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Cell Cycle-dependent Modulation of Promoter Activities of *RB* and *WAF1/Cip1* Genes

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A universal inhibitor of cyclin-dependent kinases, *WAF1/Cip1* can dephosphorylate the *RB* gene product to arrest the cell cycle at the G1 phase. Here we show that the mRNA level and the promoter activities of the *RB* and *WAF1/Cip1* genes exhibit cell cycle-dependent change when cells are released from either serum-starvation or the confluent cell state with serum. *RB* expression and promoter activity are elevated at middle to late G1. In contrast, the mRNA and promoter activity of the *WAF1/Cip1* gene increase at early G1. These results suggest that the *RB* and *WAF1/Cip1* expression and promoter activities depend not only on serum, but also on the cell cycle progression itself. Moreover, we identified the responsive region for serum-released cell cycle progression in the *RB* promoter and mapped it to the region between –4 and –182 relative to the initiating codon of the *RB* gene. The region in the *WAF1/Cip1* promoter responsible for the serum-released cell cycle progression mapped not to the *p53* binding site, but to the 374 base-pair region between –1770 and –1396 from the transcription start site.

Key words: *RB* — *WAF1/Cip1* — mRNA — Promoter activities — Cell cycle-responsive region

The retinoblastoma susceptibility gene (*RB*) was one of the first tumor suppressor genes to have been characterized,[1)] and serves as a prototype for genes in this category. The *RB* gene product (pRB) is an important component of the growth control machinery in normal cells, and the inactivation of the *RB* gene in various cancer cells[2, 3)] is likely to contribute to tumor progression. In keeping with this notion, introduction of pRB by microinjection in tumor cells lacking normal pRB has resulted in G1 arrest *in vitro*.[4)]

The *RB* gene is qualitatively regulated at the protein level by means of phosphorylation[5–8)] by cyclin-dependent kinases (CDKs). pRB undergoes cyclic changes in its phosphorylation state throughout the cell cycle, and unphosphorylated pRB in the G1 phase is thought to be active.[5–8)] Phosphorylation of pRB in the late G1 phase correlates with its conversion from an active to inactive form, and this appears to represent an important step in the G1 to S transition.[5–8)]

One of the CDK inhibitors, *WAF1/Cip1* (*p21/Sdi1*[9)]), is induced by *p53*[10)] and has also been suggested to contribute to the arrest of cell growth by inhibiting *Cdk* activity.[11–13)] However, other reports indicate that there are *p53*-independent pathways in the induction of *WAF1/Cip1* mRNA.[14)] It has been shown that *WAF1/Cip1* is induced by serum or growth factors in fibroblasts derived from *p53*-knock-out mice.[14)] This induction is transient from the early G1 to S phase without requiring protein synthesis, suggesting that *WAF1/Cip1* could be an immediate-early gene.[14)]

Although previous papers showed that mRNA of the *RB* gene[15)] and *WAF1/Cip1* gene[16)] is regulated in the cell cycle, the transcriptional mechanisms (such as promoter activation) of these two genes during the cell cycle have not been clarified. Here we demonstrate that the promoter activities of the *RB* and *WAF1/Cip1* genes show cell cycle-dependent change. The mRNA of *RB* gene and the promoter activity are elevated at middle to late G1, and those of the *WAF1/Cip1* gene increase at early G1. Fluctuations of mRNA levels and promoter activities of these genes were observed during the cell cycle progression after release of the cells from both serum-starvation and the confluent cell state. These results suggest that *RB* and *WAF1/Cip1* promoter activities are regulated not only by the serum, but also by the cell cycle progression itself. We also mapped the responsive regions in the promoters of these genes.

MATERIALS AND METHODS

Cell lines MG-63 and HOS human osteosarcoma cells were provided by Dr. Yoshizo Yanase (Wakayama Medical College, Wakayama), and Saos-2 human osteosarcoma cells were kindly provided by Dr. Rei Takahashi (Kyoto University, Kyoto). These cell lines were maintained as

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monolayers in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and incubated at 37°C under a humidified atmosphere of 5% CO_2. Stably transfected MG-63 cells were maintained in the same medium containing G418 (200 μg/ml) (Geneticin; GIBCO BRL, Rockville, MD). MG-63 and HOS cells have no abnormality of the *RB* gene.[17] The *p53* gene in MG-63 cells is rearranged and inactivated,[18] and the *p53* gene in HOS cells has a point mutation in exon 5.[19]

