J. Biochem. 2016;159(4):437–447 doi:10.1093/jb/mvv126

JB THE JOURNAL OF BIOCHEMISTRY

Characterization of the 5′-flanking region of the human *TP53* gene and its response to the natural compound, Resveratrol

Received October 5, 2015; accepted November 2, 2015; published online December 17, 2015

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Tumour suppressor p53, which is encoded by the *TP53* gene, is widely known to play an important role in response to DNA damage and various stresses. It has recently been reported that p53 regulates glucose metabolism and that an increase in p53 protein level is induced after serum deprivation or treatments with a natural compound, *trans*-Resveratrol (Rsv). In this study, we constructed a Luciferase expression vector, pGL4-TP53-551, containing 551 bp of the 5'-upstream region of the human *TP53* gene, which was then transfected into HeLa S3 cells. A Luciferase assay showed that Rsv treatment increased the promoter activity of the *TP53* gene in comparison to that of *PIF1*. Detailed deletion and mutation analyses revealed that Nkx-2.5 and E2F-binding elements are required in addition to duplicated GGAA (TTCC), for the regulation of *TP53* promoter activity. In this study, it is suggested that the transient induction of *TP53* gene expression by Rsv treatment might be partly involved in its anti-aging effect through maintenance of chromosomal DNAs.

Keywords: E2F/ETS/Nkx-2.5/Resveratrol/*TP53*.

Abbreviations: 2DG, 2-deoxy-D-glucose; CR, caloric restriction; DME medium, Dulbecco's modified Eagle's (DME) medium; FCS, fetal calf serum; Luc, Luciferase; PCR, polymerase chain reaction; Rsv, trans-Resveratrol; RT-PCR, reverse transcriptase-polymerase chain reaction; TSS, transcription start site.

Tumour suppressor protein p53, which is encoded by the *TP53* gene, is known as a DNA damage or stress responding transcription factor that binds to the consensus sequence, 5'-(A/G)(A/G)(A/G)C(A/T)(A/T)G(C/T)(C/T)(C/T)-3'(*1*). Genetic mutations on the *TP53* gene have been very frequently identified in a variety of tumour cells (*2*, *3*). Thus, p53 is regularly referred to as a 'guardian of the genome'. Significant biological functions of the p53 protein include induction of cell cycle regulatory factor-encoding genes, regulation of cellular senescence, apoptosis and autophagy (*4*). With regard to cancer generation, activation of p53 is thought to play an important role in inducing senescence to prevent aberrant fusions or breakages within telomere-shortened chromosomes (*5*). Furthermore, it has been suggested that cross-talk between telomeres and mitochondria plays a role in the regulation of aging (*6*, *7*). Moreover, p53 is known to accumulate in the cytoplasm and mitochondria in response to various stresses, suggesting that it also regulates mitochondrial functions, including glucose metabolism (*8*, *9*).

Previous studies have shown that expression of the *TP53* gene is induced after deprivation of serum from the culture medium of granulosa and HepG2 cells (*10*, *11*). Moreover, it has been reported that glucose deprivation from culture medium induces *TP53* gene expression in U2OS cells (*12*). These results suggest that reduced nutrient or energy stress may induce *TP53* gene expression. We have reported that the promoter activities of the genes encoding telomere maintenance-associated factors, including WRN and shelterin proteins, are induced after treatment with caloric restriction (CR) mimetic compounds, such as 2-deoxy-D-glucose (2DG) and *trans*-Resveratrol (Rsv) (*13*, *14*). We thus hypothesized that serum deprivation or CR mimetic compounds induce the promoter activity of the *TP53* gene. To assess the possibility, a Luciferase (Luc) expression plasmid containing 551 bp of the 5'-upstream region of human *TP53* was constructed and used for a transfection assay. The Luc reporter assay revealed that the 551-bp region responds to both serum deprivation and Rsv treatment in HeLa S3 cells. A natural polyphenolic CR mimetic compound, Rsv is known to stimulate NAD^+-dependent deacetylase sirtuin and elongate lifespan of model animals (*15–19*). A comparison of the Rsv-inducible human *WRN* and *TERT* promoter regions showed that the Sp1/GC-box is common to both (*20*). However, canonical GC-box sequences are not found in the 551 bp of the human *TP53* promoter region.

In this study, we found that deletion of the duplicated GGAA (TTCC) or c-Ets binding element drastically diminished promoter activity. Deletion analyses showed that the GGAA (TTCC) motifs and the Nkx-2.5 element both play essential roles in the regulation of *TP53* promoter activity in HeLa S3 cells. In addition, mutation of the E2F-motif apparently reduced the response to Rsv, suggesting that proteins binding

to the E2F-motif play an important role in the control of *TP53* gene expression in response to Rsv treatment.

Materials and Methods

Materials

Rsv was purchased from Cayman Chemical (Ann Arbor, MI) (*21*).

Cells and cell culture

Human cervical carcinoma (HeLa S3) cells (*13*) were grown in Dulbecco's modified Eagle's (DME) medium (WAKO Pure Chemical, Tokyo, Japan), supplemented with 10% fetal bovine serum (Biosera, East Sussex, UK) and penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Construction of luciferase (Luc) reporter plasmids

The Luc reporter plasmid pGL4-TP53-551 carrying 551 bp of the 5'-flanking region of the human *TP53* gene was constructed by a similar procedure to that previously described (*13*, *14*). Similarly, other Luc reporter plasmids were constructed by ligating a polymerase chain reaction (PCR)-amplified DNA fragment into the *Kpn*I/*Xho*I site of pGL4.10[*luc*2] (Promega, Madison, WI). The sense and anti-sense primers used for the amplification of the DNA fragments are shown in Table I. The shaded nucleotides (Table I) indicate the mutations that disrupt the c-ETS, Nkx2.5 and E2F binding elements.

