Identification of c-myc as a Down-stream Target for Pituitary Tumor-transforming Gene*

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Pituitary tumor-transforming gene (PTTG) encodes a protein implicated in cellular transformation and transcriptional regulation. To identify downstream target genes, I established cell lines with tightly regulated inducible expression of PTTG. DNA arrays were used to analyze gene expression profiles after PTTG induction. I identified c-myc oncogene as a major PTTG target. Induction of PTTG resulted in increased cell proliferation through activation of c-myc. I showed that PTTG activates c-myc transcription in transfected cells. PTTG binds to c-myc promoter near the transcription initiation site in a protein complex containing the upstream stimulatory factor (USF1). I have defined the PTTG DNA-binding site and mapped PTTG DNA binding domain to a region between amino acids 61 and 118. Furthermore, I demonstrated that PTTG DNA binding is required for its transcriptional activation function. These results definitively established the role of PTTG as a transcription activator and indicate that PTTG is involved in cellular transformation and tumorigenesis through activation of c-myc oncogene.

PTTG is a novel oncogene (1) that is overexpressed in multiple tumors including carcinomas of lung, breast, colon, leukemia, and lymphoma as well as in pituitary adenomas (2–5). The expression of PTTG in normal tissues is restricted, with highest expression in the testis (1–3). PTTG is expressed in a stage-specific manner in germ cells during spermatogenic cycle, suggesting it may play a role in male germ cell differentiation (6, 7). The level of PTTG expression is increased in rapidly proliferating cells and is regulated in a cell cycle-dependent manner, peaking in mitosis (8), suggesting that PTTG may play a role in control of cell proliferation. PTTG encodes a protein of 30 kDa without homology to other known protein (1). It was recently shown that PTTG is a mammalian securin that acts as a sister-chromatid separation inhibitor (9). PTTG protein is predominantly localized to the cytoplasm with partial nuclear localization (3, 10, 11). However, nuclear translocation of PTTG can be facilitated either by interaction with PTTG binding factor (10) or by activation of mitogen-activated protein (MAP) kinase cascade (11).

A role for PTTG as a transcriptional activator has been proposed. The C-terminal region of PTTG mediates transcriptional activation when fused to a heterologous DNA binding domain (3). Transient cotransfection of PTTG and c-myc binding factor expression constructs leads to transcriptional activation of the basic fibroblast growth factor promoter (10). In a recent study, I demonstrated that activation of the MAP kinase cascade by epidermal growth factor or an expression vector for a constitutively active form of the MAP kinase kinase (MEK1) led to stimulation of PTTG transcriptional activity (11). PTTG is phosphorylated in vitro on Ser163 by MAP kinase and is linked to the MAP kinase cascade by direct interaction with MEK1 through a putative SH3-domain binding site located between amino acids 51 and 54 (11). Both the MAP kinase phosphorylation and the MEK1-binding sites play an essential role in PTTG transcriptional function (11).

These previous studies indicate that PTTG plays a potentially important role in regulation of transcription. However, target genes whose expression is definitively regulated by PTTG remain unknown. To address the functional properties of PTTG and to examine its effect on endogenous target genes, I developed cell lines in which PTTG expression is tightly regulated by an inducible promoter (12, 13). DNA array-based gene expression profiling revealed a small number of genes exhibiting altered expression after PTTG induction. Among these candidate targets, the most dramatic induction was the c-myc oncogene. The c-myc gene has a critical role in the control of cellular proliferation. Deregulated c-myc is associated with a variety of tumors. Overexpression of c-Myc protein stimulates cell cycle progression, causes transformation, blocks differentiation, and induces apoptosis in low serum (14). I observed that induction of c-myc expression by overexpression of PTTG resulted in increased cell proliferation and colony formation, supporting the role of PTTG in regulation of cell growth.

