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Transcriptional repression of the mouse *wee1* gene by TBP-related factor 2

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Abstract

TBP-related factor 2 (TRF2), one of the TBP family proteins, is involved in various cellular functions through its transcription stimulation activity. We previously reported that TRF2 is involved in reduction of wee1 mRNA in genotoxin-treated chicken cells. In this study, we investigated the role of TRF2 in wee1 gene expression. It was found that wee1 mRNA was decreased in hydroxyurea-treated NIH3T3 cells. Mouse wee1 promoter activity was repressed by TRF2 in mouse and chicken cells. Chromatin immunoprecipitation and plasmid immunoprecipitation analyses revealed that TRF2 is recruited to the wee1 promoter in accordance with the transcriptional repression. A mutant TRF2 that lacks TFIIA-binding capacity lost its repressive function. This mutant was less recruited to the wee1 promoter than was the wild-type one, and provided a decline in promoter-recruited TFIIA. Data in this study suggest that transcription repressive activity of TRF2 to wee1 promoter needs association with the promoter and TFIIA.

Keywords: TRF2; TLP; TLF; wee1; Transcription factor

Initiation of transcription by RNA polymerase II is governed by a pre-initiation complex on a promoter including multiple general transcription factors (GTF) in addition to the RNA polymerase [1,2]. Formation of the pre-initiation complex (PIC) is initiated by transcription factor IID (TFIID), the target of which is TATA-box in a promoter [3,4]. TBP (TATA-binding protein) is an essential DNA-binding component of TFIID [2,3,5,6]. TBP is also required for TATA-less promoters though its TBP-anchoring mechanism is not fully elucidated [7,8]. Eukaryotes have three classes of TBP family proteins, TRF1, TRF2, and TRF3, in addition to the conventional TBP [9–12]. TRF2, which is also called TLP (TBP-like protein) or TLF (TBP-like factor), exists restrictedly in metazoa [13–15]. TRF2 is required for development of *Xenopus* and *Caenorhabditis elegans* [16–18] and spermiogenesis of mouse [19–21].

TRF2 consists of about 180 amino acid residues that correspond to the C-terminal conserved region of TBP with 38% identity [10,13,15]. Although TRF2 has no obvious DNA-binding ability, it exhibits transcription activation function [10,22–24]. TRF2 is required for activation of the *PCNA* gene of *Drosophila* [22]. In mammals, TRF2 facilitates transcription from the *NF1* gene [23] and core promoters of several TATA-less genes such as *TdT* and *IP$_3$1* genes [24]. When TRF2 is artificially anchored to a core promoter in a one-hybrid assay, TRF2 remarkably activates transcription despite the type of promoter [25].

Abbreviations: TBP, TATA-binding protein; TRF2, TBP-related factor 2; TLP, TBP-like protein; TLF, TBP-like factor; GTF, general transcription factor; PIC, pre-initiation complex; TFIIA, transcription factor IIA; TFIIB, transcription factor IIB; TFIID, transcription factor IID; HU, hydroxyurea; TdT, terminal deoxynucleotidyl transferase; PCNA, proliferating cell nuclear antigen; NF1, neurofibromatosis type 1; ChIP, chromatin immunoprecipitation; PIP, plasmid immunoprecipitation.

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In cells, TRF2 does not exist in cells as a component of huge complex like TBP and other TBP family proteins [6,26,27]. Nevertheless, mammalian TRF2 is stably associated with TFIIA [26,27]. In solution, TRF2 binds to TFIIA more strongly than does TBP. TRF2 forms a homodimer, which is probably transcriptionally inert [27], and the dimer is dissociated by TFIIA [27]. These lines of evidence suggest that TFIIA increases the level of transcriptionally active TRF2 in cells, recruits TRF2 to a core promoter where PIC is formed, and allows TRF2 to participate in transcriptional regulation. As described above, TRF2 works in transcriptional activation for TATA-less promoters tested as far. However, in contrast to TATA-less promoters, exogenously expressed TRF2 inhibits TATA-containing viral (adenovirus major late) [24] and cellular (c-fos) promoter [23]. In these cases, TRF2 is not recruited to a GTF-assembling promoter region [14,23]. High affinity of TRF2 to TFIIA, TRF2-dependent TFIIA titration-out, and TFIIA-governed stabilization of TFIID may be attribute to this phenomenon.

We established chicken DT40-derived cells that lack the *TRF2* gene [28]. *TRF2*$^{-/-}$ cells propagate faster than do wild-type cells and are less sensitive than are wild-type cells to genotoxic agents such as methyl methanesulfonate and UV light [28]. TRF2 is also involved in apoptosis [21,28]. These findings indicate that TRF2 functions as a checkpoint factor. TRF2 is localized in the cytoplasm [27,28]. It has been found that TRF2 migrates to the nucleus by genotoxic agents [28]. In chicken DT40 cells, it has been shown that transcript of *wee1* gene was decreased in accordance with genotoxin-induced nuclear translocation of TRF2, though it is not evident that endogenous TRF2 inhibits transcription. It was suggested that the *wee1* gene is down-regulated directly by TRF2 since the repression was observed soon after genotoxin treatment but was not observed in *TRF2*$^{-/-}$ cells [28]. However, there was no evidence as far that TRF2 directly participates in transcriptional repression of *wee1* gene.