Luc reporter plasmids, pGL4-TP53-mEts, pGL4-TP53-mNkx and pGL4-TP53-mE2F were made according to a procedure (*22*) with slight modifications. Briefly, PCR was performed with appropriate sense and antisense primers (Table I) and pGL4-TP53-551 as a template. The DNAs were denatured at 65°C for 20 min and gradually cooled down to 25°C for 20 min, then kept at 25°C for further 20 min. The double-stranded DNA products, which were treated with T4 DNA polymerase (Toyobo) and digested with *Kpn*I and *Xho*I, were introduced into the MSC of the pGL4.10[*luc*2] vector. Nucleotide sequences were confirmed by DNA sequencing service (FASMAC, Greiner Japan Inc., Atsugi, Japan) with primers Rv (TAGCAAAATAGGCTGTCCCC) and GL (CTTTATGTTTTTGGCGTCTTCC).

Transient transfection and Luc assay

Plasmid DNAs were transfected into HeLa S3 cells by the DEAE-dextran method in 96-well plates (*23*, *24*). After 24 h of transfection, the culture medium was changed to DME without serum or with 10% fetal calf serum (FCS) containing Rsv (0–40 μM). After a further 24 h of incubation, cells were collected and lysed with 100 μl of 1 x cell culture lysis reagent, containing 25 mM Tris-phospate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N',-tetraacetic acid, 10% glycerol and 1% Triton X-100, then mixed and centrifuged at 12,000 × g for 5 s. The supernatant was stored at −80°C. The Luc assay was performed with a Luciferase assay

Table I. Primer pairs used for amplifying 5'-upstream regions of the human *TP53* gene

Luc plasmid	Primer	Sequence (5' to 3')
pGL4-TP53-551	hTP53-4710	TCGGTACCTCATAAGGCTTACGTTTCCA
	AhTP53-4160	ATCTCGAGACGGTGGCTCTAGACTTTTGA
pGL4-TP53-Δ1	hTP53-4710	TCGGTACCTCATAAGGCTTACGTTTCCA
	Ahp53-4734	ATCTCGAGGCTCCTGGCACAAAGCTGG
pGL4-TP53-Δ2	hp53-5025	TCGGTACCTGATGAGAAGAAAGGATCCAG
	AhTP53-4160	ATCTCGAGACGGTGGCTCTAGACTTTTGA
pGL4-TP53-Δ3	hTP53-4710	TCGGTACCTCATAAGGCTTACGTTTCCA
	AhTP53-4210	ATCTCGAGCACATGGGAGGGGAAAACCCC
pGL4-TP53-Δ12	hp53-5025	TCGGTACCTGATGAGAAGAAAGGATCCAG
	Ahp53-4734	ATCTCGAGGCTCCTGGCACAAAGCTGG
pGL4-TP53-ΔA	hp53-4488	TCGGTACCCTTCATATTTGACACAATGCAG
	AhTP53-4160	ATCTCGAGACGGTGGCTCTAGACTTTTGA
pGL4-TP53-ΔB	hp53-4426	TCGGTACCAGCTCTGGCTTGCAGAATTTTC
	AhTP53-4160	ATCTCGAGACGGTGGCTCTAGACTTTTGA
pGL4-TP53-ΔC	hp53-4349	TCGGTACCCTCCTCCCCAACTCCATTTC
	AhTP53-4160	ATCTCGAGACGGTGGCTCTAGACTTTTGA
pGL4-TP53-ΔD	hp53-4285	TCGGTACCATGGCGACTGTCCAGCTTTGTG
	AhTP53-4160	ATCTCGAGACGGTGGCTCTAGACTTTTGA
pGL4-TP53-ΔE	hp53-4225	TCGGTACCCCTCCCATGTGCTCAAGACTGG
	AhTP53-4160	ATCTCGAGACGGTGGCTCTAGACTTTTGA
pGL4-TP53-ΔF	hp53-4333	TCGGTACCATTTCCTTTGCTTCCTCCGGC
	AhTP53-4160	ATCTCGAGACGGTGGCTCTAGACTTTTGA
pGL4-TP53-ΔG	hp53-4333	TCGGTACCATTTCCTTTGCTTCCTCCGGC
	AhTP53-4210	ATCTCGAGCACATGGGAGGGGAAAACCCC
pGL4-TP53-ΔH	hp53-4295	TCGGTACCTTACTTGTCATGGCGACTGTCC
	AhTP53-4160	ATCTCGAGACGGTGGCTCTAGACTTTTGA
pGL4-TP53-ΔI	hp53-4295	TCGGTACCTTACTTGTCATGGCGACTGTCC
	AhTP53-4210	ATCTCGAGCACATGGGAGGGGAAAACCCC
pGL4-TP53-WT	hp53-4333	TCGGTACCATTTCCTTTGCTTCCTCCGGC
	AhTP53-4193	ATCTCGAGCTTTTAGCGCCAGTCTTGAGCA
pGL4-TP53-m1	hp53-4333#1	TCGGTACCATTTGGTTTGCTTCCTCCGGCA
	AhTP53-4193	ATCTCGAGCTTTTAGCGCCAGTCTTGAGCA
pGL4-TP53-m3	hp53-4333	TCGGTACCATTTCCTTTGCTTCCTCCGGC
	AhTP53-4193#1	ATCTCGAGCTTTTAAAACCAGTCTTGAGCA
pGL4-TP53-m13	hp53-4333#1	TCGGTACCATTTGGTTTGCTTCCTCCGGCA
	AhTP53-4193#1	ATCTCGAGCTTTTAAAACCAGTCTTGAGCA
pGL4-TP53-mEts, pGL4-TP53-mEtNk	EtsM1	TCCATTTGGTTTGCTTCCTCCGGCAGGCGG
	EtsM2	AGCAAACCAAATGGAGTTGGGGAGGAGGGT
pGL4-TP53-mNkx	NkxM1	TTGCCCGAACTTGTCATGGCGACTGTCCAG
	NkxM2	GACAAGTTCGGGCAAGTAATCCGCCTGCCG
pGL4-TP53-mE2F	E2FM3	AAGACTGGTTTTAAAAGTTTTGAGCTTCTC
	E2FM4	TTTAAAACCAGTCTTGAGCACATGGGAGGG

system (Promega) and relative Luc activities were calculated as described previously (*14*, *21*, *24*).

