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# Characterization of tTA and Its Functional Domain in Tetracycline Repressor-mediated Gene Repression System

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To elucidate of role(s) of tTA as a repressor in the tTA-mediated gene repression system, we introduced mutations into the acidic domain of VP16 and examined the effects of such various mutations. In the transient repression experiment, a region containing 34 amino acids of the activation domain of VP16 (412~456) which interacts with TFIIB was found to be necessary and sufficient for the tTA-mediated repression of gene expression. However, in the experiment to investigate the fact that tTA-regulated repression is related to the activation function of VP 16, we found that the repression abilities of tTA derivatives did not correlate exactly with their activation abilities. Therefore, we conclude that increased mass of VP16 in tTA might be also important for effcient repression in addition to functional domain of VP16.

**Key words :** tTA, HCMV, HSV-1, Tetracycline repressor protein, VP16

## INTRODUCTION

Control of gene expression in a eukaryotic system by tetracycline repressor (TetR)-mediated repression was first reported in a plant system (Gatz *et al.*, 1992; Gatz and Quail, 1988) and in a mammalian system (Kim *et al.*, 1995). Binding of TetR to operator sequences when appropriately positioned in a promoter, represses the expression of the linked reporter gene. However, the presence of tetracycline can prevent the repressor from binding to its operator sequences and thereby induce the expression of the reporter gene. In contrast to the repression by the wild type TetR, transcriptional activation by a chimeric TetR-VP 16 fusion protein (tTA) was recently reported in mammalian cells (Gossen and Bujard, 1992). tTA consists of the entire TetR and the C-terminal acidic domain of herpes simplex virus type 1 (HSV-1) VP16 protein (Gossen and Bujard, 1992, 1995). In the absence of tetracycline, tTA activates the expression from a minimal promoter containing the mutiple tet operator (*tet* O) sequnces located upstream from the promoter, whereas the presence of tetracycline prevents tTA from binding to its operator sequences and thereby leads to low-level basal expression from the minimal promoter.

To study essential genes of human cytomegalovirus (HCMV) and their biological roles in the viral replicative cycle, we established TetR-based gene regulation system elsewhere (Kim *et al.*, 1995), which adapted to the HCMV US11 (early) promoter containing *tet* O sequences. Interestingly, binding of TetR to the operator sequences placed at the 3' adjacent to the TATA box of the HCMV US11 promoter inhibited the transcription of a β-glucuronidase reporter gene rather than activating the reporter gene. However, efficient repression was achieved in the presence of tTA, but not in the presence of TetR.

The mechanism by which the repression is facilitated by tTA is unknown. Simple speculation is that steric hindrance in the formation of a functional transcription initiation complex may play an important role due to the different size of tTA (~43 KDa) from TetR protein (~22 KDa). Alternatively, a functional domain of VP16 may interact with basal transcription factors or coactivators in an inappropriate manner, blocking the formation of the functional transcription initiation complex. VP16 has been shown to directly interact with the basal transcription factor TFIIB (Lin and Green, 1991) and TFIID (Ingles *et al.*, 1991; Stringer *et al.*, 1990) including the TBP (TATA binding protein) and coactivator $TAF_{II}40$ (Goodrich *et al.*, 1993).

Biochemical and genetic analysis of VP16 mutants by several groups (Berger *et al.*, 1990; Reiger *et al.*, 1993; Triezenberg *et al.*, 1988) suggest that the C-terminal acidic region (78 aa) of VP16 is functionally divided into two activation domains. $VP16_N$ (aa 412-456) when fused to the GAL4 DNA binding domain has been found to interact with TFIIB (Lin and Green,

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1991; Roberts *et al.*, 1993) and weakly with TBP (Ingles *et al.*, 1991; Kim *et al.*, 1994; Stringer *et al.*, 1990). However, VP16$_{NC}$ (aa 412-490) interacts with TFIIB more strongly than either VP16$_{N}$ (aa 412-456) or VP16$_{C}$ (aa 457-490) (Goodrich*et al.*, 1993). VP16C when fused to the GAL4 DNA binding domain has been reported to interact with TAF$_{II}$40 (Goodrich *et al.*, 1993). A point mutation in phenylalanine to proline at position amino acids 442 of VP16$_{N}$, VP$_{N}$ (FP442), abolished both the activation properties and the ability of interaction with TFIIB (Berger *et al.*, 1990; Cress and Triezenberg, 1990; Lin and Green, 1991).

