YY1 as a Regulator of Replication-dependent Hamster Histone H3.2 Promoter and an Interactive Partner of AP-2*

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In analyzing cis-regulatory elements important for cell cycle control of the replication-dependent hamster histone H3.2 gene, we discovered a binding site for the transcription factor YY1 embedded within GC-rich sequences between the two tandem CCAAT repeats proximal to the TATA element. Base mutations that specifically eliminated YY1 binding resulted in suppression of the S phase induction of the H3.2 promoter. In addition, we discovered that YY1 is an interactive partner of AP-2, which also binds the H3.2 promoter and regulates its cell cycle-dependent expression. The critical domains for YY1 and AP-2A interaction are mapped, revealing that the N-terminal portion of YY1 (amino acids 1–300) and the DNA-binding/dimerization region of AP-2A are required. Our results suggest that YY1, acting as a transcription factor binding to its site on the promoter, or through protein-protein interaction with AP-2, may be part of a regulatory network including key cell cycle regulators such as c-Myc and Rb in controlling growth- and differentiation-regulated gene expression.

The sequence motif CCAT for binding of the transcription factor Yin Yang 1 (YY1)1 occurs in a large number of cellular and viral genes, and YY1 is known to mediate the response to a variety of cell signal pathways (1, 2). YY1 is a zinc-finger-containing transcription factor highly conserved among animal species and is ubiquitously expressed in growing, differentiated, and growth-arrested cells. Although it has been established that YY1 is a multifunctional protein that can act as a transcriptional initiator, repressor, and activator, with the ability to induce DNA-bending and association with the nuclear matrix, its functional role in cell growth and differentiation is just emerging. The YY1 protein is phosphorylated with a relatively short half-life (3). In differentiating F9, 70Z/3, or U937 cells, no changes in the YY1 protein is phosphorylated with a relatively short half-life (3). In differentiating cells, YY1 activity during myogenesis correlates with changes in the differentiation of chicken embryonic myoblasts (5). The decrease in YY1 activity during myogenesis correlates with changes in the expression of two YY1-dependent genes. The gene for YY1 was previously shown that AP-2, through its binding to the H3.2 promoter and physical interaction with Rhb, may contribute to the transcriptional regulation of the H3.2 gene (17). In addition to the AP-2 site located at around 230 base pairs upstream of the transcription initiation site, the H3.2 promoter contains two CCAAT elements proximal to TATA element (18). We report here that an inverted CCAT motif, which binds YY1, was located within GC-rich sequence between the two tandem CCAAT repeats. Mutation of the YY1 binding site suppressed the cell cycle-dependent activation of the H3.2 gene. In addition, we discovered that YY1 is a novel interactive partner of AP-2, suggesting that through protein-protein interaction, YY1 could also affect the activity of cell cycle transcriptional regulatory complexes.

EXPERIMENTAL PROCEDURES
Construction of Plasmids—The construction of the wild-type pH3−254/Luc was as follows; a subfragment of H3.2 promoter spanning −254 to −6 was amplified by PCR using specific wild-type primers.
Fig. 1. Regulatory elements of the hamster histone H3.2 promoters and their interaction with putative transcription factors. The sequence of the wild-type (wt) H3.2 promoter is shown. The A base in the ATG initiation codon is numbered as +1. The localization of the major transcriptional initiation site is indicated by the arrow. The upstream cis-acting regulatory elements TATA (which binds TATA-binding protein), two tandem repeat CCAAT motifs (which bind NF-Y), an inverted YY1 binding site, an AP-2 binding site, and the X site are boxed. The specific base mutations to generate the mutant YY1 promoter (YY1m) and the mutant AP-2 promoter (AP-2m) are indicated by bold italic letters.