Western blot analysis

Western blot analysis was carried out as previously described (*13*, *21*), with antibodies against p53 (BIOSS, Woburn, MA), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) followed by the addition of horseradish peroxidase-conjugated secondary antibody (Calbiochem, Darmstadt, Germany). Signal intensities were quantified with a ChemiDoc and ImageLab System (BioRad, Berkeley, CA).

Reverse transcriptase PCR

Reverse transcriptase PCR (RT-PCR) was carried out as described previously (*13*, *21*). First-strand cDNAs were synthesized with ReverTra Ace (Toyobo, Tokyo, Japan), random primers (Takara) and total RNAs extracted from HeLa S3 cells. The sequences (from 5' to 3') of primer pairs used to amplify human *TP53*, *GAPDH* and *β-actin* cDNAs were Shp53-265; CTGCCCTC AACAAGATGTTTTG and Ahp53-436; CTATCTGAGCAGCGC TCATGG, hGAPDH556; TGCACCACCAACTGCTTAGC and hGAPDH642; GGCATGGACTGTGGTCATGAG and hbactS541; TGACGGGGTCACCCACACTGTGCCCATC and hbactA1201; CTAGAAGCATTTGCGGTGGACGATGGAG.

Conditions for the PCR were as follows: 94°C for 15 s, 55°C for 20 s and 72°C for 10 s, with 28 (*TP53*), 19 (*GAPDH*) and 20 (*β-actin*) cycles. PCR was performed with BIOTAQ DNA polymerase (BIOLINE, London, UK), and the PCR products were electrophoresed on 5% acrylamide gels and stained with ethidium bromide.

Quantitative real-time PCR

Real-time PCR analysis was carried out using the Mx3000P Real-Time qPCR System (Stratagene, La Jolla, CA) as described previously (*13*, *21*). For PCR amplification, cDNAs were amplified using Thunderbird Real-time PCR Master Mix (Toyobo) and 0.3 μM of each primer pair. The primer pairs for amplifying human *TP53* and *GAPDH* transcripts were Shp53-265/Ahp53-436 and hGAPDH556/hGAPDH642, respectively. Amplification was carried out initially for 1 min at 95°C, followed by 40 cycles (95°C 15 s and 58°C 30 s). Quantitative PCR analysis for each sample was carried out in triplicates. Relative gene expression values were obtained by normalizing C_T (threshold cycle) values of target genes in comparison with C_T values of the *GAPDH* gene using the $\Delta\Delta C_T$ method.

Results

Effects of serum deprivation on TP53 gene expression and its protein amount in HeLa S3 cells

To examine whether human *TP53* gene expression is affected by serum deprivation, total RNAs were extracted from cells after changing culture medium to serum-free DME (Fig. 1A). The relative gene expression of *TP53* compared with that of *GAPDH* began to increase slightly from 4 h after withdrawal of serum and then reached a plateau level. Western blot analysis showed that after serum deprivation, the amount of p53 protein reached a peak at 1–4 h and then decreased gradually (Fig. 1B and C). This decrease in protein amount is not only caused by the lowered mRNA level but also by degradation of the p53 protein, non-coding regulatory RNAs or another regulatory mechanism.

Upregulation of TP53 gene expression and p53 protein amount in HeLa S3 cells by CRmimetic compounds

The above results suggested that the *TP53* gene expression is transiently up-regulated by nutrient stress to HeLa S3 cells. To examine whether the expression of the *TP53* gene and its translated protein product p53 is induced by treatment with CR mimetic compounds, total RNAs and protein extracts from 2DG or Rsv-treated HeLa S3 cells were analysed by RT-PCR and Western blotting, respectively (Fig. 2).

As shown in Fig. 2A, the amount of *TP53* transcripts in the cells treated with Rsv (20 μM) and 2DG (8 mM) for 24 h increased to approximately 2-fold that of the control cells. The Western blot analyses indicated, as expected, that the relative level of the p53/β-actin increased after treatment with these two CR mimetic drugs (Fig. 2B). Next, HeLa S3 cells were collected at different times after Rsv (20 μM) treatment,