It has been established that the C-terminal portion of PTTG contains a transcriptional activation domain (3, 10, 11). However, it is not known whether PTTG directly binds to DNA, and the DNA-binding site has not been identified. I have therefore used c-myc promoter to characterize PTTG interaction with DNA. Here I show that PTTG binds to c-myc promoter near the transcription start site and forms a complex with the ubiquitous transcription activator USF1. I have mapped PTTG DNA binding domain to a region between amino acids 60 and 118. In addition I demonstrate that transcriptional activation of c-myc gene by PTTG requires its DNA binding activity.

MATERIALS AND METHODS

Antibodies—Antibodies for USF-1 and Y1 were obtained from Santa Cruz Biotechnology. C-Myc and His antibodies were purchased from Berkeley Antibody Co. and CLONTECH, respectively. Anti-PTTG anti-serum was described previously (1).
Generation of Cell Lines with Inducible PTTG Expression—The PTTG cDNA was cloned into the pCDNA3.1/His vector (Invitrogen). The coding region of PTTG and the histidine tag was then amplified by PCR with SsrI restriction site attached to both ends. The PCR product was cloned into pTRE vector (CLONTECH), resulting in pTRE-PTTG. Site-directed mutagenesis was performed to change Ser to Ala in pTRE-PTTG, using the QuikChange mutagenesis kit (Stratagene) following the manufacturer’s instructions. The resulting plasmid was termed pTRE-mPTTG. PTRE-PTTG or pTRE-mPTTG was co-transfected into HeLaS3 Tet-off cell line with pTK-Hyg vector (CLONTECH).

The cells were selected in medium containing 200 μg/ml hygromycin. Two weeks after transfection, hygromycin-resistant clones were isolated and screened for integration of PTTG by PCR. The clones that express PTTG were expanded into cell lines. The cell lines were maintained in growth medium containing 2 μg/ml doxycycline (Dox). The expression of PTTG was induced by doxycycline withdrawal. The level of PTTG induction was determined by Northern and Western blot analysis using standard protocols. The cell lines that have the highest level of induced PTTG expression after Dox withdrawal were used for latter analyses.

Expression Profiling—Total RNA was isolated from HeLaS3-PTTG cells at 0 and 24 h after PTTG induction. The RNA was labeled with [32P]dATP by reverse transcription, and the labeled cDNA was purified by column chromatography. The probe was hybridized to Atlas Human Array (CLONTECH) following the manufacturer’s instructions. In quantitative analysis, candidate PTTG targets were assembled by identifying genes that displayed an expression change of at least 2-fold. Some potential targets were verified by Northern analysis. Probes for c-myc and MEK1 were generated by PCR according to published sequences. The probe for HSP70 was kindly provided by Dr. R. Morimoto (Northwestern University).

Proliferation and Colony Formation Assays—Cell proliferation was assayed using the CellTiter 96 nonradioactive cell proliferation assay kit (Promega) according to the manufacturer’s instructions. Five thousand cells were seeded in 96-well plates (6 wells for each clone in each assay) and incubated at 37 °C for 24 to 72 h in the presence or absence of Dox. At each time point, 15 μl of the dye solution were added to each well and incubated at 37 °C for 4 h. One hundred microliters of the solubilization/stop solution were then added. After 1 h of incubation, the contents of each well were mixed, and the absorbency at 595 nm was recorded using an enzyme-linked immunosorbent assay reader. Absorbency at 595 correlates directly with the number of cells in each well. For the colony formation assay, 104 cells were plated on 35-mm plates and grown with or without Dox. After 14 days in culture, the colonies were fixed and stained with 0.1% crystal violet and counted.

Transient Transfection Assays—The c-myc promoter-luciferase reporter constructs HBM-Luc and XNM-Luc were kindly provided by Dr. Linda Penn (University of Toronto) and were described previously (15). Mutagenesis of XNM-Luc was performed using QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions (Stratagene). The construction of wild type and mutant PTTG expression plasmids was reported previously (11). NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Transfections were performed using calcium phosphate precipitation as described previously (7). All transfections were performed in triplicates, and each DNA plasmid together with pTK-Hyg was cotransfected into Tet-off HeLa S3 cell line. Hygromycin-resistant clones were selected and screened for induced expression of PTG in the absence of doxycycline. Low levels of endogenous PTG mRNA were detectable when cells were grown in the presence of doxycycline (band not visible in the photo shown in Fig. 1), and drug withdrawal led to induction of the expected 900-bp transfected PTG transcript (Fig. 1A).