Construction of plasmids The construction of the wild-type human *RB* promoter-luciferase fusion plasmid, pXRP1 has been described previously.[20] Plasmids with point mutations or internal deletions in the *RB* promoter have been described previously.[21] The construction of human cyclin A promoter-luciferase fusion plasmid was described previously[22] and we designated it as cyclin A-luc. Sp1-luc, which contains three consensus Sp1 binding sites from the SV40 promoter linked to the luciferase cDNA, was kindly provided by Dr. Peggy J. Farnham.[23] WWP-luc, a wild-type human *WAF1*/*Cip1* promoter-luciferase fusion plasmid, and DM-luc in which the *p53* binding site of the promoter has been deleted, were kindly provided by Dr. Takashi Tokino and Dr. Bert Vogelstein.[10] The deletion mutants of the *WAF1*/*Cip1* promoter was generated by the Kilo-deletion kit (Takara Co., Ltd., Kyoto) and were termed pdWAF1-2320, -1770, -1396, -1296 and -700. pD1-luc, a wild-type human cyclin D1 promoter-luciferase fusion plasmid, was kindly provided by Dr. Muller.[24] pSV2neo, a plasmid bearing the neomycin resistance gene, was provided by the Japanese Cancer Research Bank (JCRB).

Synchronization of the cells and flow cytometric analysis MG-63 cells and their transfected derivatives were synchronized at the G0 phase by serum starvation (0.05% FBS) for approximately 72 h. Cells were released from the G0 phase by exchanging the medium for fresh medium containing 10% FBS. At 4-h intervals, we monitored the cell cycle stage by flow cytometry as described before,[25] and the corresponding cell cultures were used for northern blotting and luciferase assay.

Northern blot analysis The total RNA was isolated from MG-63 cells at 4-h intervals after release from serum starvation or from the confluent cell state using the ISOGEN RNA isolation kit (Nippon Gene, Tokyo), and each sample was examined by northern blot analysis. The cDNAs of the human *RB* gene and human *WAF1*/*Cip1* gene for use as probes were obtained from p4.95BT (a kind gift of Dr. T. P. Dryja), and pCEP-WAF1 (a kind gift of Dr. Bert Vogelstein), respectively, by digesting with restriction enzymes. The probes were labeled using the Megaprime DNA labeling system (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. Northern blot analysis was performed by the standard method.[26]

Transfection and luciferase assay For stable transfections, plasmid DNAs were transfected into cells using the Chen-Okayama method.[26] Briefly, MG-63 cells (1×10^6 cells) were seeded on 100-mm-diameter culture dishes. After 16 to 20 h, cells were transfected with plasmids, such as pXRP1, cyclin A-luc, pD1-luc, cdc2-luc, WWP-luc, DM-luc or Sp1-luc, together with pSV2neo. Two days after the transfection, G418 (final concentration: 350 μg/ml) was added to the medium and neomycin-resistant colonies were pooled. These cells, termed MG-63/pXRP1, MG-63/cyclin A, MG-63/cyclin D1, MG-63/cdc2, MG-63/WWP, MG-63/DM, and MG-63/Sp1, respectively, were subjected to luciferase assay.[27] Luciferase activity was normalized with respect to the amount of protein (per 1 mg) in each cell lysate.

For transient transfections, plasmid DNAs were transfected into cells using a standard calcium phosphate method.[26] MG-63 cells (3×10^5 cells) were seeded into 60-mm-diameter dishes before transfection. In transfections, 8 or 9 μg of DNA was used per plate. Four hours after the transfection, the cells were shocked with 20% dimethyl sulfoxide for 3 min, and luciferase activity was measured 48 h after the transfection as described above.

RESULTS

Cell cycle analysis in MG-63 cells The cell cycle progression of MG-63 cell line was analyzed by flow cytometry. For synchronization, the cells were subjected to

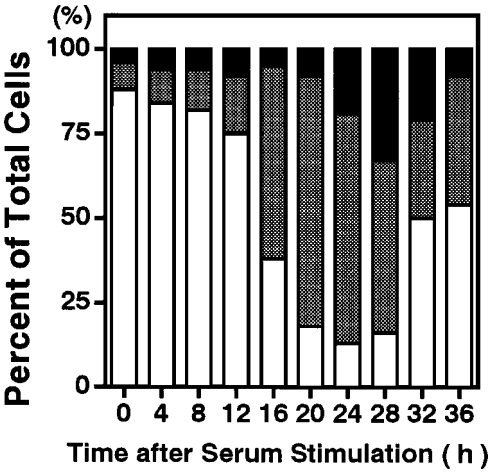

Fig. 1. Cell cycle status of MG-63 cells released from G0 by serum stimulation. MG-63 cells were serum-starved in medium containing 0.05% FBS for 72 h, then 10% FBS was added. Relative DNA content of quiescent cells (time 0), or growing cells at 4-h intervals after serum stimulation was analyzed by flow cytometry. The percentages of cells in G1/G0 (open bars), S (shaded bars), and G2/M (solid bars) phases were obtained from DNA histograms by quantitative analysis.