In this paper we describe a mechanism by which tTA induces the repression when *tet* O are present to the 3' of the minimal promoter. The functional acidic domain of VP16 was mutated and its mutants were tested to determine their abilities of repression in mammalian cells. Our repression and acitivation data suggest that mass of VP16 in tTA derivatives as well as the functional domain of VP16 could be important for the efficient repression possibly due to steric hinderance to the formation of the transcriptional preinitation complex.

## MATERIALS AND METHODS

### Cells and virus

Human foreskin fibroblast (HFF) cells were isolated in this laboratory and used below passage 20. U373-MG astrocytoma cells were obtained from the American Type Culture Collection. Both cell types were grown in Dulbecco's modified Eagle medium (DMEM; Mediatech) containing 10% fetal bovine serum (GIBCO) and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). HCMV strain AD169 was obtained from the American Type Culture Collection; wild type and recombinant HCMV were propagated according to standard protocols.

### tTA expression plasmids

In order to make constructs expressing tTA derivatives, pUHD15-1 (Fig. 1A) was used as the parental plasmid, which encodes tTA (TetR-VP16) fusion protein, as described previously by Gossen and Berjard (1992). To construct pUHD15-1 (Δ456), the oligonucleotide 5'GATCCTAGGTACCTACGGACCC3' and its complement were inserted between the *Sma* 1 site and the *BamH* 1 site of pUHD15-1. To construct pUHD 15-1 (FP442), the oligonucleotide 5'CCGACGCGCTAGACGATCCGGATCTGGACATGTTGGGGGACGGGGATTCCCC3' and its complement were cloned between the *Sph* 1 site and the *Sma* 1 site of pUHD15-1. To construct pUHD15-1 (Δ456-442FP), The 583 bp of *Sma* I and *Hind* III fragment from pUHD15-1 (Δ456) was cohesively ligated into pUHD15-1 (442FP). pIE(s) was