RESULTS

Inducible Expression of PTTG—To study the functional properties of PTTG, I established inducible, tetracycline-regulated expression of PTTG in HeLa S3 cells. I cloned the His-tagged PTTG into pTRE expression vector (pTRE-PTTG). This plasmid together with pTK-Hyg was cotransfected into Tet-off HeLa S3 cell line. Hygromycin-resistant clones were screened for induced expression of PTTG in the absence of doxycycline. Low levels of endogenous PTG mRNA were detectable when cells were grown in the presence of doxycycline (band not visible in the photo shown in Fig. 1), and drug withdrawal led to induction of the expected 900-bp transfected PTG transcript (Fig. 1A).

The inducible protein expression was analyzed by immunoblotting using anti-His antibody against the N-terminal epitope tag. As shown in Fig. 1B, anti-His antibody detected a protein of about 30 kDa only in induced cells (i.e. doxycycline). These results indicate that PTTG expression is tightly regulated by tetracycline in the established cell line (designated HeLaS3-PTTG). I also generated cell lines with inducible expression of a transcriptional-defective mutant PTTG (11) containing Ala substitution of Ser to Ala. These cell lines (designated HeLaS3-mPTTG) also demonstrated tightly regulated mutant transcript (Fig. 1A) and protein (Fig. 1B).

Gene Expression Profiling to Identify PTTG Targets—A potential advantage of PTTG as a transcriptional regulator has been suggested by its ability to enhance transcription of transiently transfected reporter constructs (3, 10, 11). However, target genes whose expression level is hindered after inductive expression of PTTG. Total RNA was isolated from HeLaS3-
PTTG at 0 and 24 h after PTTG induction, labeled with 

of c-myc expression. However, there was no change in either c-

of the transactivation-defective mutant PTTG. These include c-myc oncogene, MEK1, MEK3, protein kinase C β-1 (PKCβ-1), and the heat shock protein HSP70. The increased expression of c-myc, MEK1, and HSP70 was confirmed by Northern blot analysis (Fig. 2).

Induction of c-Myc by PTTG—Myc is an important regulator of many cellular processes, including growth promotion, differentiation, and apoptosis (17–19). Identification of c-myc as a potential target for PTTG is of particular interest; I therefore investigated further the relationship between these two oncogenes. I performed a time course study on the expression of c-myc after induction of PTTG by tetracycline withdrawal. As shown in Fig. 3, both c-myc mRNA (Fig. 3A) and protein (Fig. 3B) were induced within 6 h of induction of wild type PTTG expression. However, there was no change in either c-myc mRNA (Fig. 3C) or protein (Fig. 3D) expression after induction of the transactivation-defective mutant PTTG expression. These results indicate that transcriptional activation function of PTTG is required to induce c-myc expression.

Many studies have shown that the c-myc gene has a critical role in the control of cellular proliferation. Overexpression of c-Myc protein stimulates cell cycle progression, causes transformation, blocks differentiation, and induces apoptosis in low serum (14). I sought to test whether induction of PTTG expression in HeLaS3-PTTG cells has any effect on growth property of these cells. As shown in Fig. 4A, when cells were grown in the presence of tetracycline, no significant differences in growth rate were observed between HeLaS3-PTTG and HeLaS3-pCMV (cell line that expresses the vector). When induced with tetracycline withdrawal, the growth rate of cells expressing PTTG was increased more than 2-fold. In contrast, in HeLaS3-mPTTG cells, induction of mutant PTTG after tetracycline withdrawal led to slightly retarded cell growth (Fig. 4A). I then measured the ability of these cells to form colonies in the presence or absence of tetracycline. A 2-fold increase in colony formation was observed after the induction of PTTG (Fig. 4B). In contrast, withdrawal of tetracycline had no effect on colony formation in HeLaS3-pCMV or HeLaS3-mPTTG. These results suggest that PTTG may exert its effects on cell proliferation through activation of the c-myc oncogene.