In this study, we investigated the effect of hydroxyurea on wee1 gene expression in mouse cells, and found that HU induces migration of TRF2 to the nucleus. TRF2 was associated with the upstream region of the wee1 promoter and decrease transcription from the wee1 promoter. Furthermore, a mutant TRF2 that has defect in TFIIA binding showed no repressive property to the wee1 promoter. In this paper, we present a novel role of TRF2, namely TRF2 could behave as a transcriptional repressor.

Materials and methods

Cells. Mouse NIH3T3 cells were maintained in DMEM-low medium (Sigma) containing 10% fetal calf serum at 37 °C. L2-8 [27] is a derivative of NIH3T3 that constitutively expresses N-terminal Flag/His-tagged TRF2 (FH-TRF2). Chicken B lymphoma cell-derived DT40 cells were maintained as suspension culture in RPMI1640 medium (Sigma) at 39.5 °C supplemented with 10% fetal calf serum and 1% chicken serum. P17 is a DT40-derived strain that lacks the *TRF2* gene [28].

Plasmids. A mouse DNA fragment containing mouse wee1 promoter region was amplified by PCR and inserted into a pGL3-basic (Promega) at *Xho*I and *Bgl*II sites via TA-cloning using pGEM-T-Easy plasmid (Promega). To construct a reporter plasmid, the mouse wee1 promoter fragment from −310 to +260 relative to the transcription start site was inserted into a pGL3-basic containing the fire fly luciferase gene to construct pGW−310/+260. This plasmid was used as a parental construct for its deletion derivatives. The numbers given to the name of the pGW plasmids represent an included wee1 promoter sequence. As for the TRF2-expressing effector plasmid, mouse TRF2 cDNA was inserted into an *Eco*RI site of pCIneo-FH, which was derived from pCIneo (Promega), to construct FH-TRF2. This plasmid produces a fusion protein having an N-terminal Flag/His_6-tagged TRF2. Point mutants of TRF2 in the pCIneo-FH vector were also used [27]. For example, A32E means that the 32nd alanine of TRF2 is changed with glutamic acid. pGL3/mG2p[−432 to +67] and pGL3/mG2p[−15 to +34] are luciferase reporter plasmids of mouse cyclin G2 promoter (Y. Tanaka, manuscript in preparation). pGL3/mG1p is a luciferase reporter plasmid containing mouse cyclin G1 promoter from −451 to +61 (Y. Tanaka, manuscript in preparation). All plasmids used in this study were propagated in *Escherichia coli* JM109 and purified with a plasmid purification kit (Qiagen).

DNA transfection and luciferase assay. The reporter plasmids used in transfection experiments were cleaved at a unique *Sal*I site located just downstream from the *luciferase* gene in the vector. In the standard luciferase assay, NIH3T3 cells were inoculated in 24-well plates (Falcon) (5×10^4/well), cultured for 24 h, and transfected with DNAs. We used 120 ng of reporter plasmid, 25 ng of effector plasmid, and 10 ng pRL-TK (Promega) as a control luciferase plasmid that has the *Renilla* luciferase gene. DNA was introduced into cells with Lipofectamine-Plus reagent and OPTI-MEM (Invitrogen). Twenty-four hours after transfection, cells were harvested and treated with a lysis solution (Promega), and 5 μl of the supernatant fraction was used for luciferase assay. Luciferase activity was assayed by using a Dual-Lucifease Assay System (Promega). As for DT40 cells, 2.5×10^5 cells/well were transfected with 120 ng of reporter plasmid and 25 ng of effector plasmid by using DMRIE-C DNA transfection reagent (Invitrogen). Obtained luciferase activities were normalized with protein concentrations. The same experiment was performed twice and luciferase activity was determined from three independent samples for each assay.

Western blotting. Whole cell extract (20 μg) was separated by SDS–polyacrylamide gel electrophoresis. Proteins in a gel were transferred to a PVDF membrane, and TRF2 was detected by using an anti-TRF2 antibody and an ECL Plus kit (GE healthcare Bioscience) as previously described [27].