The wild-type 5’ primer was overhung with a XhoI site (5’-ctagctgAAGCAGAACCCTGGCCAGTCAGC-3’) and the 3’ primer with a HindIII site (5’-ctagagctgAGACGGGAAATCTGGAGCC-3’). The PCR-amplified fragment was digested with XhoI and HindIII and inserted into a pGL3-basic luciferase vector (Promega, Madison, WI) to generate the pH3[254]Luc. The pH3[254AP2m]Luc construct was generated by PCR amplification with an AP-2 mutant primer (5’-ctagctgAGCA-GAACCTTGGCCAGTCAGC-3’) and subcloned into pGL3 vector as the wild-type promoter pH3[254]Luc. Site-directed mutagenesis to generate pH3[254YY1m]Luc was performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenic oligonucleotide primers, each complementary to opposite strands of the vector, were designed and synthesized for introducing specific mutation in YY1 site as shown in Table I. The orientation and sequence of the wild-type and mutated promoter constructs were confirmed by DNA sequence analysis.

Cell Synchronization—K12 Chinese hamster fibroblast cells were grown in DMEM with 10% bovine calf serum (Gemini, Calabasas, CA) and 1% antibiotics (consisting of 50 μg/ml penicillin and streptomycin, 100 μg/ml neomycin). For cell synchronization, subconfluent cultures were growth arrested by serum deprivation in low glucose DMEM containing 0.5% serum for 48–72 h. The cells were released from the cell cycle.

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Promoter constructs were confirmed by DNA sequence analysis.

Selection of Stable Transfectants—Stable cell lines harboring the wild-type or mutant H3.2 promoter driving luciferase reporter gene were established by cotransfection using the calcium phosphate precipitation method (19). A total of 10 μg of test plasmid DNAs with 2 μg of neomycin expressing vector under the control of the SV40 promoter (pSV40-Neo3) were used for both reporter constructs. The transfectants were selected for resistance to G418 (250 μg/ml) for about 4 weeks. The colonies harboring the respective constructs were pooled and maintained as stable cell lines. Cell lysates were assayed for luciferase activities using a luciferase assay kit (Promega, Madison, WI) in a TD-20/20 Luminometer (Turner Design, Sunnyvale, CA) according to the manufacturer’s protocol. The luciferase activities of the stable transfectants were determined from the same number of cells throughout the experiment.

Electrophoretic Mobility Shift Assays (EMSA)—Double-stranded oligonucleotides were end-labeled and purified, and conditions for EMSAs have been described previously (17).

Antisera—The anti-AP-2A N-terminal peptide rabbit polyclonal antibody is a gift of Dr. M. Tainsky (University of Texas, Houston, TX). The anti-YY1 monoclonal antibody, anti-ATF-1, and anti-IRF-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Glutathione S-Transferase (GST) Pull-down Assays—GST or GST-fusion proteins were induced in Escherichia coli with isopropyl-1-thio-β-D-galactopyranoside and purified by affinity chromatography. Protein yields were quantitated by Coomassie Blue staining in comparison with bovine serum albumin after SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein-protein interactions were assayed with equal amounts of in vitro translated and 35S-labeled proteins and purified GST proteins bound onto beads as described (17). Proteins bound onto beads were released and resolved by 10% SDS-PAGE.

Immunoprecipitation and Immunoblot—Subconfluent K12 hamster fibroblasts or 293T human kidney fibroblast cells were transiently co-transfected with various expression plasmids or their empty vectors (20 μg total in each transfection mixture) using SuperFect reagent (Qiagen, Hilden, Germany). The cells were then lysed in ELB buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, and 0.5% Nonidet P-40) in situ at 4 °C for 10 min, and used for immunoprecipitation as described previously (17). The immunoprecipitated proteins were resolved by a 10% SDS-PAGE and transferred to Immunobilon-PVDF membrane. The blots were preincubated in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20, containing 2% bovine serum albumin, at 4 °C overnight. The individual primary antibodies were diluted at 1:500 to 1:2000 and incubated for 2 h at 4 °C. The horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Roche Molecular Biochemicals) was diluted at 1:10,000. The protein band was visualized by chemiluminescence (ECL) detection (Amersham Pharmacia Biotech).