PTTG Activates c-Myc Transcription in Transfected Cells—To test whether PTTG induces c-myc expression by activating its transcription, I transfected NIH3T3 cells with fusion constructs containing either 2.5 kilobase pairs (HBM-Luc) or 142 bp (XNM-Luc) of c-myc 5′-flanking region (15) fused to the reporter gene luciferase. As shown in Fig. 5, the luciferase activity of both HBM-Luc and XNM-Luc was increased 2.5-fold when cotransfected with a wild type PTTG expression plasmid, whereas there was no change in reporter gene activity when the cells were cotransfected with either the pCMV vector or transactivation-defective mutant Ser162→Ala (11). These results suggest that PTTG is able to transactivate c-myc transcription and that the DNA sequences required for PTTG transactivation are located within 142 bp of the c-myc 5′-flanking region.

PTTG Binds to c-Myc Promoter in a Complex with USF1—To determine whether PTTG transactivates c-myc transcription by directly interacting with its promoter, I performed DNaseI footprinting experiments using nuclear extract prepared from HeLaS3-PTTG cells. Purified Sp1 protein was used as a control for experimental conditions. As shown in Fig. 6, Sp1 protected two regions on c-myc promoter corresponding to its consensus binding sites. In the presence of the nuclear extract, a region between −20 and +28 in the c-myc 5′-flanking region was protected from DNaseI digestion (Fig. 6, plus strand DNA was labeled), suggesting that nuclear proteins in HeLaS3-PTTG cells specifically interact with this DNA sequence. Similar results were obtained when minus-strand DNA was labeled (data not shown).

To determine whether the nuclear proteins that interact with this specific DNA sequence in the c-myc gene include PTTG, I performed gel mobility shift assays using the sequence between −20 and +28 as the probe. As shown in Fig. 7A, incubation of HeLaS3-PTTG nuclear extract with the probe resulted in two mobility-shifted bands (lane 2) that were competed with unlabeled, homologous oligonucleotide (lane 3) but not by unrelated oligonucleotide (lane 4). The addition of antipeptide antibody to the reaction led to super shift of the upper band (lane 6), whereas pre-immune serum had no effect (lane 5). These results suggest that PTTG is present in the protein complex that interacts with the c-myc 5′-flanking sequence.

The sequence between −20 and +28 of the c-myc gene includes two adjacent initiator elements (consensus: YYANTYY) (20), Inr-A (−4 to +7) and Inr-B (−7 to −17), that are potential binding sites for transcription factors USF-1 (21), YY1 (22), and TFII-I (21). I therefore tested whether any of these factors are...
present in the mobility-shifted bands. Fig. 7A shows that inclusion of anti-USF1 antibody resulted in the disappearance of the upper band of the two mobility-shifted bands (lane 7), suggesting that USF1 is part of the protein complex that interacts with the 48-bp c-myc sequence. The addition of both anti-PTTG and USF1 antibodies resulted in the disappearance of the upper band as well as a super-shifted band (lane 8), suggesting that USF1 was in the same protein complex with PTTG. The addition of anti-YY1 antibody did not cause any change in the mobility-shifted bands (lane 9).

To test whether PTTG by itself could also bind to this DNA sequence in the c-myc promoter, PTTG was expressed in and purified from Escherichia coli as a glutathione S-transferase fusion protein. After thrombin protease digestion to remove glutathione S-transferase from the fusion protein, purified PTTG was used in gel mobility shift assays. As shown in Fig. 7B, incubation of PTTG with the probe resulted in a mobility-shifted band (lane 2) that was competed with unlabeled, homologous oligonucleotide (lane 3) but not by unrelated oligonucleotide (lane 4). The addition of anti-PTTG antibody to the reaction led to the appearance of a super-shifted band (lane 5). These results suggest that PTTG is capable of DNA binding by itself.