Plasmid immunoprecipitation. Protocols of plasmid immunoprecipitation (PIP) to detect DNA that bounds to TRF2 *in vivo* TRF2 were established from those of a chromatin immunoprecipitation (ChIP) assay described previously [23]. NIH3T3 cells were transfected with the wee1 promoter fragment (−310 to +260) together with TRF2-expressing FH-TRF2 vector. Cells were treated with 1% formaldehyde for 20 min at 37 °C. Glycine was added 0.13 M and cells were collected by brief centrifugation. Cells were lysed with PBS(−) containing 1% NP40, 1 mM benzamidine–HCl, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.5 mM PMSF. Chromatin was sheared by passing through a 20 G needle 10 times and a 26 G needle 10 times. The supernatant of centrifugation (13 krpm, 5 min) was mixed with M2 agarose beads (Sigma). Protein–DNA complexes were eluted from the beads by a method similar to that used for immunoprecipitation. Samples eluted with flag peptides in PBS were adjusted to 0.5 M NaCl, incubated at 65 °C for 3 h, and treated with proteinase K (0.1 mg/ml) at 37 °C for 1 h. DNA was recovered by ethanol precipitation and amplified by PCR. Wide-range and narrow-range primers that amplify DNA from −310 to +260 and from −120 to +30 of wee1 sequences, respectively, were used for PCR. PCR (20–25 cycles) was performed in a standard procedure, and amplified DNA was quantified densitometrically.

Hydroxyurea treatment and chromatin immunoprecipitation. L2-8 cells were inoculated in a 150-mm dish in 20% confluent and cultured for 24 h. Cells were treated with 1.5 mM hydroxyurea (HU) for 12 h. ChIP assay

was carried out according to the manufacture's instruction (Upstate) with slight modifications. Ten percent of glycerol was added to all the buffers and SDS was replaced by NP-40. Cells were treated with 1% formaldehyde for 20 min and 0.13 M glycine for 5 min and lysed with a ChIP lysis buffer. The supernatant of centrifugation (13 krpm for 10 min) of the lysate was diluted 10 times with a ChIP dilution buffer. Blocking of the M2 agarose beads and immunoprecipitation were the same as those described for the PIP assay. ChIP products were eluted with 125 μg/ml flag peptides by incubation at 4 °C for 30 min. DNA in the sample was purified and amplified by PCR with the narrow-range primers (see above).

Immunostaining. Cells were fixed with paraformaldehyde as described previously [28]. TRF2 was detected with rabbit anit-TRF2 antibody and FITC-conjugated secondary antibody, as previously described [28]. Nuclei were stained with DAPI.

RT-PCR. Total RNAs were converted to cDNAs, and amounts of wee1 cDNA were determined by PCR using a set of primers that generate a 478-bp DNA fragment. Sequences at 767–791 (around the junction of exons 2 and 3) and 1245–1221 (in exon 7) relative to the first methionine of the mouse wee1 cDNA were used as forward and reverse primers, respectively.

Results

Expression of wee1 mRNA in HU-treated cells

TRF2 is present predominantly in the cytoplasm. In our previous work, it was found that TRF2 is translocated to the nucleus by treatment with DNA-damaging genotoxic agents such as methyl methanesulfonate and UV [28]. Nuclear translocation of TRF2 was also observed when cells were treated with HU [29]. TRF2 emerged as small dots mainly in the cytoplasm (Fig. 1A). Furthermore, Fig. 1A shows that HU treatment significantly increased the proportion of TRF2-concentrating nucleus from 8% to 42%. Amount of wee1 mRNA was determined by semi-quantitative RT-PCR. We found that wee1 gene expression in HU-treated NIH3T3 cells was weakly but significantly decreased to 70% of that in control cells (Fig. 1B). On the other hand, expression of *cyclin G2* and *gapdh* genes were slightly increased and not affected, respectively, by HU treatment (Fig. 1B). These results suggest that endogenous wee1 gene expression is specifically repressed by TRF2.

Ectopically expressed TRF2 represses the wee1 promoter

To investigate effects of TRF2 on wee1 gene expression, promoter region of the mouse *wee1* gene was isolated by PCR-assisted amplification from mouse genomic DNA and a luciferase reporter plasmid was constructed. The isolated fragment encompassed a promoter sequence from −310 to +260 relative to the transcription start site. Transcription start point of the mouse *wee1* gene has been determined [30,31]. It was found that the mouse wee1 promoter does not contain a TATA-box element but does contain a GC-box in the proximal promoter region (data not shown). The reporter plasmid was introduced into NIH3T3 cells together with TRF2-expressing effector plasmid. Exogenous TRF2 in transfected cells reached at a level threefold higher than that of endogenous TRF2 (Fig. 2A, upper panel). The reporter assay revealed that the wee1 promoter was repressed to 68% of that in the control experiment by TRF2 overexpression. This TRF2-mediated transcriptional repression was augmented dose-dependently by the effector plasmid (Fig. 2B-a). On the other hand, transcription from mouse cyclin G2 promoter was enhanced by the exogenous TRF2 suggesting that repressive activity of TRF2 to the wee1 promoter is specific (Fig. 2B-b).