serum starvation (0.05% FBS) for 72 h, resulting in enrichment of cells in G1/G0 up to 88% (Fig. 1). Afterwards the cells were stimulated with serum and the cell cycle was monitored at 4-h intervals for 36 h. More than 75% of the cells remained at the G1/G0 phase at 16 h after serum stimulation (Fig. 1). This time point, between 12 and 16 h, was surmised to be the G1/S boundary. Subsequently, the cells entered the S phase and maximum

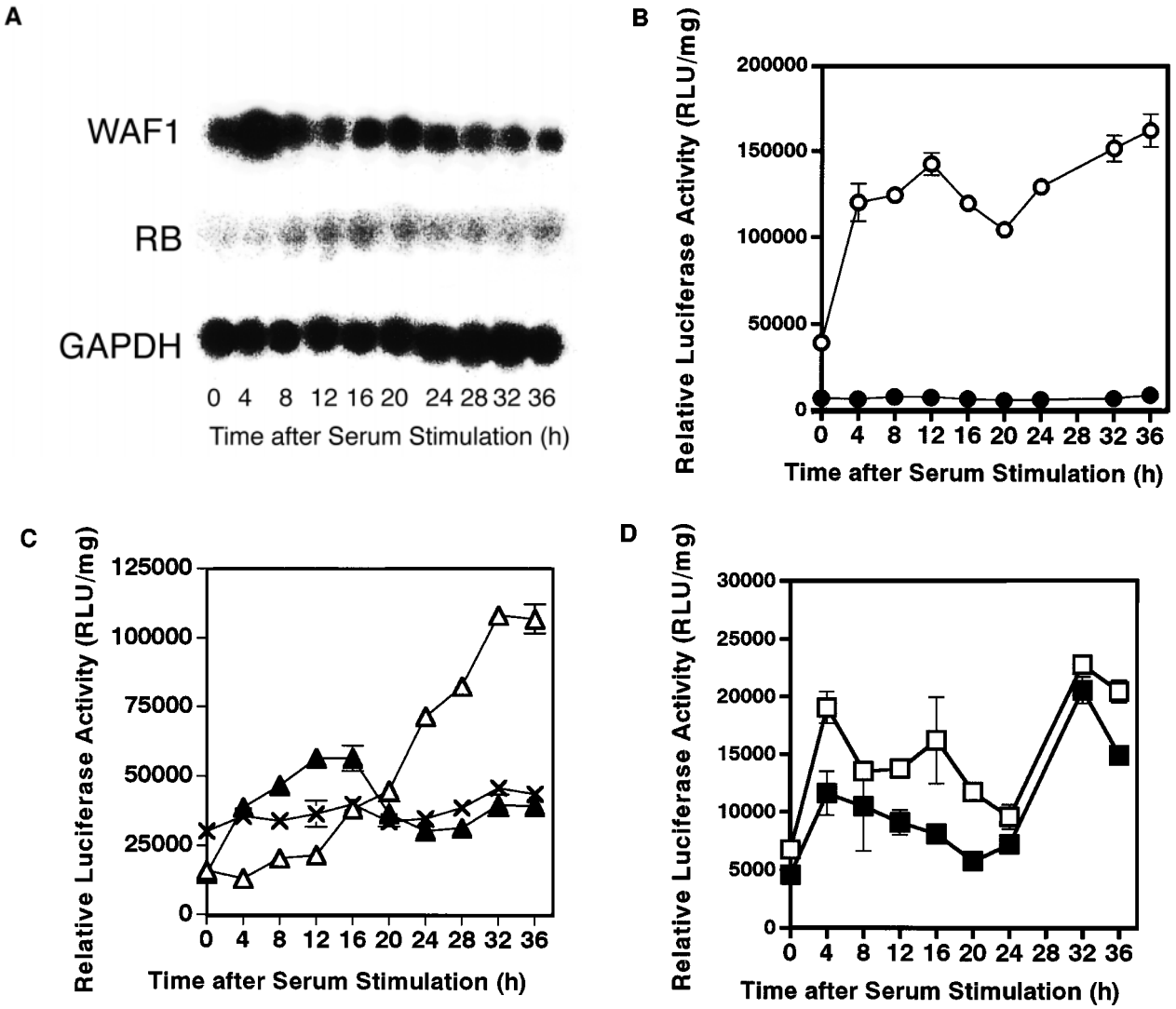

Fig. 2. Transcriptional expression of *RB* and *WAF1/Cip1* during cell cycle progression by serum stimulation. A. MG-63 cells were serum-starved in medium containing 0.05% FBS for 72 h, then 10% FBS was added, and total RNA was isolated at the indicated times after release and analyzed by northern hybridization. B and C. Cell cycle-dependent regulation of promoter activity of pXRP1 (*RB* promoter-luciferase plasmid) (○), Sp1-luc (●) during the cell cycle (B). Promoter activity of cyclin A(△), cyclin D1(▲), and cdc2(×) during the cell cycle (C). Stably transfected MG-63 cells of MG-63/pXRP1, MG-63/cyclin A, MG-63/cyclin D1, MG-63/cdc2 and MG-63/Sp1 were serum-starved using medium containing 0.05% FBS. After 72 h of serum starvation, the cells were stimulated by the addition of medium containing 10% FBS. Luciferase activity was monitored at 4-h intervals after release from serum-starvation. The activity is shown as raw light unit (RLU) per 1 mg protein in the cell lysate. The luciferase assay was performed in triplicate. Data are shown as means±SD. D. Cell cycle regulation of wild-type (WWP-luc) (□) and *p53* binding site-deleted (DM-luc) (■) *WAF1/Cip1* promoters. Stably transfected MG-63 cells of MG-63/WWP and MG-63/DM were serum-starved using medium containing 0.05% FBS. After 72 h of serum-starvation, the cells were stimulated by the addition of medium containing 10% FBS. Luciferase activities were monitored at 4-h intervals after release from serum-starvation. Luciferase activity is shown as RLU per 1 mg protein in the cell lysate. The luciferase assay was performed in triplicate. Data are shown as means±SD.