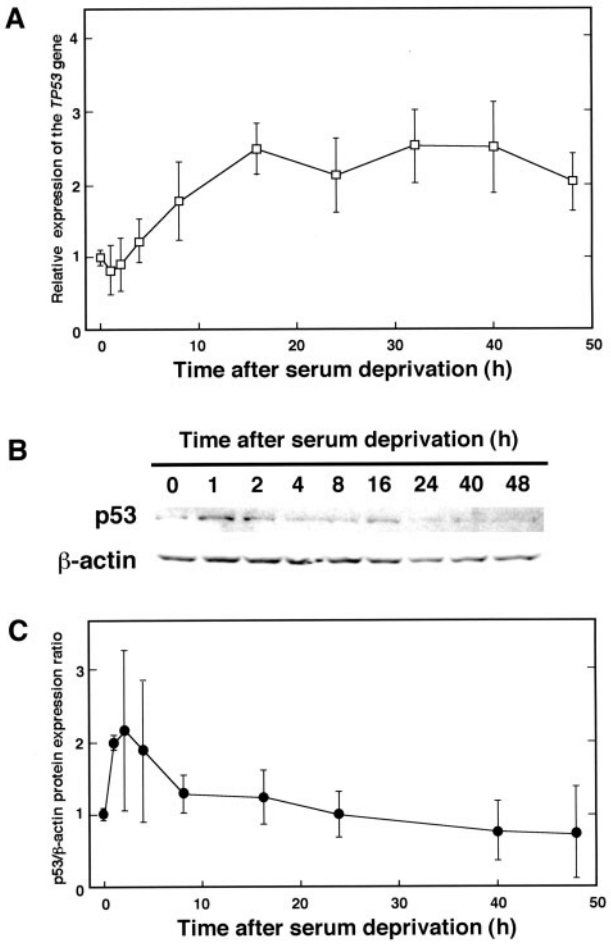

Fig. 1 Changes in *TP53* gene expression and p53 protein level after serum deprivation. (**A**) HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium without FCS for 0, 1, 2, 4, 8, 16, 24, 32, 40 and 48 h (lanes 1–10, respectively), then harvested. Total RNAs were extracted from cells, and synthesized cDNAs were subjected to quantitative real-time PCR with appropriate primer pairs to amplify *TP53* and *GAPDH* cDNAs. Results show relative *TP53*/*GAPDH* gene expression ratios that were compared with that of untreated cells (0 h). Three independent experiments were done, and results show means ± SD. (**B**) Similarly treated HeLa S3 cells as in (A) were collected after 0, 1, 2, 4, 8, 16, 24, 40 and 48 h of serum withdrawal. Proteins extracted from cells were separated by a 15% SDS-PAGE, and Western blotting was performed with primary antibodies against p53 and β-actin (upper and lower panels, respectively). (**C**) Each band was quantified and the results are shown by relative p53/β-actin protein expression ratio. Results are shown as means ± SD from at least three independent experiments.

and total RNAs were extracted. As shown in Fig. 2C, the relative expression of the *TP53* gene reached its peak level in comparison to the *GAPDH* gene (approximately 3-fold) at 16 h after the Rsv treatment (Fig. 2C).

Isolation and characterization of the 5′-flanking region of the human TP53 gene

To examine whether the induction of the *TP53* transcripts by Rsv occurs with the activation of the promoter, we isolated 551 bp of the 5'-upstream region of