Repression of the mouse wee1 promoter by TRF2 in chicken cells

The mouse wee1 promoter activity was further determined by using chicken DT40 cells, in which TRF2-mediated repression of the endogenous *wee1* gene was originally found [28]. It was found that wee1 promoter-

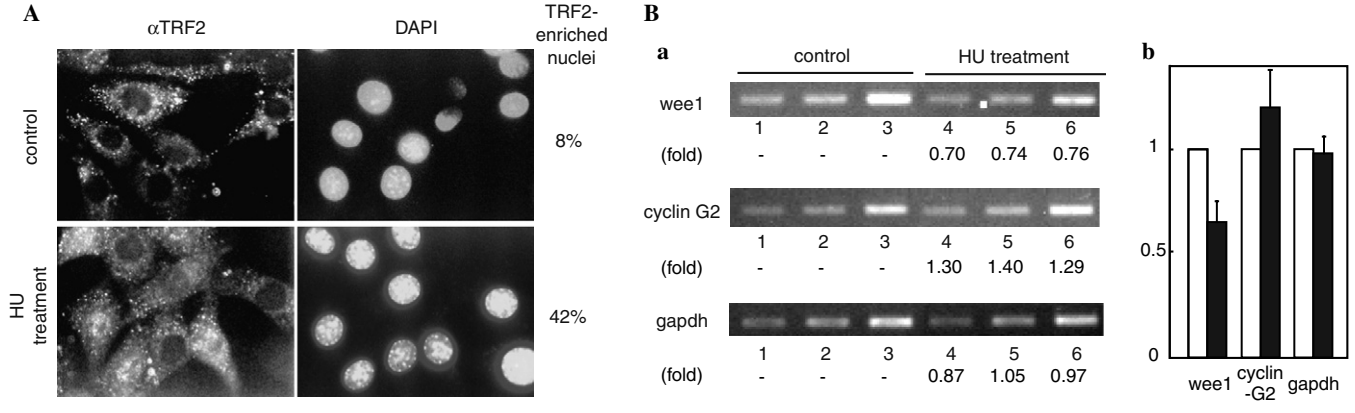

Fig. 1. Hydroxyurea depresses expression of the mouse wee1 gene. (A) NIH3T3 cells were treated with (HU treatment) or not treated with (control) hydroxyurea (HU) and stained with αTRF2 antibody. Nuclei were stained with DAPI. Proportion of TRF2-enriched nuclei-containing cells was calculated by counting 300 cells. (B) Gene expression of wee1, glycerolaldehyde-3-phosphate dehydrogenase (gapdh), and cyclin G2 was determined by RT-PCR using RNAs from control and HU-treated cells (a). Cycle numbers of PCR were 27 (lanes 1 and 4), 28 (lanes 2 and 5), and 29 (lanes 3 and 6) for *wee1*, 27 (lanes 1 and 4), 28 (lanes 2 and 5), and 29 (lanes 3 and 6) for *cyclin G2*, and 20 (lanes 1 and 4), 21 (lanes 2 and 5), and 22 (lanes 3 and 6) for *gapdh*. Relative band intensities of HU-treated cell-derived samples to the control ones at each amplification cycle were determined and depicted with a graph (b).

