YY1 Transcription Factor is Not Responsible for the Negative Regulation of Hamster Muc1 Transcription

AKINORI HISATSUNE1, SANG WON HYUN1, INSONG JAMES LEE1, STEVEN GEORAS2 and K. CHUL KIM1

1Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, 20 Penn Street, Baltimore, Maryland; 2Department of Medicine, Division of Pulmonary and Critical Care Medicine, and Division of Allergy and Clinical Immunology, The Johns Hopkins Asthma and Allergy Center, Baltimore, Maryland, U.S.A.

Abstract. Muc1 is the cell surface glycoprotein abundantly expressed in cancer cells and has been shown to be involved in tumor metastasis and promotion. Recently, we identified a 37 bp segment on the hamster Muc1 promoter with the ability to suppress Muc1 transcription. This 37 bp putative negative regulatory element (NRE) binds to a transcriptional regulator Yin Yang 1 (YY1). In the present study, we examined whether binding of YY1 is responsible for the negative regulatory effect by the 37 bp segment using a hamster pancreatic cancer cell line, HP-1 cells, transfected with various expression plasmid constructs. Our results showed that: (1) overexpression of YY1 up-regulated the transcriptional activity of the full-length hamster Muc1 promoter in a dose-dependent manner; (2) the mutation of the YY1 binding site did not affect either the basal transcriptional activity or the increased transcriptional activity by YY1; and (3) even the deletion of the 37 bp NRE segment could not abrogate the increased transcriptional activity by YY1. We conclude that the NRE acts in a YY1-independent manner and that YY1 instead enhances Muc1 transcriptional activity. Further study of the precise mechanism by which YY1 augments Muc1 gene expression should be worthwhile.

MUC1 (MUC for human and Muc for non-human) mucin is a large membrane-tethered glycoprotein overexpressed in many adenocarcinoma cells and has been shown to be responsible for tumor metastasis (1,2), immune suppression (3) and tumor progression (4,5). A great deal of effort has been invested by a number of laboratories in an attempt to characterize the mechanism of MUC1 expression in tumor cells. Expression of the MUC1 gene has been shown to be influenced by a number of factors, including hormones and retinoids (6-12), cytokines (13,14), tyrosine kinase inhibitors (15,16) and possibly by gene modifications such as DNA methylation or DNA conformational status (17-20). Although the molecular mechanisms of the regulation of MUC1 transcription by these factors are not well defined, cis-acting elements within the MUC1 promoter mediating the effects have been identified (14,21-24). Recently, we identified a 37 bp segment as a putative negative regulatory element (NRE) in the hamster Muc1 promoter (25). Computer assisted sequence analysis of this NRE revealed the presence of a potential binding site for Yin Yang 1 (YY1) transcription factor.

YY1, also called NF-E1, δ or UCRBP, belongs to the GLI-krüppel family of transcription factors and can initiate, activate, or repress transcription depending on the promoter context (26-29). YY1 is a constitutive nuclear phosphoprotein that appears to be expressed in most cell types (30). Targeted disruption of the YY1 gene in mice results in peri-implantation lethality (31). Since its original isolation as a repressor of the P5 promoter of adeno-associated virus (28), a large number of cellular and viral genes are now known to be regulated by YY1 (29,32).

In this study, we intended to see whether YY1 binding to the 37 bp segment is responsible for the negative regulation of Muc1 gene by this NRE.

Materials and Methods

Materials. All the chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified. HP-1 cells (hamster pancreatic cancer cell line) were a generous gift from Dr. Michael Hollingsworth (Eppley Institute, University of Nebraska Medical
An expression construct encoding the full length of human YY1 was cloned into pSG5 vector as we previously described (33). Anti-YY1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of Muc1 promoter-reporter constructs. Both the full-length of wild-type hamster Muc1 promoter/reporter plasmid and its mutant construct lacking the 37 bp NRE site were prepared as previously described (25). Mutation of the YY1 binding site on the 37 bp NRE site was carried out by introducing point mutations at -1618 (G for C) and -1617 (G for A) using PCR. The oligonucleotide sequence of the mutagenic primer was 5’-AAGTGCGCGCGCTTGCTCTTTAAATACTCAGACTGAGAGGCGCGGAGCTATTGC-3’, where mutated nucleotides are indicated in bold with underline, and that of the reverse primer was 5’-TCAGCGCGCCCACAAGAATTTAGG-3’. The amplification consisted of denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec and extension at 72°C for 1 min, with final elongation at 72°C for 10 min. The resulting fragments were digested with BssHII, and then ligated into the full-length of wild-type hamster Muc1 promoter/reporter construct that had been digested with BssHII. The sequence of this construct was confirmed by nucleotide sequencing (Biopolymer Core Facility, University of Maryland, Baltimore, USA).