Identification of the DNA-binding Site for PTTG—To further define the DNA sequence required for PTTG DNA binding, I generated oligonucleotides that contain various mutations within the 48-bp c-myc gene (see “Materials and Methods”)
sequence and performed gel shift assays to test PTTG interaction with these sequences.

Initially, I tested whether mutations within the initiator elements (Inr) affected PTTG DNA binding. In the adenovirus-2 major late promoter (AdMLP) Inr, which shares substantial homology with c-myc Inrs, mutation of residues −1 and −2 or +2, +3, and +4 relative to the transcription start site reduced USF1 binding to Inr element without significantly affecting basal transcription levels (23). Based on these observations, I made analogous mutations (Fig. 8A) within the c-myc Inr to determine whether these elements play a role in PTTG DNA binding. As shown in Fig. 8B, incubation of oligonucleotide containing point mutations within Inr-A (Myc M1, panel A) and recombinant PTTG resulted in a mobility-shifted band (lane 2) that was competed by unlabeled homologous (lane 3) but not by unrelated (lane 4) oligonucleotide. The addition of anti-PTTG antibody resulted in a super-shifted band (lane 5). When HeLaS3-PTTG nuclear extracts were included, two mobility-shifted bands were present (lane 6). They were competed by unlabeled Myc M1 (lane 7) but not by unrelated (lane 8) oligonucleotide. The addition of anti-USF1 antibody, however, did not lead to the disappearance of the upper band (lane 9) as observed when wild type oligonucleotide was used (Fig. 7A, lane 7). A super-shifted band was observed in the presence of anti-PTTG antibody (lane 10). These results suggest that mutations within Inr-A do not affect PTTG DNA binding, whereas they abolish USF1 DNA binding. I have also tested the effects of mutations within Inr-B (Fig. 8A, Myc M2) on PTTG and USF1 DNA binding, and the results indicated that mutations within this initiator element did not affect DNA binding of either PTTG or USF1 (data not shown).

I then divided the 48-bp c-myc sequence into two parts; probe GH1 contains sequence −20 to +4, and GH2 contains +5 to +28. Fig. 9A shows that incubation of GH1 probe with either recombinant PTTG (lane 2) or HeLaS3-PTTG nuclear extracts (lane 6) resulted in a mobility-shifted band. This band was competed by unlabeled GH1 (lanes 3 and 7) but not by GH2 (lanes 4 and 8). The addition of anti-PTTG antibody resulted in a super-shifted band (lanes 5 and 9). One of the other hand, incubation of GH2 probe with either recombinant PTTG (lanes 11–13) or HeLaS3-PTTG nuclear extracts (lane 14–16) resulted in no specific mobility-shifted band. These results indicate that the DNA-binding site for PTTG resides between position −20 to +4.

I then made point mutations within this 24-bp pair sequence (Fig. 9B). As shown in Fig. 9C, incubation of wild type oligonucleotide with HeLaS3-PTTG nuclear extracts resulted in a mobility-shifted band (lane 2) that was competed by 100× unlabeled homologous sequence (lane 3) and was super-shifted by anti-PTTG antibody (lane 6). The addition of 100× excess of cold GH1 m1 resulted in very little competition (lane 4), and addition of the same molar excess of GH1 m2 had no effect (lane 5). When GH1 m1 was used as a probe, it generated a mobility-shifted band in the presence of nuclear extracts (lane 8). The band was competed by both unlabeled wild type and m1 (lanes 9 and 10) but not by m2 oligonucleotide (lane 11). The affinity of nuclear protein binding to m1 probe was lower than that to the wild type probe (compares lanes 2–6 to 8–12). When GH1 m2 and m3 were used as the probes, no specific mobility-shifted band was observed (Fig. 9C, lanes 13–20). These results suggest that the sequences between −3 and −5 (mutated in m2) and between −15 and −20 (mutated in m3) are required for PTTG DNA binding.