accumulation in the S phase was 74% at 20 h after the release (Fig. 1). At 24 h, cells in the G2/M phase increased, and reached a peak of 33% at 28 h after serum stimulation (Fig. 1). MG-63 cells exited the cell cycle to the G1 phase at approximately 32–36 h after the release. These results are in reasonable agreement with a previous report that the doubling time of MG-63 cells was 38 h.[28)]

Expression of *RB* and *WAF1*/*Cip1* mRNA during cell cycle progression We next investigated *RB* and *WAF1*/*Cip1* gene expression during cell cycle progression. MG-63 cells were serum-starved for 72 h and then stimulated with 10% serum. We monitored the *RB* and *WAF1*/*Cip1* mRNA levels at 4-h intervals after serum stimulation. The data plotted in Fig. 2A showed that the *RB* mRNA level was elevated until 12 h and declined thereafter, while the *WAF1*/*Cip1* mRNA was transiently induced at 4 h and declined thereafter (Fig. 2A). These results in MG-63 cells are consistent with previous reports[15, 16)] on other cell lines.

Promoter activities of *RB* and *WAF1*/*Cip1* genes are regulated during the cell cycle To examine whether the *RB* promoter is regulated in the cell cycle progression, we stably transfected MG-63 cells with pXRP1, carrying the wild-type *RB* promoter fragment. The stable cell line was termed MG-63/pXRP1. MG-63/pXRP1 cells were also arrested at the G1/G0 phase in medium containing 0.05% FBS for 72 h (data not shown). Cell extracts were collected at this 0 h point of the G1/G0 phase and at 4-h intervals after the addition of medium containing 10% FBS. The cell cycle status at each time point was analyzed by flow cytometry, and the same histograms as those of the parent cells were obtained (data not shown). Interestingly, the *RB* promoter activity in MG-63/pXRP1 cells increased 3-fold at 4 h after the serum stimulation,