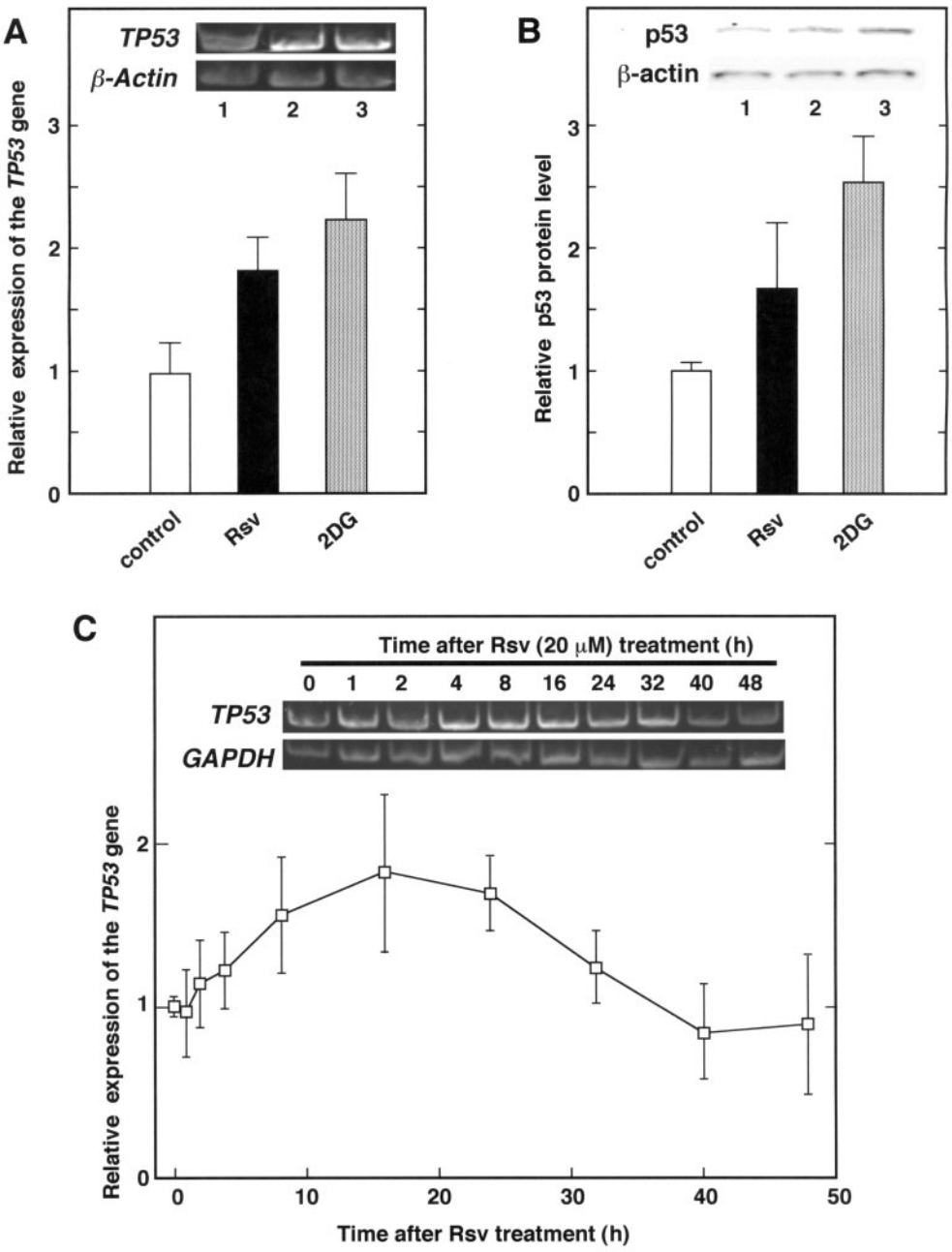

Fig. 2 Effects of CR mimetic compounds on *TP53* gene expression and p53 protein level in HeLa S3 cells. (**A**) The culture medium of HeLa S3 cells was changed to Dulbecco's modified Eagle's (DME) medium containing 10% FCS with 20 μM of Rsv or 8 mM of 2DG (lanes 2 and 3, respectively). Total RNAs were extracted from cells, and synthesized cDNAs were subjected to PCR with appropriate primer pairs to amplify *TP53* (upper panel) and *β-actin* (lower panel) cDNA. The histograms show relative *TP53*/*β-actin* gene expression ratio. Results are shown as means ± SD from three independent experiments. (**B**) HeLa S3 cells similarly treated to (A) were collected, then extracted proteins were separated by a 15% SDS-PAGE, and Western blotting was performed with primary antibodies against p53 and β-actin (upper and lower panels, respectively). Each band was quantified and results show relative p53/β-actin protein expression ratio. Results are shown as means ± SD from three independent experiments. (**C**) The culture medium of HeLa S3 cells was changed to DME containing 10% FCS with 20 μM of Rsv and harvested after 0, 1, 2, 4, 8, 16, 24, 32, 40 and 48 h. Total RNAs were extracted from cells and synthesized cDNAs were subjected to PCR with primer pairs to amplify *TP53* (upper panel) and *GAPDH* (lower panel) cDNA. Real-time quantitative RT-PCR was carried out to analyse *TP53* and *GAPDH* gene expression in HeLa S3 cells after 20 μM of Rsv treatment for 0 to 48 h. The results show relative *TP53*/*GAPDH* gene expression ratio compared with that of Rsv non-treated cells. Results are shown as means ± SD from at least three independent experiments.

the *TP53* gene by PCR. Sequence analysis revealed that the pGL4-TP53-551 contains a nucleotide identical to NCBI Sequence ID, NC_018928.2 (nucleotides from 7600471 to 7599921), and that it covers the sequence of the most-upstream 5'- end of the cDNA (Sequence ID; NM_001276760.1, NM_001276761.1, NM_001276696.1, NM_001276695.1 and NM_001126118.1 for variant 1, 2, 3, 4 and 8 of the *TP53* mRNA, respectively; GENE ID, 7157 TP53). Interestingly, this 551-bp region also contains a 5'-upstream end of variant 3 of the *WRAP53* mRNA (Accession No.NM_001143991.1; GENE ID, 55135 WRAP53) in a reverse orientation to that of the *TP53* gene. The transcription start site (TSS) was tentatively set as +1 at the most-upstream 5' of the *TP53* transcripts shown in the database. The TF-SEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html) suggested that the characteristic recognition sequences of several known transcription factors were found in the 551-bp region (Fig. 3). Although no obvious sequences similar to the TATA or CCAAT boxes were found, putative binding sites for HNF-3β (−430 to −419), GATA-x (−422 to −413, −413 to −400), GATA-1/2/3 (−382 to −374, −3 to +12), GATA-1/2 (−366 to −357), Oct-1 (−316 to −303), HSF-1/2 (−170 to −161), C/EBPβ (−175 to −163), c-Ets (−90 to −79), Nkx-2.5 (−52 to −45), c-Rel/NF-κB (+10 to +19) and E2F (+41 to +48) are contained in the 551-bp region (Fig. 3).

Effect of Rsv on the TP53 promoter activity

To examine whether the human *TP53* promoter is affected by the natural compound Rsv, Luc reporter plasmid pGL4-TP53-551 and its derivative deletion constructs (Fig. 4A–C, left panel) were transiently transfected into HeLa S3 cells. First, the Luc activities of pGL4-TP53-551 and pGL4-TP53-Δ1, Δ2, Δ3 and Δ12-transfected cells were compared to the Luc activity of the pGL4-PIF1-transfected cells (*14*, *21*). As shown in Fig. 4A, the relative Luc activity of the pGL4-TP53-551-transfected cells increased after the addition of Rsv (20 μM) to the cell culture. This induction by Rsv was also observed in pGL4-TP53-Δ2- and Δ3-transfected cells but not in the pGL4-TP53-Δ1- and Δ12-transfected cells. Luc activities of pGL4-TP53-Δ1- and Δ12-transfected cells were almost background level, probably due to their lack of TSSs. The results suggested that the sequence of the 91-nucleotides from −9 to +83 is primarily required for the *TP53* promoter activity.

We made further serial deletions from the 5'-upstream by PCR, and these reporter plasmids were transiently transfected into HeLa S3 cells. As shown in Fig. 4B, the deletion of the −105 to −44 region completely abolished the promoter activity, suggesting that the 61 bp, which contains the putative Nkx-2.5 binding sequence, 5'-CTTACTTG-3', is an essential region for the *TP53* promoter function. To narrow the core promoter sequence, additional deletions were made by PCR with the 188-bp fragment of the pGL4-TP53-ΔC (Fig. 4C). Although, Luc activity from cells that were transfected with pGL4-TP53-ΔH was comparatively lower than that of the pGL4-TP53-ΔC-transfected cells (Fig. 4C, right panel), it showed a positive response to Rsv. The result suggests that the region from −53 to +83 contains sets of essential transcription elements, including the Nkx-2.5 and the E2F motifs, respond to Rsv in HeLa S3 cells. Moreover, deletion of 50 bp (+34 to +83) from ΔH abolished the response to Rsv (Fig. 4C, compare ΔI and ΔG, with ΔH and ΔF, respectively). Complete loss of promoter activity was observed by a deletion from nucleotide −53 to −44 (Fig. 4C, compare ΔD with ΔH). These results suggest that Nkx-2.5 element from nucleotide −52 to −45 is essentially required for the *TP53* promoter activity and that putative E2F binding sequence (+41 to +48) plays a role in the response to Rsv. While it remains to be shown whether *TP53* gene expression is controlled during cardiogenesis, the homeobox protein Nkx-2.5 has been shown to regulate the differentiation of myocardial lineage or cardiogenesis (*25*, *26*).