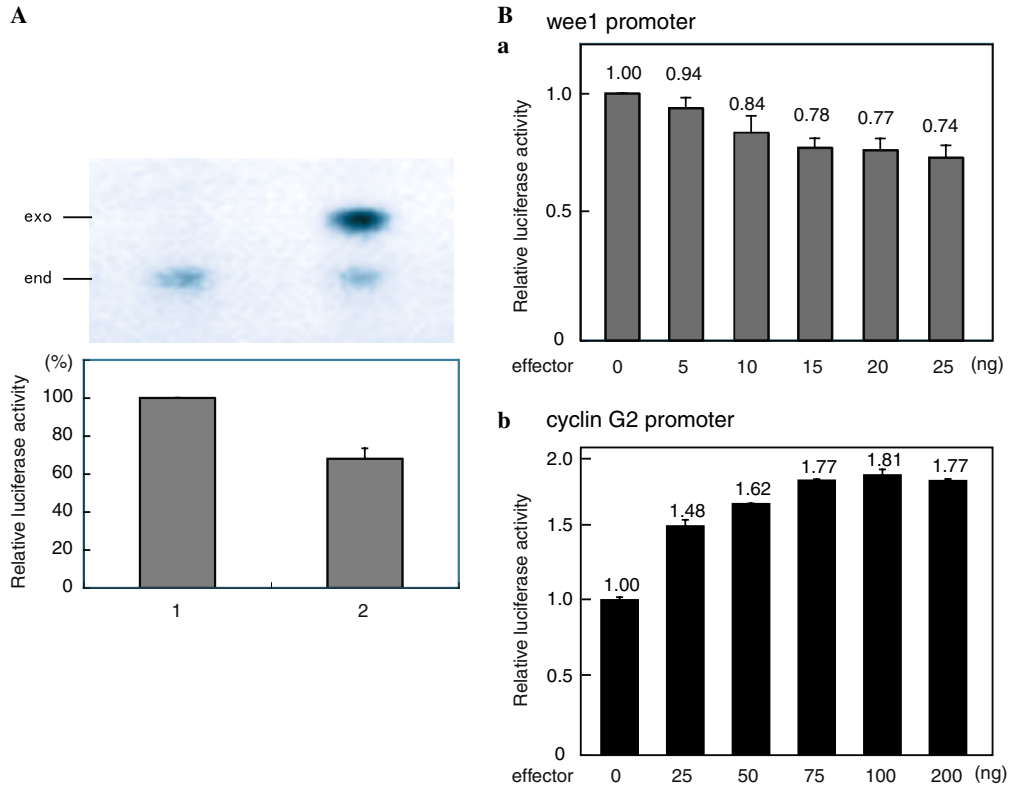

Fig. 2. Repression of wee1 promoter by exogenously expressed TRF2. (A) NIH3T3 cells were transfected with 25 ng of TRF2-expressing effector plasmid (lane 2) or a vacant vector (lane 1) together with 120 ng reporter plasmid. Expression levels of the exogenous (exo) and endogenous (end) TRF2 determined by Western blotting are shown above the panel. Results are presented as relative luciferase activities to lane 1. (B) Dose dependency of the TRF2-expressing plasmid on the wee1 promoter (a) and cyclin G2 promoter (pGL3/mG2p[−432 to +67]) (b).

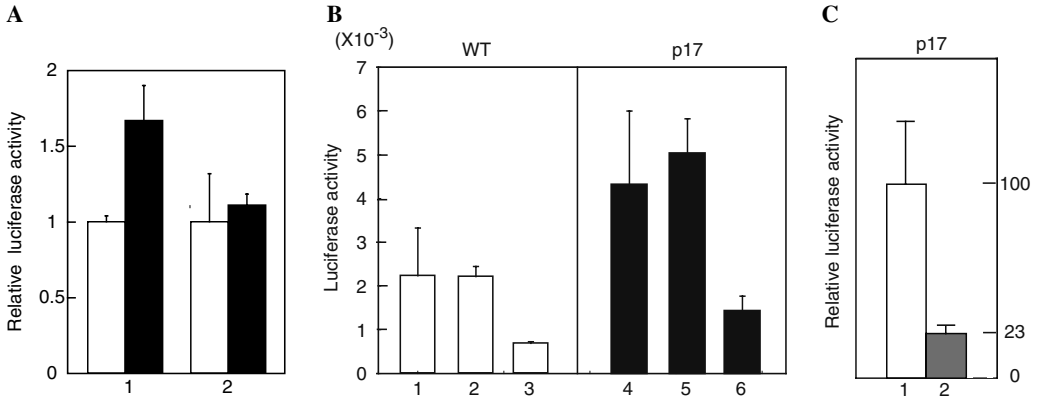

Fig. 3. Promoter function of wee1 in chicken DT40 cells. (A) Endogenous transcription machinery-driven promoter activity in DT40 cells. Wee1 [pGW−310/+260] (lane 1)and cyclin G1 (lane 2) reporter plasmids were introduced into wild-type (open column) and *TRF2*-knockout (p17) DT40 cells (solid column), and luciferase activity was measured. Enzyme activity was normalized with protein concentration, and demonstrated as relative activity to that in the wild-type cells. (B) Deletion analysis of the wee1 promoter using wild-type (WT) and p17 cells. The wee1 reporter construct (lane 1: pGW−310/+260, lane 2: pGW−203/+260, lane 3: pGW−108/+260) was introduced into cells, and promoter activity was measured. Luciferase activity was presented as raw luminometer units. (C) The wee1 reporter activity of pGW−310/+260 was determined in p17 cells co-transfected with (lane 2) or not co-transfected with (lane 1) TRF2-expressing effector plasmid.

driven luciferase activity in wild-type DT40 cells was selectively repressed compared to cyclin G1 promoter and 60% of that in p17 cells (Fig. 3A). Relatively low promoter activity in wild-type cells was also observed in the case of two 5′-deletion versions: pGW−203/+260 and pGW−108/+260 (Fig. 3B, lanes 2 and 3 vs. lanes 5 and 6). We found that pGW−108/+260 exhibited a considerable promoter activity compared to other two longer versions in both cells (e.g., lane 3 vs. lanes 1 and 2 of Fig. 3B), suggesting that the DNA stretch between −310

and −108 contains an enhancer element of the *wee1* gene. We overexpressed TRF2 in p17 cells to examine the effect of TRF2 on the wee1 promoter. It was found that the wee1 promoter was remarkably repressed by TRF2 (23% of the control promoter activity) (Fig. 3C). From these findings, we concluded that the mouse wee1 promoter is repressed by TRF2 in vertebrate cells.