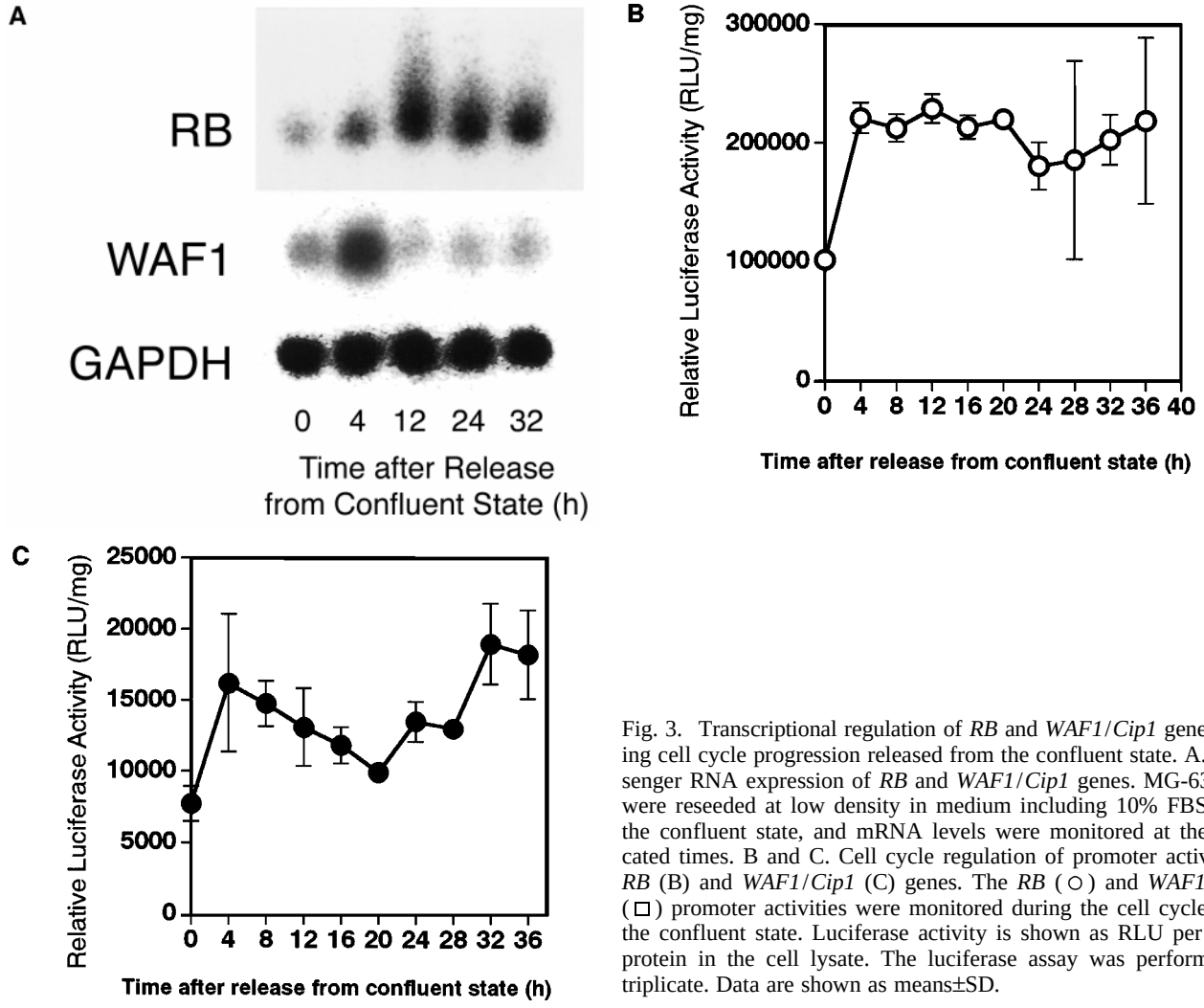

Fig. 3. Transcriptional regulation of *RB* and *WAF1*/*Cip1* genes during cell cycle progression released from the confluent state. A. Messenger RNA expression of *RB* and *WAF1*/*Cip1* genes. MG-63 cells were reseeded at low density in medium including 10% FBS from the confluent state, and mRNA levels were monitored at the indicated times. B and C. Cell cycle regulation of promoter activity in *RB* (B) and *WAF1*/*Cip1* (C) genes. The *RB* (○) and *WAF1*/*Cip1* (□) promoter activities were monitored during the cell cycle from the confluent state. Luciferase activity is shown as RLU per 1 mg protein in the cell lysate. The luciferase assay was performed in triplicate. Data are shown as means±SD.

corresponding to the early G1 phase, and the activity increased 4-fold by 12 h after the release (late G1 phase) (Fig. 2B). The *RB* promoter activity started to decline at 16 h (early S phase), then began to increase again at 24 h and reached the maximum activity at 32–36 h (Fig. 2B), which corresponds to G2/M to early G1 phase. As controls, we monitored the promoter activities of various other genes during the cell cycle. We generated stable transfectants of Sp1-luc, cyclin A-luc, cyclin D1-luc, and cdc2-luc in MG63 cells. The promoter activity of Sp1-luc did not change significantly during the cell cycle (Fig. 2B). The promoter activity of cyclin A gene started to increase when the cells entered the S phase (Fig. 2C), as previously shown in NIH3T3 cells.[29] In the case of the cyclin D1 promoter, luciferase activity increased gradually until the G1/S boundary, then declined (Fig. 2C). This result is consistent with the results of a previously reported study in which the expression of cyclin D1 mRNA increased gradually after serum stimulation until the G1/S boundary.[30] *Cdc2* promoter activity remained at almost the same level during the cell cycle (Fig. 2C). These results indicate that the *RB* promoter activity is specifically regulated in the cell cycle, and that the activity is stimulated in the early G1 phase, and reaches the highest level at the G1/S boundary.