Above results showed that the region from +34 to +83, which contains a putative E2F binding sequence, responds positively to Rsv in HeLa S3 cells. To examine the Rsv-responding elements in detail, point mutations were introduced in the pGL4-TP53-WT plasmid. As shown in Fig. 4D, the mutation on the c-Ets element greatly reduced basal promoter activity with its response to Rsv (pGL4-TP53-mt1). Although the mutation on the E2F element does not affect the basal promoter activity, the response to Rsv was abolished (pGL4-TP53-mt3). Moreover, cells that were transfected with pGL4-TP53-m13, which carries mutations on both c-ETS and E2F elements, showed an apparent promoter activity, but they do not show response to Rsv. Moreover, we performed transfection experiment with plasmids carrying mutations on these elements in the pGL4-TP53-551 plasmid (Fig. 5). The result indicated that point mutations on Nkx-2.5 and E2F binding element reduced the response to Rsv. Introduction of mutations on the c-Ets element did not alter the response to Rsv, suggesting that other responsive element(s) in the region from −468 to −97 is also contributing for the response. Taken together, these results suggest that the *TP53* promoter is regulated by c-Ets, Nkx-2.5 and E2F binding elements under the control of the other sequence(s) to respond to Rsv in HeLa S3 cells.

Discussion

In this study, we showed that both serum withdrawal and the addition of CR mimetic drugs up-regulate *TP53* gene expression in HeLa S3 cells. Moreover, deletion and mutation analyses of the 5'-upstream region of the *TP53* gene revealed that c-Ets, Nkx-2.5 and E2F elements are essential for the activation of the promoter in response to Rsv.

Previous studies indicated that various transcription factors, including NF-κB, CREB, c-Myc and c-Ets, are involved in the regulation of *TP53* gene expression (*12*, *27*, *28*). A palindromic c-Ets element, which is located in very close proximity to the TSS of the *TP53* gene (*29*), has been shown to play an essential role in controlling its promoter function (*30*). We have reported

that the duplicated GGAA (TTCC) motif is frequently found in the promoter regions of the DNA repair factor-encoding human genes (*23*), including *PARP* and *PARG*, which encode poly(ADP-ribose) metabolism regulating enzymes (*31*, *32*). A microarray study of HIV-1 infection in human primary $CD4^+$ T cells revealed interferon-dependent up-regulation of *TP53* gene expression (*33*). However, the proteins that bind to the duplicated GGAA motifs to regulate the *TP53* promoter have not been correctly shown. The opposite