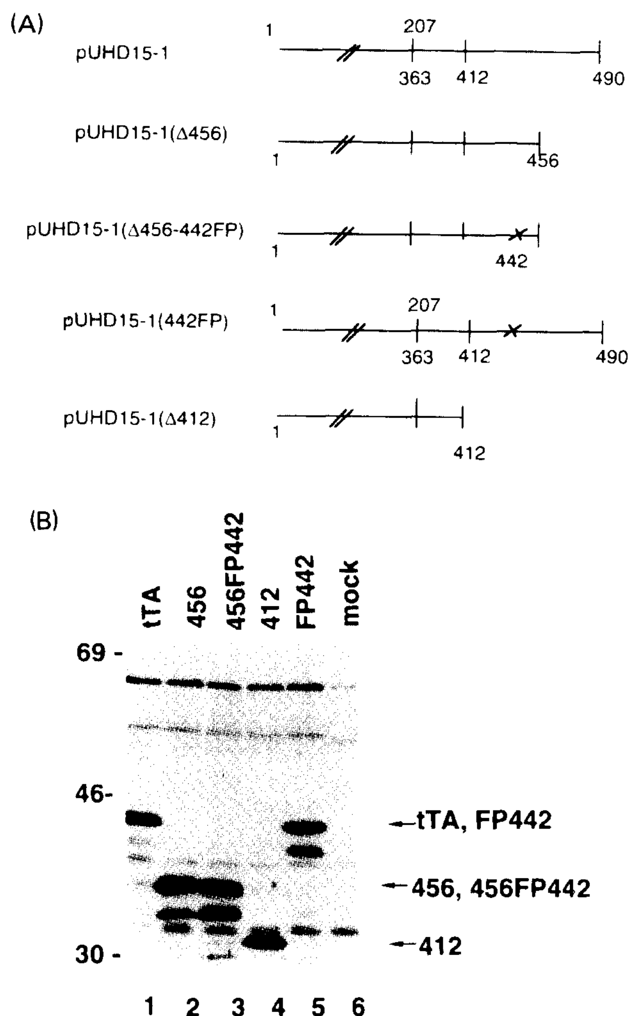



**Fig. 1.** Expression of tTA and derivatives. (A) A schematic representation of the structures of tTA (pUHD15-1) and derivatives used in this study. The coding regions of TetR amino acids (upper number) and VP16 amino acids (lower nimber) are indicated. x mark represents the point mutation (phenyl alanine to proline) site 442 in VP16 coding region. (B) Western blot analysis of tTA and derivatives. HFF cells were transfected with plasmid encoding tTA or derivatives. The cells were infected with HCMV at 24 h posttransfection, and total cellular proteins were harvested at 48 h posttransfection. Following SDS-PAGE, Western blot analysis was performed using the TetR polyclonal antibody. The location of the tTA and derivatives are indicated on the right; the position of molecular weight markers (in KDa) on the left.

derived from pIE (containing sequentially the HCMV immediate-early promoter, a poly linker, and the HSV-1 thymidine kinase polyadenylation signal) and contains a universal stop codon in all three frames at the distal end of the polylinker, adjacent to the polyadenylation signal. pUHD15-1 (Δ412) was constructed from pIE(s) and pUHD15-1 and encodes a TetR-VP16 fusion protein truncated after 50 amino acids of the VP16 domain [tTA(Δ412)]. Essentially, the region encoding TetR and the first 50 amino acids of VP16

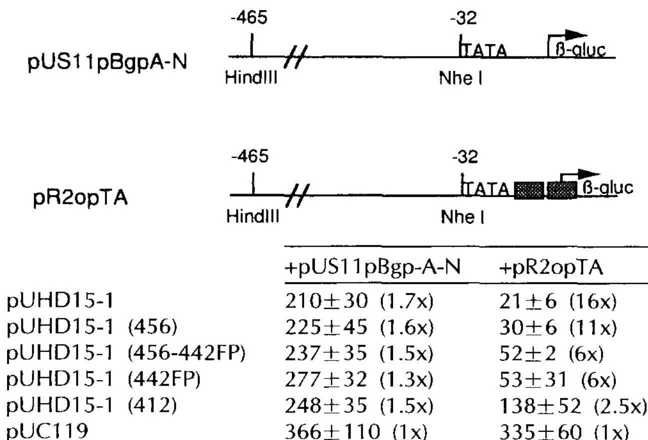
was cloned just upstream of the stop codons of pIE(s). All plasmid DNA manipulations were done according to standard protocols (Sambrook *et al.*, 1988).

### Reporter gene plasmids

For the tTA mediated-repression assay, pR2opTA (Fig. 2) as described by Kim *et al.* (1995) was used. Seqentially, the plasmid consists of the US11 promoter containing the two *tet* operators, β-glucuronidase gene, and HCMV US10 poly A signal. For the tTA mediated-activation assay, pMIEBg7op (Fig. 3) was used. To constuct pMIEBg7op, a 500 bp of the *Xba* I- *Xho* I fragment from pUHC13-3 (Gossen and Bujard, 1992) containing the seven tet O seuences and the minimal HCMV IE promoter was inserted into pBg10pA. The plasmid (pBg10pA) consists of promoterless β-glucuronidase gene and HCMV US10 poly A signal.