Recruitment of TRF2 to the promoter region

We investigated whether TRF2 binds to wee1 promoter sequence in cells. First, ChIP assay was performed using L2-8 strain of NIH3T3 cells that stably express FH-TRF2 [27]. Cells were treated with HU to induce nuclear migration of cytoplasmic TRF2, and wee1 promoter sequence in DNA–protein immunocomplexes was detected by PCR. As seen in Fig. 4A, amounts of amplified DNA for the HU-treated cells were much greater than those for non-treated cells (lane 5 vs. lane 6). DNA was poorly amplified when control IgG was used (Fig. 4A, lanes 3 and 4). We further performed PIP assay by using NIH3T3 cells transfected with FH-TRF2-expression plasmid and a reporter plasmid that contains a wee1 promoter sequence from −310 to +260 (Fig. 4B). Immunocomplexes containing the wee1 promoter DNA and TRF2 were precipitated with M2 beads, and the promoter sequence was detected by PCR. DNA was significantly amplified when we overexpressed an FH-TRF2-expressing plasmid was used but not vacant plasmid (Fig. 4B, lane 3 vs. lane 4). Experiments using vacant plasmid (lane 3) and control IgG (lanes 5 and 6) yielded no DNA band (Fig. 3B). These data suggest that TRF2 associated with the mouse wee1 promote *in vivo*.

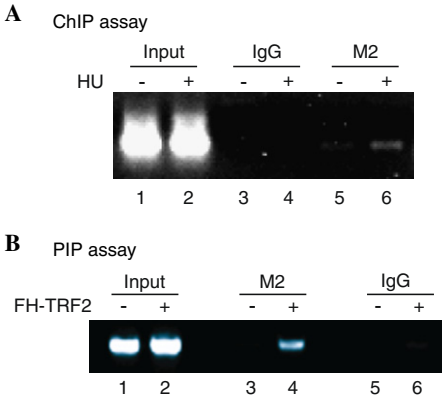

Fig. 4. Association of TRF2 with wee1 promoter region in NIH3T3 cells. (A) ChIP assay to detect association of TRF2 with the endogenous wee1 promoter. Normal (−) and HU-treated cells (+) were examined using anti-flag antibody (M2) beads or control IgG beads. Input: 0.3% of the material used for immunoprecipitation. (B) PIP assay to detect association of TRF2 with wee1 promoter region of the exogenously introduced wee1 promoter. Cells were transfected with TRF2-expressing plasmid (FH-TRF2: lanes 2, 4, and 6) or a vacant vector (lanes 1, 3, and 5) together with wee1 reporter plasmid (pGW−310/+260). Immunoprecipitation was performed with M2 beads or control IgG beads. The wide-range primer set was used to amplify the DNA. Input: 2% of the material used for immunoprecipitation.

TFIIA-binding capacity of TRF2 and TRF2-mediated transcriptional repression

As described above, TFIIA plays a role in localization of TRF2 and its participation in transcriptional regulation. In a previous study, we prepared several mutation of TRF2 some of which, namely TFIIA-type mutants, have a defect in TFIIA-binding ability [27]. In this study, we examined how these mutations affect the TRF2-dependent transcriptional repression of the wee1 promoter. Wild-type TRF2 brought a 26% decrease in the level of promoter activity as stated above (WT TRF2), whereas four TFIIA-type mutants (M1, N37E, R52E, and R86S) did not exhibit a repressive function (Fig. 5A). M1 has two point mutations of A32E and L33A. Two TFIIA-unrelated mutants such as R55E and M3 (E74R and E75A) exhibited an equivalent promoter activity as wild-type TRF2. We further investigated effects of those mutants on another promoter using mouse cyclin G2 promoter that is activated by TRF2 (Y. Tanaka, manuscript in preparation). Different from the wee1 promoter, 4 TFIIA-type mutants of TRF2 displayed no stimulating activity or rather repressive activity to the cyclin G2 promoter (Fig. 5B). Taken together, it is suggested that effect of TFIIA-type mutations on TRF2-repressive wee1 promoter is specific.