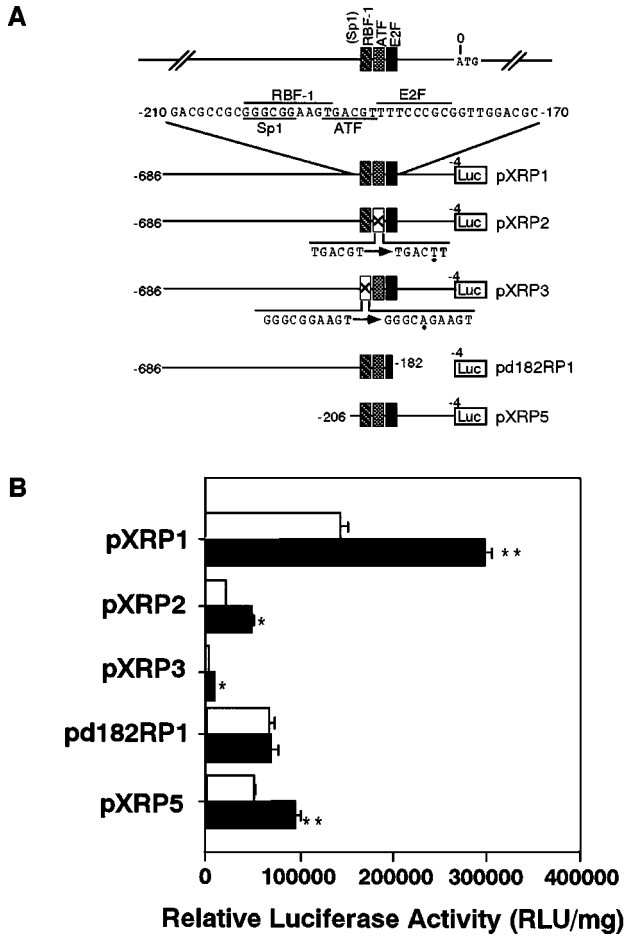

Fig. 4. Mapping of the responsive region of cell cycle progression in the *RB* promoter. A. Schematic representation of wild-type (pXRP1), and mutant *RB* promoter-luciferase fusion plasmids. The boxes in the scheme show the location of the *cis*-acting elements designated as RBF-1 (Sp1), ATF, and E2F sites. The sequence is numbered relative to the start site of translation. pXRP2 and pXRP3 have point mutations in the ATF and RBF-1 of the *RB* promoter, respectively.[20, 21] In pXRP5, the upstream region of the RBF-1 site was deleted, and in pd182RP1, the 3′ side of the *RB* promoter including a part of the E2F site was deleted.[21] B. Mutant *RB* promoter-luciferase constructs were stably transfected into MG-63 cells, and the luciferase activities of serum-stimulated cells were measured in RLU per 1 mg in cell lysate. Open columns indicate the promoter activities from control cells and closed columns indicate the promoter activities from 12-h serum-stimulated cells. The luciferase assay was performed in triplicate. Data are shown as means±SD. * $P<0.05$, ** $P<0.01$.

In order to compare the regulation of *WAF1*/*Cip1* promoter activity with that of the *RB* promoter during the cell cycle, we generated MG-63 cell lines stably transfected with a wild-type *WAF1*/*Cip1* promoter-luciferase fusion plasmid (WWP-luc) or mutant *WAF1*/*Cip1* promoter-luciferase fusion plasmid, in which the *p53* binding site had been deleted (DM-luc). The stable cell lines were termed MG-63/WWP and MG-63/DM, respectively, and we monitored their promoter activities throughout the cell cycle as described above. The fluctuation of the *WAF1*/*Cip1* promoter activity from WWP-luc (wild-type) showed a similar pattern to that of DM-luc (a deletion mutant of the *p53* binding site) (Fig. 2D). In both MG-63/WWP and MG-63/DM cells, the *WAF1*/*Cip1* promoter activities increased about 3-fold at 4 h after serum stimulation, corresponding to the early G1 phase, started to decline gradually until 20–24 h (middle S phase), then began to increase again (Fig. 2D). Reaccumulation of luciferase activity was observed to a high level at 32–36 h after serum stimulation (late G2/M to early G1 phase) (Fig. 2D). The same fluctuation was observed in HOS human osteosarcoma cells stably transfected with WWP-luc or DM-luc (data not shown).