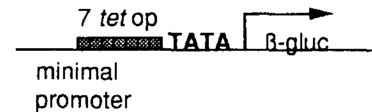
### Transient expression and β-glucuronidase assay

U373-MG cells were transfected in 60 mm plates by the standard calcium phosphate method (Sambrook *et al.*, 1988). Ten μg of supercoiled plasmid DNA was transfected per plate. After 7 h at 37°C, the cells were shocked with 15% glycerol in 1X HeBS (pH 7.05) for 2 min. The cells were washed twice with PBS and growth media were added. Cell extracts were made at 48 h post-transfection and β-glucuronidase assay




| | +pUS11pBgp-A-N | +pR2opTA |
|---|---|---|
| pUHD15-1 | 210±30 (1.7x) | 21±6 (16x) |
| pUHD15-1 (456) | 225±45 (1.6x) | 30±6 (11x) |
| pUHD15-1 (456-442FP) | 237±35 (1.5x) | 52±2 (6x) |
| pUHD15-1 (442FP) | 277±32 (1.3x) | 53±31 (6x) |
| pUHD15-1 (412) | 248±35 (1.5x) | 138±52 (2.5x) |
| pUC119 | 366±110 (1x) | 335±60 (1x) |

**Fig. 2.** Repression assay for tTA derivatives. The schematic shows the US11 promoter region of the two plasmids reported previously (Kim *et al.*, 1995). The US11 promoter sequence, -465 to + 1 (relative to the transcription initiation site), controls expression of the β-glucuronidase (β-gluc) reporter gene in the plasmids. The locations of the TATA box (TATA) and *tet* O (sheded rectangles) are indicated. β-glucuronidase activity (pmole of product per 0.1 μg protein per minute) was determined following cotransfection of the indicated plasmids in the HCMV-infected transient expression system. Cotransfection with pUC119 was done as a negative control. The results shown are the means of three independent transfections. Fold repression (in parentheses) was calculated relative to the pUC119 data.




| plasmid | +pMIEBg7op |
|---|---|
| pUHD-15-1 | 153±67 (19x) |
| pUHD15-1 (456) | 35±9 (4.4x) |
| pUHD15-1 (456-442 FP) | 11±3 (1.4x) |
| pUHD15-1 (442FP) | 38±8 (4.8x) |
| pUHD15-1 (412) | 9±2 (1x) |
| pUC119 | 8±2 (1x) |

**Fig. 3.** Activation assay for tTA derivatives. The schematic shows the HCMV minimal promoter region containing seven tandom *tet* O sequences. The locations of the TATA box (TATA) and tet O (sheded rectangles) are indicated. β-glucuronidase activity (pmole of product per 0.1 μg protein per minute) was determined following cotransfection of the indicated plasmids in the U373-MG transient expression system. Cotransfection with pUC119 was done as a negative control. The results shown are the means of three independent transfections. Fold repression (in parentheses) was calculated relative to the pUC119 data.

was done, as previously described (Jones *et al.*, 1991). In addition, HFF cells were transfected in 60 mm plates by DEAE-dextran method (Kim *et al.*, 1995). Ten μg of supercoiled plasmid DNA was transfected per plate in 2 ml of DMEM containing 200 μg of DEAE-dextran (Mw=500,000; Pharmacia) per ml. After 4 h at 37°C, the cells were shocked with 20% dimethyl sulfoxide in 1X HeBS (pH 7.05) for 2 min. The cells were washed twice with PBS and growth media were added. After incubation for 24 h, these plates were infected with HCMV wild-type strain AD169 at a multiplicity of infection of 3 PFU/cell. Cell extracts were made at 20 h postinfection and β-glucuronidase assay was done as described above. Total protein in the cell extracts was determined by the Bradford method as described in the kit (Bio-Rad).

### Western blot analysis

Proteins were extracted from cells and analyzed by Western blotting using an ECL kit (Amersham) as described previously (Roeder, 1991). The primary anti-TetR rabbit polyclonal antibody (Kim *et al.*, 1995) was diluted 1:500 for use; the donkey anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (Amersham) was diluted 1:10,000 for use.