PIP assay was performed to determine how TFIIA-type mutant is recruited to the wee1 promoter. The amount of a wee1 promoter-containing amplified DNA derived from M1 mutant-transfected cells was significantly small compared to that from wild-type TRF2 transfected cells (Fig. 6, lane 5 vs. lane 6). This result indicates that the TFIIA-type mutant is recruited to the target region only little. To examine whether TFIIA is also recruited to the promoter region together with TRF2, a PIP-re-PIP assay was performed. PIP products obtained in lanes 4–6 of Fig. 6 were immunoprecipitated again with anti-TFIIA antibody, and DNA in the 2nd PIP products was amplified for the wee1 promoter sequence (Fig. 6, lanes 7–9, respectively). Large amount of amplified DNA was detected, though a non-specific band was observed when M1 mutant was used as an effector protein (Fig. 6, lane 9). However, specifically amplified DNA was not observed when wild-type TRF2 was replaced with M1 mutant (Fig. 6, lane 9). These findings suggest that TFIIA is recruited to the wee1 promoter region together with TRF2, and the recruitment of TRF2 requires its TFIIA-binding ability.

Discussion

In our previous work, it was found that cytoplasmic TRF2 migrates to the nucleus when cells were treated with genotoxic agents such as UV and methyl methanesulfonate [28]. We found that this migration also takes place by different agents such as HU [29]. Fig. 1 represents HU-mediated nuclear migration of TRF2. Since TRF2 is believed to function in the nucleus, TRF2 migration-inducing agent seems to be a stimulator for TRF2. We found that HU

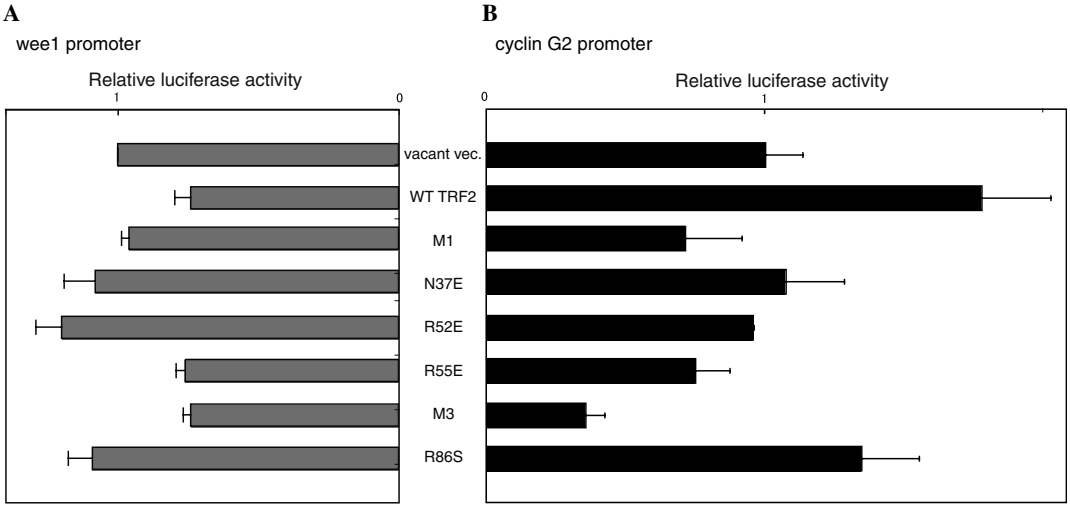

Fig. 5. Mutation analysis of TRF2 in TRF2-mediated repression of the wee1 promoter. TRF2-expressing plasmid, vacant vector, or each mutant TRF2-expressing plasmid was introduced into NIH3T3 cells together with wee1 reporter plasmid (pGW−310/+260) (A) or cyclin G2 reporter plasmid (pGL3/mG2p[−15 to +34]) (B), and the luciferase activity was measured. The results were displayed as relative activities to that using a vacant vector. WT TRF2: wild-type TRF2. M1: A32E plus L33A. M3: E74R puls E75A. M1, N37E, R52E, and R86S have a TFIIA-type mutation.

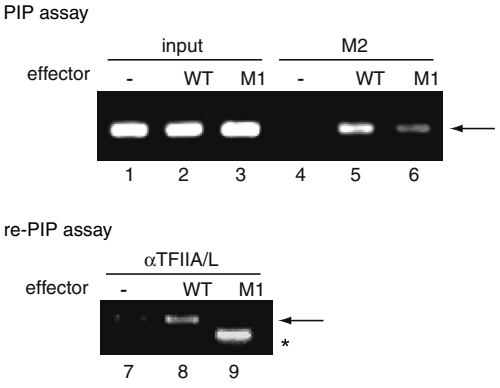

Fig. 6. Association of TRF2 with the wee1 promoter region and TFIIA-binding ability of TRF2. PIP assay was carried out as described in the legend to Fig. 4B by using the narrow-range primer set and M2 beads (lanes 4–6). Wild-type TRF2 (WT) or TFIIA-type mutation-carrying M1 mutant was introduced into NIH3T3 cells together with the wee1 reporter plasmid (pGW−310/+260). (−) Transfection of vacant vector. A re-PIP assay was performed to detect association of the proximal promoter region with TFIIA in addition to TRF2. The first PIP products of lanes 4–6 containing DNA and TRF2 were immunoprecipitated with anti-TFIIA antibody, and TFIIA-containing DNA amplified by 40-cycle PCR were shown in lanes 7–9, respectively. Arrow: specific amplified band. Asterisk: non-specific band.

treatment results in repression of transcription of the endogenous *wee1* gene (Fig. 1B) and that TRF2 is associated with the wee1 promoter (Fig. 4A) in NIH3T3 cells. These results suggest that the endogenous *wee1* gene is specifically down-regulated by TRF2. Since decrease in wee1 transcript has been found in genotoxic agent-treated chicken cells as well [28], TRF2-directed transcriptional repression of the *wee1* gene is likely a general phenomenon in vertebrates.