To rule out the possibility that the promoter activities of *RB* and *WAF1*/*Cip1* genes are activated by serum itself, MG-63 stable transfectants were arrested at the G1/G0 phase by keeping the cells confluent in the medium containing 10% FBS, and the cell cycle was released by reseeding. Messenger RNA expression of these two genes in cells reseeded from the confluent state showed similar patterns to those of the serum-stimulated cells monitored by northern blotting (Fig. 3A). Furthermore, the *RB* (Fig. 3B) and *WAF1*/*Cip1* (Fig. 3C) promoter activities were

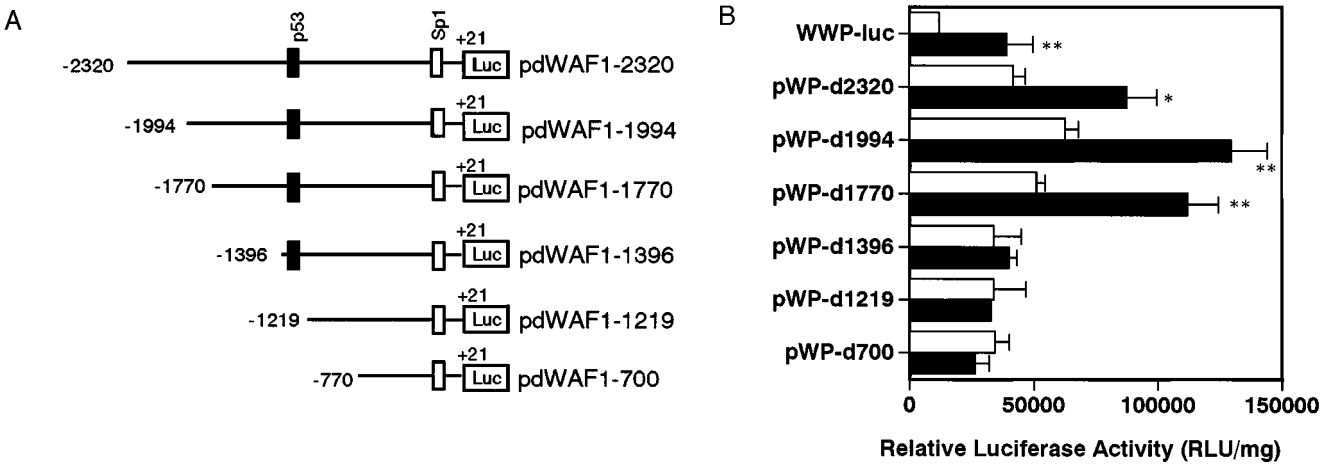

Fig. 5. Mapping of the responsive region to cell cycle progression in *WAF1*/*Cip1* promoter. A. A schematic representation of luciferase constructs with 5′ deletion plasmids of *WAF1*/*Cip1* promoter. The deletion panel pWAF-2320 to pWAF-700 was created using the exonuclease III based system, Kilo deletion kit (Takara). The sequence is numbered relative to the start site of transcription. Plasmids from pWAF-2320 to pWAF-1770 included the *p53* binding site. B. Deletion plasmids of *WAF1*/*Cip1* promoter-reporter constructs were transiently transfected into MG-63 cells, and the luciferase activities of serum-stimulated cells were measured in RLU per 1 mg in cell lysate. Open columns indicate the promoter activities from control cells and the closed columns indicate the promoter activities from 4-h serum-stimulated cells. The luciferase assay was performed in triplicate. Data are shown as means±SD. * $P<0.05$, ** $P<0.01$.

monitored, and they also showed almost the same pattern as the serum-stimulated cells. These results indicate that the *RB* and *WAF1*/*Cip1* promoters are regulated specifically by the cell cycle progression itself.