RESULTS

Identification of a Functional YY1 Binding Site in the H3.2 promoter—The promoter sequence of the hamster histone H3.2 gene spanning –254 to +3 is shown in Fig. 1. Located within this sequence are the canonical TATA sequence, two CCAAT binding sites for NF-Y binding, an AP-2 binding site, and an AP-1 like binding site termed X. An ATGG sequence motif, which represents the inverted CAT sequence found to be a predictor of high affinity YY1 binding site (20), was found between the NF-Y binding sites and is embedded within GC-rich motifs resembling that of the Sp1 binding site. To test for YY1 binding to the ATGG motif, synthetic oligonucleotides encompassing the YY1 site (spanning from –188/-131) of H3.2 promoter were used as probe in EMSAs (Fig. 2). The sequence and size of the binding sites used in EMSAs are shown in Table I.
I. With HeLa nuclear extract, two predominant complexes were observed. Both complexes are specific for the H3.2 sequence as revealed by self-competition with molar excess of the unlabeled probe and by the failure of competition with a heterologous AP-2 sequence (Fig. 2A, lanes 1–5). The slower electrophoretic mobility complex represented NF-Y binding to the probe, since this complex was abolished by competition with a NF-Y consensus site (Wu and Lee, data not shown). Likewise, the faster electrophoretic mobility complex contained YY1, whose binding was completely abolished with molar excess of a consensus YY1 site (Fig. 2A, lanes 6 and 7). Since the consensus YY1 oligomer used also contains a low affinity NF-Y binding site CCGG (21), partial competition for the NF-Y complex was also observed.

The ability of YY1 to bind directly to the H3.2 promoter (pter) was demonstrated by using the bacterially expressed GST-YY1 protein (Fig. 2B, lanes 6 and 7). Since the consensus YY1 oligomer used also contains a low affinity NF-Y binding site CCGG (21), partial competition for the NF-Y complex was also observed. The ability of YY1 to bind directly to the H3.2 promoter (pter) was demonstrated by using the bacterially expressed GST-YY1 protein (Fig. 2B, lanes 6 and 7). Since the consensus YY1 oligomer used also contains a low affinity NF-Y binding site CCGG (21), partial competition for the NF-Y complex was also observed.

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identical to the wild-type promoter with the exception of the two-base change converting the ATGG sequence to CTCG, thereby destroying specifically the YY1 binding site (Fig. 1). Both wild-type and mutated promoter sequences were linked to a luciferase reporter gene, and stably transfected into K12 cells by cotransfection with a neomycin resistance gene. The promoter activities during the cell cycle after serum release were determined by measurement of the luciferase activity. The level of luciferase activity of the wild-type H3.2 promoter at time 0 was set as 1. The relative promoter activity at each time point was derived from equal number of cells and was plotted as a function of time after serum release.

![Fig. 3. Effect of YY1 and AP-2 binding site mutations on the cell cycle induction of the H3.2 promoter. Stable transfectants containing the wild-type (○), YY1 mutant (□), and AP-2 mutant (△) H3.2 promoter driving a luciferase gene were synchronized by serum starvation. Following serum release, the promoter activities at various time points were determined by measurement of the luciferase activity. The level of luciferase activity of the wild-type H3.2 promoter at time 0 was set as 1. The relative promoter activity at each time point was derived from equal number of cells and was plotted as a function of time after serum release.

**Regulation of Histone H3.2 Promoter by YY1**

The kinetics of S phase induction was considerably delayed when the YY1 binding site was mutated (Fig. 3). The final induction level was reduced to about 6-fold. In contrast, mutation in a previously identified AP-2 binding site of H3.2 promoter resulted in increased promoter activity several hours prior to the onset of the S phase (Fig. 3). These results showed that, although the AP-2 site appears to be required for promoter repression during G1 phase prior to the G1/S transition, the YY1 site is required for optimal transcriptional activation of the H3.2 promoter during G1/S transition.
Physical Association between YY1 and AP-2 in Vitro—AP-2 has been shown to be an interactive protein of Rb (17, 22). Further, we identified AP-2 as a major transcription factor binding to the cell cycle regulatory domain of the H3.2 promoter referred here as the H3 core (17). The H3 core is located about 70 base pairs upstream of the YY1 binding site (Fig. 1).