Transient transfection and luciferase assay. HP-1 cells were grown in Minimum Essential Medium with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). At about 70% confluence, transfection was carried out with SuperFect (QIAGEN, Valencia, CA, USA) according to the manufacturer’s instruction. Briefly, HP-1 cells were plated in 24-well plates at 50,000 cells/well 24 h before transfection. Transfection was carried out by adding a 5:1 (v/w) SuperFect /DNA mixture in a final volume of 410 μl/well into the cultured cells. The DNA sample consisted of 500 ng of reporter construct, 500 ng of expression plasmid and 20 ng of an internal control plasmid pRL-TK. After 3 h of transfection, the transfection mixture was removed and replaced with fresh growth media. After incubation for 48 h at 37°C, cells were harvested and assayed for luciferase activity. Luciferase activities were determined using the Dual-luciferase assay system (Promega, Madison, WI, USA) in which Firefly luciferase activity was used for measuring the expression of a specific gene whereas Renilla luciferase activity was used for internal control. The light intensity was measured by a luminometer (Wallac Victor2, PerkinElmer, Wellesley, MA, USA). The total amount of expression plasmids added to each well was balanced to be equal by adding the empty expression construct pSG5. The gene-specific luciferase activity was normalized based on internal control. For each construct, at least two different DNA preparations were tested and each transfection was repeated at least three times in order to verify the reproducibility.
Real-time PCR. Total cellular RNA was extracted from HP-1 cells by using RNasea (QIAGEN), according to the manufacturer’s instruction. One μg of total RNA was reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) in a total volume of 20 μl. Each RNA sample was incubated at 25°C for 5 min, at 42°C for 30 min and then at 85°C for 5 min. The quantitative PCR was carried out in a total volume of 25 μl according to the manufacturer’s manuals for iQ SYBR Green Supermix (Bio-Rad). An aliquot of 2 μl cDNA or control plasmid was used as template for amplification in iCycler (Bio-Rad) with primer set for YY1 (5’- GGATAACTCGGCCATGAGAA-3’ and 5’- CGCAAATTGAA GTCCAGTGA-3’), and GAPDH (5’- CCCATCACCATCTTGAGGAG-3’ and 5’- GTTGTCATGGATGACCTTGGC -3’). Each primer concentration was 300 nM. To avoid problems associated with genomic DNA contamination, primers were selected that span one intron of the genomic sequence. PCR cycles (n = 40) consisted of a 15 sec melt at 95°C, followed by an annealing at 60°C for 30 sec and an extension at 72°C for 30 sec. All reactions were performed in triplicate. The Ct value was defined as the number of PCR cycles required for the specific fluorescence signal to exceed the detection threshold value set by the software installed in the iCycler. Standard curves for YY1 and GAPDH (internal control) were generated by the serial dilution of pSG5 vector containing human YY1 cDNA and pBluescript vector containing GAPDH cDNA. The expression of YY1 and GAPDH mRNA was calculated based on each of the standard curves.

Statistics. The difference between groups was assessed using Student’s t-test for unpaired samples. p<0.05 was considered significantly different.

Results

Promoter analysis of hamster Muc1 gene. Various plasmid constructs used in this study are shown in Figure 1B together with the 37 bp segment containing the YY1 binding site. Figure 1C confirms our previous finding that deletion of the 37 bp region between -1651 and -1614 augmented the promoter activity, suggesting that it contains an NRE. Sequence analysis of the hamster Muc1 promoter revealed the presence of 7 potential YY1 binding sites including the one on the 37 bp NRE region (Figure 1A).

Levels of YY1 mRNA following transfection of the YY1 expression vector. Transient transfection of the varying amounts of YY1 expression plasmid into HP-1 cells resulted in a dose-dependent increase in the levels of YY1 mRNA
as judged from quantitative real-time PCR analysis (Figure 2). YY1 mRNA levels increased by 300-fold and 470-fold over control following transfection with 62.5 ng/well and 125 ng/well of YY1 expression plasmid, respectively.

