells. Currently, we do not know whether these different species are differentially processed forms of YY2 or whether they are gene products similar to YY2. It is also unclear why YY2, which has a predicted molecular mass of 41.4 kDa, migrates with an apparent molecular mass of 58.4 kDa in SDS-polyacrylamide gels. Interestingly, YY2 is expressed in all of the human cell lines and tissues we have examined thus far (data not shown) with the exception of colon tissue. Further studies are required to determine the mechanisms that repress the expression of YY2 in the colon.

As predicted from the close homology in the DNA-binding domains between YY1 and YY2, YY2 binds specifically to an oligodeoxynucleotide containing the consensus YY1-binding site. However, YY2 binds to some but not all of the oligodeoxynucleotides derived from promoters containing YY1 sites. We speculate that in addition to the core YY1-binding site, additional DNA sequences dictate the binding of YY2 to DNA. In this case, to fully understand the functions and mechanisms of YY2 action, it would be important to identify the DNA

![Diagram](image-url)
sequences that favor or exclude the binding of YY2 to DNA.

Similar to Gal4-YY1, Gal4-YY2 represses transcription when tethered to a promoter containing Gal4-binding sites. Also, similar to YY1, YY2 contains both transcriptional activation and repression domains. However, in contrast to YY1, YY2 does not contain the acidic-rich domain that maximizes the transcriptional activation capacity of YY1 (1, 42, 44). This finding suggests that the mechanisms of transcriptional activation are different for the two proteins.

Overexpression of YY2 increased p53 and c-Fos promoter activity. In addition, we found that YY2 increased the activity of the c-Myc and CXC4R promoters when expressed in cells at lower concentrations and decreased the activity of these promoters when expressed in higher concentrations. However, depletion of endogenous YY2 in HeLa cells consistently reduced the activity of all four promoters examined. This finding suggests that YY2 functions as a transcriptional activator on these promoters. We suggest that the effects of YY2 on transcription are promoter-dependent, concentration-dependent, and perhaps dependent on other transcription factors such as YY1.

The discovery of YY2 introduces a new layer of complexity to the regulation of genes containing YY1-binding sites. Besides REX-1 and YY2, do additional promoters with a similarity to YY1 exist? In our search of the GenBank™ data base, we found at least one more novel cDNA that potentially encodes a human protein with 75% amino acid identity within the zinc finger motifs of YY1 and YY2 (data not shown). Outside of the zinc fingers, no homology exists between this protein and YY1 or YY2.

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