## RESULTS AND DISCUSSION

### Expression of tTA derivatives

To express tTA derivatives, mammalian expression plasmids encoding various forms of tTA were constructed (Fig. 1A). pUHD15-1 was used as a parental plasmid ; it encodes a tTA (TetR-VP16) fusion protein

in which TetR is fused to the 128 amino acid C-terminal acidic domain of the HSV-1 VP16 protein (Gossen and Bujard, 1992). pUHD15-1 (Δ412) encodes a truncated form of tTA, which lacks the C-terminal 78 amino acids of VP16. pUHD15-1 (Δ456) encodes a truncated form of tTA, which lacks the C-terminal 34 amino acids of VP16. pUHD15-1 (Δ456FP442) encodes a point mutation in phenylalanine to proline on amino acids 442 of tTA (Δ456). pUHD15-1 (FP442) encodes a point mutation in phenylalanine to proline on amino acids 442 of tTA. tTA expression from these plasmids was monitored by Western blot analysis after transient expression in HFF cells. High amounts of tTA proteins were detected in each tTA derivative (Fig 1B).

### Repression of the two operator-modified US11 promoter by tTA derivatives

To determine the effect of the mutated tTA proteins on the expression from the two operator-modified US 11 promoter (pR2opTA) previously displayed efficient repression (Kim *et al.*, 1995), cotransfection assays were performed in HFF cells. The results of three independent experiments are summarized in Fig. 2. In the absence of tTA protein (i.e., the pUC119 cotransfection), HCMV-induced expression was detected in the intact US11 promoter as well as in the two operator-modified US11promoter. In the presence of tTA, the gene expression was repressed to about 16 fold. The absence of 78 amino acids of the acidic VP16 domain in tTA (Δ412) resulted in only 2 to 3-fold repression in the HCMV-induced US11 gene expression, suggesting that 78 amino acids of acidic VP16 domain are required for the efficient repression. However, tTA (Δ456) repressed the gene expression up to about 11-fold, indicating that the 34 amino acids of the VP16 activation domain (412-456) are necessary to the sufficient repression. The mechanism of tTA-regulated repression could be related to binding ability of the transcriptional cofactors by VP16 functional domains. Several groups (Goodrich *et al.*, 1993) previously demonstrated that VP16 (412-456) fused to the GAL4 DNA binding domain had ability to interact with TFIIB (Lin and Green, 1991; Roberts *et al.*, 1993) but lost ability to interact with TAFII40 (Goodrich *et al.*, 1993). The requirerment of the 34 amino acids of the VP16 activation domain (412-456) for the repression suggests that TFIIB but not TAFII40 is involved in the tTA-regulated repression. A point mutation from phenylalanine to proline at position 442 of tTA (Δ456) showed 6-fold repression, which is about half of the repression level of tTA (Δ456). This result is explained by the possibility that tTA (Δ456FP442) has lost its capability to bind to TFIIB due to the point mutation, as GAL4-VP16 (Δ456FP442) fusion protein loses its ability to bind to TFIIB in its point mutant (Lin and Green, 1991). This implies that inhibition of interaction of the functional domain of VP16 with TFIIB affects the repression level. tTA (Δ456FP442) still has 6 fold repression, which is about two times higher than that of tTA (Δ412). This result suggests that increased mass of tTA (Δ456FP442) (~37 KDa) compared to tTA (Δ 412) (~32 KDa) may hinder the formation of functional transcription initiation complex in addition to inhibition of the interaction between functional domain of VP16 and TFIIB. It is particularly noteworthy that tTA (FP442) showed only 6-fold repression as tTA (Δ 456FP442) did, even though the size of tTA (FP442) is ~5 KDa larger than that of tTA (Δ456FP442) indicating that the increase after a certain size of the VP 16 domain does not elicit the increase in repression level correspondively. Therefore, 37 KDa of tTA derivatives seems to be the optimum size to achieve the maximum repression level in this case.