Since both YY1 and AP2 appear to be regulators of the S phase induction of the H3.2 promoter, we tested the interaction between these two transcription factors. Using GST pull-down assays with [35S]methionine-labeled AP-2A protein and various GST fusion proteins, we confirmed that AP-2A can form homodimeric complexes (Fig. 4A, lane 3). In addition, we showed that AP-2A can interact with both GST-YY1 and GST-YY1D with C-terminal domain deleted (Fig. 4A, lanes 4 and 5), but not with GST alone or GST-Ha-Ras (Fig. 4A, lanes 2 and 6). Thus, the YY1 domain interacting with AP-2 lies in its N-terminal portion outside the C-terminal repression and zinc finger DNA-binding domain (Fig. 5B).

To further map the interaction domains of AP-2A with YY1, full-length and various truncated forms of AP-2A (N262, ΔN165, ΔN227, ΔC390) were in vitro transcribed and translated and GST pull-down assays were performed with GST-YY1. The radiolabeled AP-2A proteins, as shown in Fig. 5A, were mixed with equal amounts of GST-YY1 and subjected to GST pull-down assays as described above. Strong interaction with YY1 was observed with both AP-2A (ΔN165) and full-length AP-2A. In contrast, little interaction was detected with N262, which is devoid of the dimerization domain; ΔN227, which has deleted part of the basic DNA binding domain; and ΔC390, which has lost part of its dimerization domain and is not able to bind DNA (Fig. 5B). These results show that AP-2 binding to YY1 requires an intact DNA binding and dimerization domain of AP-2.

In Vivo Association of YY1 with AP-2—To determine whether YY1 and AP-2 can associate in vivo, two independent approaches were taken. First, in vivo co-immunoprecipitation experiments were performed. Two mammalian cell lines, K12 and 293T cells, were used. Because AP-2A was present in low abundance in mammalian cells, to enhance detection between nonspecific IgG band cross-reacting with the antibody. The positions of the protein size markers (in kDa) are indicated. In C and D, the same lysates used in A and B were immunoblotted with anti-AP-2 antibody. The position of AP-2A is indicated.

**Fig. 5.** Mapping the interaction domain of AP-2 with YY1. A. in vitro translated [35S]-labeled full-length or truncated AP-2A was used. The input AP-2A protein samples (10%) were shown on the left panel. The positions of the protein size markers (in kDa) ran in parallel are indicated. At the right panel, GST pull-down assays were performed with the various [35S]-labeled AP-2A proteins (full-length or subfragments as indicated on top) and with bacterial expressed and purified GST-YY1. The proteins bound onto the GST-YY1 were eluted, applied to a 10% SDS-PAGE, and detected by autoradiography. B. schematic drawing of the functional domains of AP-2 protein and a summary of the AP-2 domains required for YY1 binding.