In reporter gene assay for the mouse wee1 promoter, overexpressed TRF2 repressed the promoter function in both mouse and chicken cells (Figs. 2 and 3B). Relatively strong wee1 promoter activity was observed in TRF2-knockout DT40 cells compared with that in wild-type cells (Fig. 3A and B). Moreover, PIP and ChIP analyses revealed that TRF2 is concentrated on a wee1 promoter sequence in a situation in which wee1 transcription is repressed (Fig. 4). Taken together with the results shown in Fig. 1, the data obtained in this study suggest that TRF2 represses transcription of the *wee1* gene. Previous studies demonstrated that TRF2 activates several cellular TATA-less genes such as *IP_3R1, TdT, PCNA, NF1*, and *cyclin G2* [22–24,28]. On the other hand, it is also known that certain TATA-containing promoters are repressed by TRF2 in a reporter gene assay when TRF2 is exogenously expressed [23,24]. However, there is no report as far for TRF2-dependent transcriptional repression of cellular TATA-less genes. The present study provides a novel example of TRF2 function.

This study suggests that the repression is associated with the binding of TRF2 and a target promoter sequence. However, at present time, it is not fully understood where and how TRF2 is associated with wee1 promoter. Although TRF2 is generally known not to have an obvious DNA-binding sequence, Chong et al. [23] suggested a 24-bp DNA stretch as a potential TRF2 target within the human NF1 promoter. We could not detect a significant homology with the 24-bp sequence in the mouse wee1 proximal promoter. Deletion analysis revealed that TRF2 target region for the *wee1* gene is included in a sequence from −108 to +260 (Fig. 3A). Moreover, ChIP (Fig. 4A) and PIP assays (Fig. 6) with the narrow-range primer set suggested an association of TRF2 and wee1 DNA from −120 to +30. Taken together, these results suggest that TRF2 is recruited to the wee1 proximal promoter that contains a core promoter function. Core promoter of *TdT* gene

[24] and *cyclin G2* gene (Y. Tanaka, manuscript in preparation) is considered to be a target of TRF2 in TRF2-directed transcriptional activation.

TRF2 may bind to a proximal promoter with weak affinity and low specificity. We speculate that the DNA-binding specificity of TRF2 varies dependent on promoter sequence and existence of a DNA-bound factor on the proximal promoter. TFIIA is the best candidate of such a factor because TRF2 binds strongly to TFIIA and is recruited to the promoter together with TFIIA (Fig. 6, lanes 7–9). TFIIA was demonstrated to dissociate TRF2 dimer that may be transcriptionally inert [27]. Results of the present study suggest that TFIIA-binding ability of TRF2 is required for entry of TRF2 (Fig. 6). We have shown the importance of TFIIA for TRF2-directed transcriptional activation of the TdT promoter [24]. Thus, TFIIA is suggested to be a critical factor for TRF2 in its both positive and negative roles.

It is not well elucidated how TRF2 inhibits transcription when it is recruited to the wee1 proximal promoter. Since the wee1 proximal promoter is thought to be a target of transcriptional repressor, TRF2 may function as a co-repressor if some repressor proteins bind to a DNA stretch near the TRF2 entry site. Alternatively, TRF2 might disturb a stimulation signal from unknown activators by steric hindrance or TRF2 itself might destabilize PIC formed on the wee1 proximal promoter.

In this study, we found that wee1 gene expression is negatively regulated by TRF2. TRF2 is known to be required for development [16–18]. Moreover, we have demonstrated that TRF2 represses cell-cycle progression via inhibition of entry to the M phase [28]. Wee1 is one of the Cdk inhibitors that negatively regulates cell cycle by inhibiting G_2-M transition [32,33]. Hence, it is strange that TRF2-induced growth inhibition is attributed to TRF2-directed repression of the *wee1* gene if wee1 has an only cell-cycle repressive function [28]. However, recent studies clarify that cells in which wee1 gene expression is repressed undergo cell-cycle arrest and apoptosis [34,35], indicating that wee1 has multiple functions in cell-cycle regulation. TRF2-directed repression of wee1 gene expression might be responsible for TRF2-mediated cell-cycle arrest and apoptosis.

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