Mapping PTTG DNA Binding Domain—PTTG does not contain any typical DNA binding motif, and the amino acids required for PTTG DNA binding have yet to be identified. I therefore sought to determine the DNA binding domain for PTTG. Various parts of PTTG were expressed in and purified from E. coli. After thrombin protease digestion, the purified...
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Proteins were used in gel mobility shift assays. The integrity of the proteins were verified by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining. As shown in Fig. 10, truncated proteins containing either the N-terminal 118 amino acids or amino acids between 61 and 199 generated a mobility-shifted band (lanes 2 and 6) that was competed by unlabeled homologous oligonucleotide (lanes 3 and 7) but not by unrelated oligonucleotide (lanes 4 and 8). The addition of anti-PTTG antibody resulted in a super-shifted band for PTTG (1–118) (lane 5). Antibody was not included in reactions with PTTG (61–199) because the epitope is located at the N-terminal 17 amino acids. PTTG deletion mutant containing amino acids 119–199 did not generate mobility-shifted band (lanes 9–11). These results indicate that the PTTG DNA binding domain resides between amino acids 61 and 118.

PTTG DNA Binding Is Required for Its Transactivation of c-Myc Gene—To test whether binding of PTTG to DNA is required for its transactivation function, I first determined the effect of mutations within PTTG-binding site on PTTG transactivation of c-myc gene. The same nucleotide substitutions between position -3 and -5 (m2) and -15 to -20 (m3) were made in XNM-Luc by site-directed mutagenesis. Fig. 11A shows that cotransfection of PTTG expression vector resulted in a 3-fold increase in wild type c-myc promoter activity (XNM-Luc); mutations between -15 and -20 (m3XNM-Luc) abolished this activation without affecting the basal promoter activity. Mutations between -3 and -5 (m2XNM-Luc) not only abolished PTTG-activated transcription but also reduced basal promoter activity. The decrease in c-myc basal promoter activity in m2XNM-Luc may be a result of mutations near the transcriptional initiation site. I then tested whether DNA binding-defective mutant PTTG (119–199) could still activate c-myc transcription. As shown in Fig. 11B, PTTG (119–199) by itself had little effect on c-Myc promoter activity. When cotransfected with wild type PTTG, it inhibited activation of c-myc promoter by wild type PTTG. These results indicate that PTTG DNA binding is required for its transactivation function.
PTTG has been implicated in many types of human neoplasm. However, the mechanisms involved in PTTG cellular transformation and tumorigenesis remains to be elucidated. In this study, by generating cell lines with tightly regulated inducible expression of PTTG and expression profiling using DNA arrays (24, 25), I have identified a down-stream target gene. Induction of PTTG expression in HeLaS3 cells resulted in increased cell proliferation and colony formation. This effect involves induction of another oncogene c-myc.

The product of the c-myc proto-oncogene is a critical regulator of cellular proliferation (26). Overexpression of c-Myc contributes to the transformation of primary fibroblasts in culture (27) as well as development of neoplasia in a wide variety of tissues (28). The c-Myc protein exhibits sequence-specific DNA binding when dimerized with its partner Max (29–31). The interaction with Max is necessary for most of the physiological effects of c-Myc (32). c-Myc has been implicated in both activation and repression of transcription (33–35). The expression of the c-myc gene is closely correlated with proliferation. c-myc expression is absent in quiescent cells but is rapidly induced upon the addition of growth factor (36–38). The expression pattern of c-myc is similar to that of PTTG, which is also induced by mitogenic treatment such as serum (8). Identification of c-myc as a target for PTTG is therefore consistent with the potential role PTTG plays in controlling cell proliferation. In my previous study, I have shown that PTTG is phosphorylated by MAP kinase and that activation of this signal transduction pathway by growth factors such as epidermal growth factor enhances PTTG transactivation function (11). c-Myc is also a target of MAP kinase cascade and is phosphorylated on Ser15 by MAP kinase (39, 40). Phosphorylation at this site is associated with enhanced transactivation function of c-Myc (40–42). In this study, I have identified MEK1 as another potential PTTG target. Taken together, these results suggest a functional pathway that includes MAP kinase cascade and c-Myc for PTTG-mediated effects on cell proliferation.