**Fig. 6.** Co-immunoprecipitation of AP-2 with YY1 in vivo. Expression vectors for human YY1, human AP-2A, either alone or in combination as indicated on top, were transiently transfected into K12 (A) or 293T cells (B). In lane 1, the cells were co-transfected with the empty expression vector and served as negative control. The cell lysates prepared from the transfected cells (lanes 1–4) were immunoprecipitated with a rabbit polyclonal anti-AP-2A N-terminal peptide antibody. The immunoprecipitates were applied onto a 10% SDS-PAGE. The filters were immunoblotted with an anti-YY1 antibody. In A, a whole cell lysate (WCL) prepared from the K12 cells was also applied to the SDS-PAGE to provide a positive control for the electrophoretic mobility of YY1, as indicated by the closed arrow. The open arrow indicates the
AP-2A and YY1, expression vectors for AP-2A and YY1 were transfected either singly or in combination into K12 and 293T cells. After transfections, whole cell lysates were prepared and immunoprecipitated with an anti-AP-2 antibody. The immunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted with an anti-YY1 antibody. For both K12 (Fig. 6A) and 293T (Fig. 6B) cells, YY1 were immunoprecipitated by the anti-AP-2 antibody (Fig. 6, A and B, lanes 2 and 4). The expression of AP-2A in cells transfected with the AP-2A expression vector was confirmed by immunoblotting the cell lysates with the anti-AP-2A antibody (Fig. 6, C and D, lanes 3 and 4).

In the second approach to determine for AP-2 and YY1 association in vivo, we investigated the composition of a novel multiprotein complex (X'), which was formed in EMSAs using K12 nuclear extract and a H3 core (~253 to ~214) as probe (Fig. 7A). The X' complex was observed when highly concentrated cell lysates were used. The H3 core (~254 to ~214) contains the AP-2 binding site and the X binding site (Table I). Previously we have shown that mutation of site X and the first G residue of the AP-2 site severely diminished the ability of AP-2 to bind to the H3 core (17). Likewise, the H3 core mutation resulted in the reduction of its ability to compete for X' binding (Fig. 7A). In determining the components of the X' complex, the K12 nuclear extract was pre-incubated with antibodies against a large number of transcription factors prior to addition of the H3 core probe. As expected, one component of X' is AP-2A (Fig. 7B). We discovered X' also contains YY1 since antibody against YY1 was also able to inhibit the formation of X' (Fig. 7C). Other antibodies such as those directed against ATF-1, IRF-1 and other transcription factors were without effect (Fig. 7, B and C, and data not shown). These results demonstrated that YY1 and AP-2 can associate in vivo and are components of a multiprotein complex binding to the cell cycle regulatory domain of the H3.2 promoter.

**DISCUSSION**

Through deletion mutation analysis, the critical region for S phase induction of the hamster H3.2 promoter has been mapped to a region between ~241 and ~210, which contains a conserved AP-1-like site (X) adjacent to an AP-2 binding site (17, 18, 23). Although the promoter sequence downstream of this region by itself is not sufficient to confer cell cycle regulation to the H3.2 gene (18), it may contain sequences that are necessary for optimal induction of the H3.2 gene during G1/S transition. By DNA sequence comparison between H3.2 and the replication-dependent human thymidine kinase (htk) promoter, a YY1 binding site core (5'-ATGG-3') was discovered between the two tandem CCAAT repeats in both promoters (15). Similar to the H3.2 promoter, the two CCAAT motifs of the htk promoter are downstream of the cell cycle regulatory region and bind NF-Y (24, 25). The YY1 binding site within the htk promoter is also immediately adjacent to GC-rich motifs resembling the Sp1 binding site. This high degree of conservation within the promoter organization of H3.2 and htk, both of which are replication-dependent, suggests functional significance. This prediction was borne out through mutation analysis of the YY1 binding site on the H3.2 promoter. In this report, we showed that the ATGG motif represents an inverted binding site for YY1. The binding of YY1 to this sequence motif does not require other nuclear proteins as purified recombinant YY1 is able to bind this site. Further, mutation of the YY1 site by two base changes eliminated YY1 binding and suppressed the S phase induction of the H3.2 promoter. These results suggest that a simple mode of action by YY1 toward regulation of the H3.2 promoter is through direct binding to its
site and activate transcription (Fig. 8A). This mechanism may also be employed for the activation of the histone H4 promoter by YY1 through its multiple binding sites on the histone H4 regulatory elements (14).