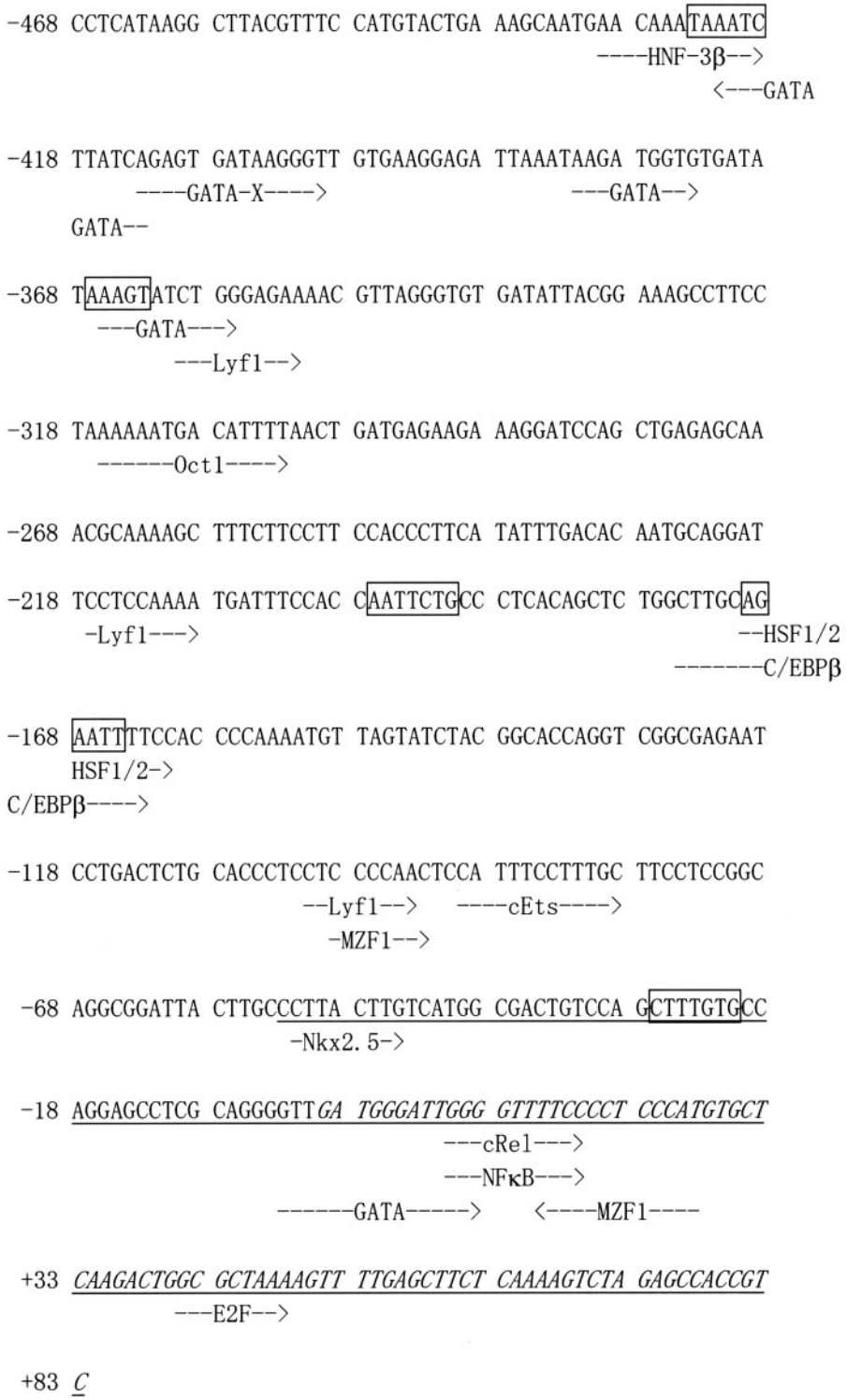

Fig. 3 Nucleotide sequence of the 5'-flanking region of the human *TP53* gene. The nucleotide sequence of the 551-bp fragment that was obtained from PCR is shown. The most-upstream 5'-end of the *TP53* cDNA (NM_001276760.1, NM_001276761.1, NM_001276696.1, NM_001276695.1 and NM_001126118.1) is designated nucleotide +1. Putative transcription factor-binding sites (TF-SEARCH score > 87) are indicated by dotted arrows. Boxes represent CdxA sequences. Italic and underlined characters represent the cDNA sequences that overlap the human *TP53* (Gene ID: 7157) and *WRAP53* (GENE ID: 55135) genes, respectively.