**Effect of overexpression of YY1 on the Muc1 promoter activity.** Muc1 promoter deletion constructs, D and N (Figure 1), which differ only by the presence of the 37 bp NRE, were prepared and their sequences confirmed. When these constructs were transiently co-transfected with varying amounts of the YY1 expression vector, the luciferase activities of both constructs increased in a dose-dependent manner (Figure 3). However, the activity of construct D was always significantly lower than that of construct N in all amounts of YY1 used; by 47%, 60% and 50% for 0, 62.5 ng/well and 125 ng/well, respectively (Figure 3).

**Discussion**

Recently we showed that the 37 bp segment located between -1651 and -1615 of the hamster Muc1 promoter serves as an NRE and that YY1 specifically binds to this segment (25). Since YY1 has been shown to be present ubiquitously in cells and also to play an important role in transcriptional regulation in various cell types, we hypothesized that the NRE effect by the 37 bp segment may be mediated by binding of YY1 transcription factor to this segment. In testing this hypothesis, we took two approaches. First, we wanted to see whether the NRE effect is enhanced by increasing cellular YY1 levels. In order to increase cellular YY1 levels, we used transient transfection of an YY1 expression plasmid (33), which resulted in a proportionate increase of YY1 mRNA following transfection (Figure 2). Co-transfection of the constructs with the YY1 expression vector resulted in a drastic yet dose-dependent increase in the promoter activity in both of the constructs (Figure 3). However, the promoter activity of construct D was lower that of construct N in all amounts of YY1, indicating that the suppressive effect of the 37 bp remains unchanged regardless of excess YY1. These results seem to suggest one of two possibilities. The binding of YY1 to the 37 bp segment had already been saturated by the endogenous YY1 and the excess YY1 acted on the low affinity YY1 binding sites, which resulted in an increase in the promoter activity. In fact, nucleotide sequence analysis using a transcription factor binding site data base revealed the presence of seven putative YY1 binding sites on the Muc1 promoter (Figure 1A). Alternatively, the results also suggest that binding of YY1 to the 37 bp is not responsible for its NRE effect.

As a second approach, we decided to see whether the mutation of the YY1 binding site on the 37 bp could abolish the NRE effect of the segment. The consensus core sequence of the YY1 binding site has been reported to be CCAT in...
which CA has been shown to be crucial for the binding (26). We have shown previously that, when these two bases were mutated to GG, the mutated NRE segment completely lost its ability to bind to YY1 and, furthermore, when anti-YY1 antibody was added to the reaction mixture, a supershift was manifest indicating that YY1 binds specifically to the 37 bp NRE site (25). Based on these results, we constructed an YY1 binding site mutant from the wild-type, in which the two critical bases of the 37 bp were mutated to GG in order to prevent YY1 binding to the 37 bp NRE site. We also prepared a deletion mutant of the wild-type only by the absence of the 37 bp. When these three different constructs (the wild-type, the YY1 mutant, the 37 bp deletion mutant) were transiently co-transfected with varying amounts of the YY1 expression plasmid, we still observed significant increases in the promoter activity by YY1 in all the constructs. However, no significant difference was seen in the promoter activity between the wild-type and the YY1 mutant in all the amounts of YY1 (Figure 4) although a clear NRE effect was observed with the 37 bp deletion mutant (no-NRE group) in all the amounts of YY1. Thus, it is not likely that YY1 binding to the 37 bp accounts for the suppressive effect on Muc1 transcription by this 37 bp NRE segment and we are currently characterizing other factors that bind to this NRE region for their repressing activities.

The term YY1 (Yin Yang 1) for the transcription factor was derived based on its ability to both activate and repress transcription depending on the context of the nucleotide sequences around its binding site. Thus, the multiple actions of YY1 are explained mostly by its ability to interact with other sequence-specific factors (34-37), viral proteins (38), co-factors (39,40) and basal transcriptional components (27,30,41). Furthermore, YY1 interacts within multiple nuclear compartments (42) and associations with cofactors can be modulated by acetylation as well as deacetylation (43). Given that there are 7 potential YY1 binding sites on the Muc1 promoter (Figure 1A), the increase in promoter activity by exogenous YY1 seems to suggest that these sites are probably involved either alone or in combination in enhancing Muc1 gene expression. Further studies elucidating the molecular mechanism by which YY1 augments Muc1 expression should be worthwhile.

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