### Activation of minimal promoter by tTA derivatives

To investigate that the tTA-regulated repression is related to the activation function of VP16, we tested the ability of tTA derivatives to activate the gene expression from an indicator plasmid. This indicator plasmid (pMIEPBg7op) consists of a β-glucuronidase gene under the control of the HCMV minimal promoter lacking its enhancers but linked to 7 *tet* operators (Gossen and Bujard, 1992) and the HCMV US10 poly A signal. The indicator plasmid and each tTA derivative construct were cotransfected into HFF cells, and transient expression was analyzed. The data shown in Fig. 3 represent the means of three independent experiments. In the presence of tTA (i.e., pUHD15-1), expression from the *tetO*-minimal promoter activated ~19-fold, whereas essentially no activation was observed in the presence of mock (i.e., pUC119). No activation was also observed in the presence of tTA (412) and tTA (Δ456FP442) as several groups previously demonstrated that GAL4-VP16 (Δ412) and GAL4-VP16 (Δ456FP442) fusion proteins lost transcriptional activation ability due to impairment of their TFIIB binding ability (Berger *et al.*, 1990, Roberts *et al.*, 1993). However, these two mutants [tTA (Δ412) and tTA (Δ 456FP442)] showed the repression level 2.5- and 6-fold, respectively, suggesting that the increase of mass in tTA derivatives is necessary for the efficient repression by steric hindrance to the formation of the preinitiation complex with basal transcription factors. It is particularly noteworthy that tTA (FP442) caused 5-fold activation level as tTA (Δ456) did but tTA (FP442) showed about half the repression level of tTA (Δ456) (Fig. 2). This difference in repression levels between the two mutants may not be explained by the activation ability of VP16. Their different repression levels may come from their different TFIIB binding abi-

lity. But tTA (FP442) binding ability to TFIIB should be measured.

### A model for transcriptional activation and repression by tTA

The results from experiment and previous study (Kim *et al.*, 1995) indicate that either activation or repression by tTA could occur, depending on the positioning of the *tet* operator in a promoter. A schematic diagram of potential differences between transcriptional activation and repression by tTA is shown in Fig. 4.

In the activation scheme (Fig. 4A), tTA binds *tetO* located upstream of the TATA box at a distance in which its VP16 domain is in a favorable position to stabilize the interaction of certain basal factors and coactivators of the TFIID complex, as well as factor TFIIB, with the promoter. This leads to the formation of a functional preinitiation complex (Roeder, 1991; Zawel and Reinberg, 1992), resulting in stimulation of transcription (Goodrich *et al.*, 1993; Lin and Green, 1991). In the repression scheme (Fig. 4B), tTA binds *tetO* just downstream of the TATA box. We hypothesize that the functional VP16 domain in tTA is in an unfavorable position and impairs the formation of a functional preinitiation complex, causing transcriptional repression. Our cumulative data suggest that this impairment could be caused by steric hindrance to the formation of the preinitiation complex, as well as by inappropriate binding by the VP16 activation domain to TFIIB which positions it in a nonfunctional conformation. 34 amino acids of VP16 domain (412-456) are necessary and sufficient for tTA-mediated repression (Fig. 2), suggesting that TFIIB but not TAFII 40 is involved in the repression. However, there might be additional coactivator(s) which is involved in the tTA-mediated repression.

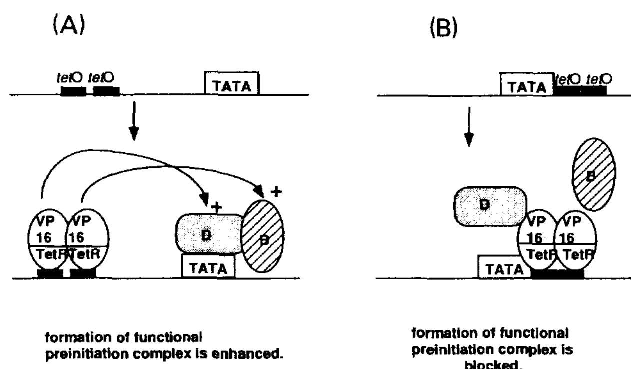



**Fig. 4.** (A) In the acivation scheme, *tet* operators upstream of TATA box in an optimal position to activate transcription when bound by TetR-VP16 (tTA). In this favorable position VP16 stabilizes TFIID and TFIIB binding to the TATA box region and thereby stimulates the formation of a functional preinitiation. (B) In the repression scheme, *tet* operators downstream of TATA box in an optimal arrangement for repression when bound by TetR-VP16 (tTA). In this unfavorable position, the TetR-VP16 binding to the TetR operators interferes the formation of a functional preinitiation complex, resulting in transcriptional repression.

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