Since the binding motif of YY1 is widely distributed in a large number of genes, specific regulation conferred by YY1 is likely related to its ability to associate with other proteins. The ability of YY1 to interact with components of the basal transcription machinery such as TATA-binding protein, transcription factor IIB, and TATA binding protein associated factor (TAF) II 55 suggest that YY1 could act as a bridging protein between the upstream regulatory elements and the core polymerase complex binding to the TATA element (2). The interactions between YY1, c-myc, and E1A strongly imply the importance of YY1 as a transcriptional regulator in growth and differentiation. It has been proposed that one mechanism for YY1 action is through the recruitment of chromatin-modifying enzymes such as histone deacetylases and histone acetyltransferases, leading to chromatin alteration (26). Both cyclin E/Cdk2 kinase and the viral oncoprotein E1A have been shown to associate with and activate the histone acetyltransferase activity of CBP/p300 during the mammalian G1/S transition (27). This could lead to alteration in chromatin topology at specific cell cycle stages. Additionally, YY1 has been shown to associate with the nuclear matrix, which is known to play a role in defining the higher order structure of chromatin (28). Under these conditions, YY1 activity might be altered and the transcription of YY1 target genes could be affected.

In this study, we discovered that AP-2 is a novel interactive protein of YY1. Previously, we have identified that AP-2 can bind to the cell cycle control region of H3.2 promoter (17). More interestingly, AP-2 physically interacts with the important growth suppressing region of Rb C-terminal domain (17 and data not shown). In addition, AP-2 has been shown to associate with both c-myc and E1a oncoproteins. These observations raise a possibility that AP-2 and YY1 function at overlapping regulatory pathways. To explore this possibility, we demonstrated that co-immunoprecipitation and co-existence within a multiprotein complex that AP-2 and YY1 physically associate with each other in vivo. The important domains for their interactions have been mapped to the C-terminal basic and dimerization domain of AP-2 and a larger N-terminal portion of YY1 outside most of the zinc finger regions. Although the exact region of the YY1 N-terminal portion (amino acids 1–330) required for AP-2 interaction remained to be determined, since this portion of YY1 is devoid of repression or DNA binding activities, it is most likely that the modulation of AP-2 activity by YY1 is mediated through physical interaction. In an attempt to analyze the effect of overexpression of YY1, either alone and in combination with AP-2A, on the native H3.2 promoter, we found that the promoter activity was repressed (data not shown). The mechanism for repression is currently unclear and could be due to direct or indirect effects associated with overexpression, such as squelching of basal factors or interference of other critical factors such as proteins binding to the GC-rich sequence adjacent to the YY1 site (29). Here we show, through direct mutagenesis of the YY1 binding sites, that YY1 acts as an activator within the context of the H3.2 promoter. In case of the AP-2 binding site, its mutation resulted in increase in H3.2 promoter activity prior to the G1/S transition, suggesting functional consequence of the interaction between AP-2 and Rb as proposed in Fig. 8B.

In summary, the discovery of a functional YY1 binding site required for optimal S phase induction of a replication-dependent gene such as H3.2 provides direct evidence for YY1 involvement in the regulation of a replication-dependent gene promoter. Our study predicts that other growth-regulated genes bearing YY1 binding site around transcriptional control region (11) may also be targets for YY1 regulation. The identification of interaction between YY1 and AP-2 and their interactive network with c-Myc and Rb suggests that YY1 and AP-2 may play important roles in mediating the pleiotropic effects of c-Myc and Rb on cell growth and differentiation (Fig. 8B). In support, targeted disruption of the mouse YY1 gene results in pre-implantation lethality, strongly implying that YY1 may regulate genes essential for rapid proliferation and differentiation of mouse embryos in early development (8). Similar to AP-2 gene disruption (30), YY1 heterozygotes exhibit severe developmental abnormalities and neurulation defects, supporting the notion that the two factors through their functional interaction may converge upon similar developmental regulatory pathways. Future investigations into the possible role of YY1 in the control of other growth- and differentiation-regulated genes will put these hypotheses to test.

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