Identification of cell cycle-responsive region of the *RB* promoter The RBF-1 and ATF sites are core elements of the *RB* promoter.[20] The RBF-1 site is located from position −192 to −188 and the ATF site from position −201 to −198 from the start site of translation in the *RB* promoter (Fig. 4A). The E2F site, a silencer element of the *RB* gene,[21] is located from −187 to −180, directly downstream of the RBF-1 and ATF sites (Fig. 4A). To identify the responsive element to cell cycle progression in the *RB* promoter, several mutant plasmids of *RB* promoter were stably transfected into MG-63 cells and the promoter activities of serum-starved cells and serum-stimulated cells were compared for 12 h. The promoter activity of pXRP1, a wild-type *RB* promoter, and pXRP2, ATF site-mutated *RB* promoter, pXRP3, RBF-1 site-mutated *RB* promoter and pXRP5, in which the upstream region of the RBF-1 site had been deleted, were stimulated about 2-fold (Fig. 4B). The promoter activity of pd182RP1, in which the 3′ side of the *RB* promoter including a part of E2F site had been deleted, was not stimulated by the serum (Fig. 4B). These results suggest that the *RB* promoter activity is stimulated by the serum-released cell cycle through the region between −4 and −182 relative to the initiating codon of the *RB* gene.

Identification of cell cycle-responsive element of the *WAF1*/*Cip1* promoter To investigate the responsive element to cell cycle progression in the *WAF1*/*Cip1* promoter, we examined the activity of a series of 5′ deletion mutants of *WAF1*/*Cip1* promoter-luciferase fusion plasmids (Fig. 5A). The luciferase activity of these mutant plasmids was assayed in *p53*-deficient MG-63 osteosarcoma cells by means of transient transfection assays. Several deletion mutants of the *WAF1*/*Cip1* gene promoter were generated and transiently transfected, and serum was depleted for 72 h. Thereafter, the transfected cells were serum-stimulated for 4 h, and the promoter activities were compared to that of serum-starved cells. The promoter activities of pdWAF1-2320, pdWAF1-1994 and pdWAF1-1770 in serum-stimulated cells were activated (Fig. 5B). However, the promoter activities of pdWAF1-1396, pdWAF1-1219 and pdWAF1-700 were not stimulated by cell cycle progression at 4 h after the serum stimulation (Fig. 5B). These results suggest that the responsive element for cell cycle progression in the *WAF1*/*Cip1* promoter exists in the 374 base-pair region between −1770 and −1396 from the transcription start site.

DISCUSSION

Our findings are consistent with previous reports that the *RB* mRNA level reaches the maximum at the G1/S boundary,[15] and that the *WAF1*/*Cip1* mRNA level reaches

a peak in early G1 in the cell cycle progression released by serum.[16] Furthermore, similar fluctuation of promoter activity during the cell cycle was detected, suggesting that the expression of the *RB* and *WAF1*/*Cip1* genes in cell cycle progression is regulated at the transcriptional level. These modulations of *RB* and *WAF1*/*Cip1* promoter activity during the cell cycle were also observed in cell cycle progression after release from the confluent cell state. These results suggest that *RB* and *WAF1*/*Cip1* promoter activities change dependently on cell cycle progression itself, not only on serum. Also, as MG-63 cells are known to be *p53*-deficient,[18] our results show that *WAF1*/*Cip1* promoter activity is regulated through a *p53*-independent pathway in cell cycle progression.

Recently, several types of *p53*-independent regulation of *WAF1*/*Cip1* gene expression have been reported. TGF-β1,[31] phorbol myristate acetate,[32] okadaic acid,[32] butyrate[33] and trichostatin A[34] can stimulate the *WAF1*/*Cip1* gene promoter through Sp1 sites. In addition, transcription factors such as signal transducers and activators of transcription protein,[35] $1\alpha,25(OH)_2$ vitamin D_3 ($1,25(OH)_2D_3$) receptor[36] and C/EBPβ[37] can act through their specific binding sites in *WAF1*/*Cip1* promoter. Our data on transient transfections indicate that the activity of the *WAF1*/*Cip1* gene is regulated through its promoter by the cell cycle, and that a region from −1770 to −1396 from the transcription start site of the *WAF1*/*Cip1* promoter can respond to cell cycle progression induced by serum in *p53*-deficient MG-63 cells. This 374 bp region could contain a serum cell cycle-responsive element which is independent of the *p53* binding site. Macleod *et al*.[38] reported a 711-bp serum response region in the *WAF1*/*Cip1* promoter. Our 374-bp region is included in that 711-bp region. We speculate that the major region responsible for serum-released cell cycle progression might be included within this 374 bp.

In summary, we have identified the region in the *RB* and *WAF1*/*Cip1* genes which responds to cell cycle progression. Identification of the transcriptional factors that induce *RB* or *WAF1*/*Cip1* gene expression during cell cycle progression could be the next important step.

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