The identification of c-myc as an endogenous gene that is rapidly induced after PTTG expression provides a physiological target to study the potential role of PTTG in transcriptional regulation. Our results showed that transient transfection of a PTTG expression plasmid led to activation of the proximal c-myc promoter, whereas expression of a transactivation-defective PTTG mutant had no effect on c-myc promoter activity. In addition, in the cell line that expresses transactivation-defective PTTG, c-myc expression was not induced after induction of mutant PTTG expression. These results indicate that activation of c-myc expression requires PTTG transactivation function.

I have provided evidence that PTTG transactivates c-myc by directly interacting with its promoter. DNaseI protection assays showed that nuclear proteins from HeLaS3 cell overexpressing PTTG interact with the c-myc gene between −20 and +28. Results from gel mobility shift assays using oligonucleotides containing various mutations of this region indicated that the DNA-protein complexes contain at least two proteins. One of these proteins is the ubiquitous basic helix-loop-helix-leucine zipper transcription factor USF1 that binds to the initiator element between −4 and +7 (InrA). Although USF was shown to bind to Inr of the AdMLP and activates transcription through specific cooperation with TFII I (20, 21), the data presented here show for the first time that USF1 also binds to the Inr in c-myc promoter. The DNA-binding site for PTTG was located between +4 and −20, and the nucleotides important for PTTG binding include the residues between −3 and −5 as well as between −15 and −20. Importantly, mutations within these nucleotides not only prevented PTTG from binding DNA but also abolished PTTG-mediated transcriptional activation of the c-myc gene. It is possible that binding of PTTG and USF to adjacent sites on c-myc promoter could result in cooperative binding of both factors to DNA, or alternatively, transcriptional synergy may occur by the two transcriptional activators contacting the general transcriptional machinery simultaneously. These exciting possibilities will be explored in the future studies.

A typical transcription activator protein is a modular protein composed of DNA binding and activation domains (43). PTTG does not contain any common DNA binding motifs such as homeodomain, zinc fingers, basic leucine zipper (bZIP), and basic helix-loop-helix (44). Combining mutagenesis with gel mobility assay, I have mapped the DNA binding domain of PTTG to a region between amino acids 61 and 118 adjacent to the Inr. Although USF was shown to bind to Inr of the AdMLP and activates transcription through specific cooperation with TFII I (20, 21), the data presented here show for the first time that USF1 also binds to the Inr in c-myc promoter. The DNA-binding site for PTTG was located between +4 and −20, and the nucleotides important for PTTG binding include the residues between −3 and −5 as well as between −15 and −20. Importantly, mutations within these nucleotides not only prevented PTTG from binding DNA but also abolished PTTG-mediated transcriptional activation of the c-myc gene. It is possible that binding of PTTG and USF to adjacent sites on c-myc promoter could result in cooperative binding of both factors to DNA, or alternatively, transcriptional synergy may occur by the two transcriptional activators contacting the general transcriptional machinery simultaneously. These exciting possibilities will be explored in the future studies.

**DISCUSSION**

PTTG DNA binding is required for its transactivation.

NIH 3T3 cells were transiently transfected with the indicated plasmids and assayed for luciferase reporter gene activity (represented as fold of NIH 3T3 cells were transiently transfected with the indicated plasmids and assayed for luciferase reporter gene activity (represented as fold of induction over promoter-less pGL2). Values represent mean ± S.E. (n = 9, average of three independent experiments). A. effect of mutations within PTTG DNA-binding site on PTTG transactivation of c-myc promoter. B. deletion of PTTG DNA binding domain (DBD) abolishes its transactivation.
lished the important role of PTTG in the regulation of transcrip
tion. Although the definitive requirement for PTTG in c-myc activation will need to be confirmed once PTTG-null cells are available, the data presented here clearly indicate that c-myc is likely a physiological target for PTTG and, thus, links PTTG to a functional pathway involved in cellular proliferation and transformation.

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