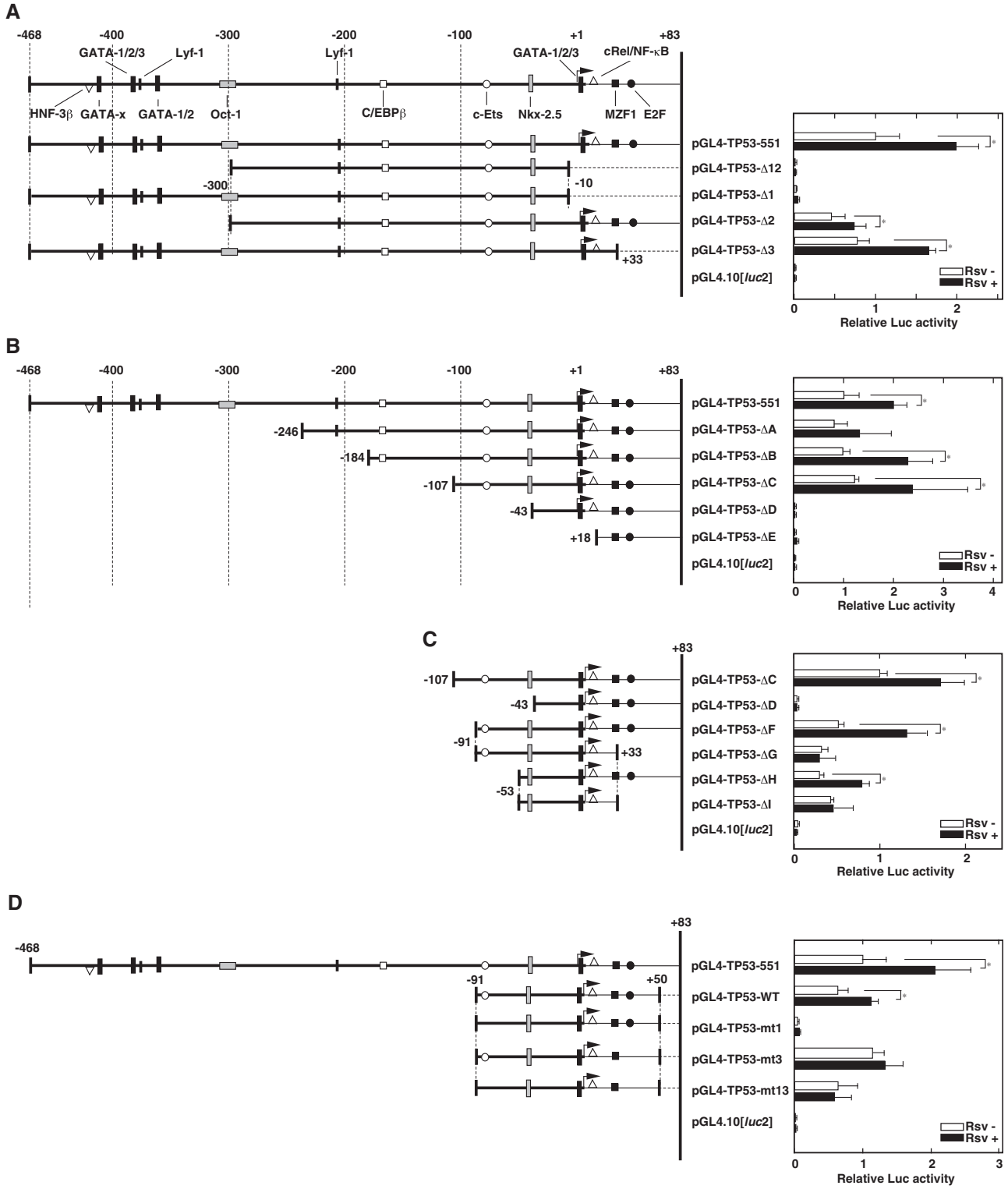

Fig. 4 Effect of Rsv on human *TP53* promoter activity. (**A–D**) (Left panel) The 5'-flanking region of the human *TP53* gene, which has been ligated upstream of the *Luciferase* gene of the pGL4.10[*luc*2], is shown. The 5'-end of the cDNA is designated +1. Putative transcription factor binding elements that were found from the TF-SEARCH program (score > 87) are schematically shown. The sequence of the *TP53* promoter contained in each deletion construct is schematically indicated. (A) Luc reporter plasmids were transiently transfected into HeLa S3 cells and treated with (closed bars) or without (open bars) Rsv (20 μM) for 24 h, then they were collected after further 24 h incubation. Luc activities were normalized to that of the pGL4-PIF1-transfected cells. Histograms show relative Luc activities of deletion construct transfected cells comparing with that of the pGL4-TP53-551-transfected cells without Rsv treatment. (B–D) Similar experiments as described under (A) were carried out with pGL4-TP53-551, -ΔA, -ΔB, -ΔC, -ΔD, -ΔE, -ΔF, -ΔG, -ΔH, -ΔI, -WT, -mt1, -mt3, -mt13 and pGL4.10[*luc*2]. Results are shown as means ± SD from five independent experiments. Statistical analysis was performed with the Student's t test and asterisks indicate values of $P < 0.05$.

strand nucleotide sequences from −92 to −79 and −83 to −70 are 5'-GCAAAGGAAATGGA-3' and 5'-CCGGAGGAAGCAAA-3', respectively (Fig. 3). Therefore, Ets family class III proteins, such as Spi1, SpiB and SpiC, which recognize the sequence 5'-(A/T/C)(G/A)(A/G/C)GGAA(G/C)(T/C)N-3' (*34*), may be candidate binding factors. We have proposed that tandem repeated or duplicated GGAA-motifs serve as a fine-tuning system to respond to a variety of stresses that induce apoptosis and DNA damage/interferon responses (*35–37*). Thus, in this context, the duplicated GGAA motif in the region from −92 to −70 might be a competitive target for various transcription factors, including the Ets family proteins. It should be noted that the duplicated GGAA (TTCC) plays important roles in the regulation of human *RB1* and mouse *Rb1* promoter (*23*, *38*). Of note, it has been experimentally shown that GABP α/β protein targets the GGAA motif in the mouse *Rb1* promoter region (*38*).

The response to Rsv was diminished only when the E2F element was eliminated or mutated (Fig. 4), suggesting that the putative E2F binding sequence 5'-TTTAGCGC-3' (inverted nucleotide sequence from +41 to +48), which is located downstream of the TSS (Fig. 3), is the *cis*-element that is primarily required to respond to Rsv. Although the TF-SEARCH program did not indicate a tandem repeated element, the nucleotide sequence from +51 to +58 is 5'-TTTTGAGC-3'. Thus, the 18-bp nucleotide from + 41 to +58 consists of a palindromic tandem inverted repeat of the E2F element. It should be noted that the tandem E2F consensus sites are located in the promoter region of the human *p107* gene, which encodes Rb-related protein (*39*). A variety of biological functions, including DNA replication, DNA repair, DNA-damage check point and G1-S transcriptional activation, are regulated by the E2F family proteins, which are encoded by eight distinct genes (*E2F1* to *E2F8*) (*40*, *41*). Given that the E2F family includes both positive and negative transcription regulators (*41*), the tandem repeated E2F element could serve as a fine-tuning modulator for *TP53* transcription in response to cell cycle progression or DNA-damage-inducing signals in a similar mechanism, which is regulated by the duplicated GGAA motifs (*35–37*). E2F family proteins are known as regulators for p53, and E2F1-p53 cooperation in particular has a significant role in the regulation of apoptosis (*40*, *41*). Recent studies have suggested that p53 up-regulates the transcription of the *E2F7* gene, which in turn causes cellular senescence linking the Rb and p53 pathways (*42*, *43*). It was shown that E2F1 binds to E2F element of the *E2F7* promoter to activate its transcription (*44*). Moreover, overexpression of NF-Y up-regulates E2F1, which leads to an increase in the amount and the activity of p53 in mouse embryonic fibroblasts and human cells (*45*). These lines of evidences imply that Rsv-induced expression of the *TP53* gene is controlled by the E2F family proteins. Importantly, duplicated GGAA (TTCC) motifs are present near the TSS of the human *RB1* gene (*23*). Moreover, the RB protein has been suggested to affect and modulate the transcription from E2F target gene promoters (*46*). Taken together, these findings suggest that the Rsv-induced activation of the *TP53* promoter might be associated with interaction between RB and E2F. Therefore, the effect of Rsv to induce cell cycle arrest (*47*, *48*) could be partly explained by the activation of E2Fs that affect G1-S cell cycle progression.

Rsv activates p53 to induce apoptosis and up-regulates p53 protein level in mouse epidermal cells and bovine pulmonary artery endothelial cells (*49*, *50*). It was reported that Rsv induces the expression of the *ASPP1* (*PPP1R13B*) gene, which encodes the apoptosis stimulation protein of p53, *via* the induction of E2F1 in breast cancer cells (*51*). The observation suggests a possible mechanism through which Rsv effectively evokes apoptosis by the up-regulation of *TP53* and *ASPP1* in an E2F1-dependent manner. We have reported that Rsv induces *SIRT1* promoter activity in HeLa S3 cells (*52*, *53*). It is noteworthy that the human *SIRT1* promoter is activated by serum deprivation in a p53-dependent manner (*54*). This suggests that the transient induction of the *SIRT1* gene occurs as a result of the effect of p53 after Rsv treatment. It is

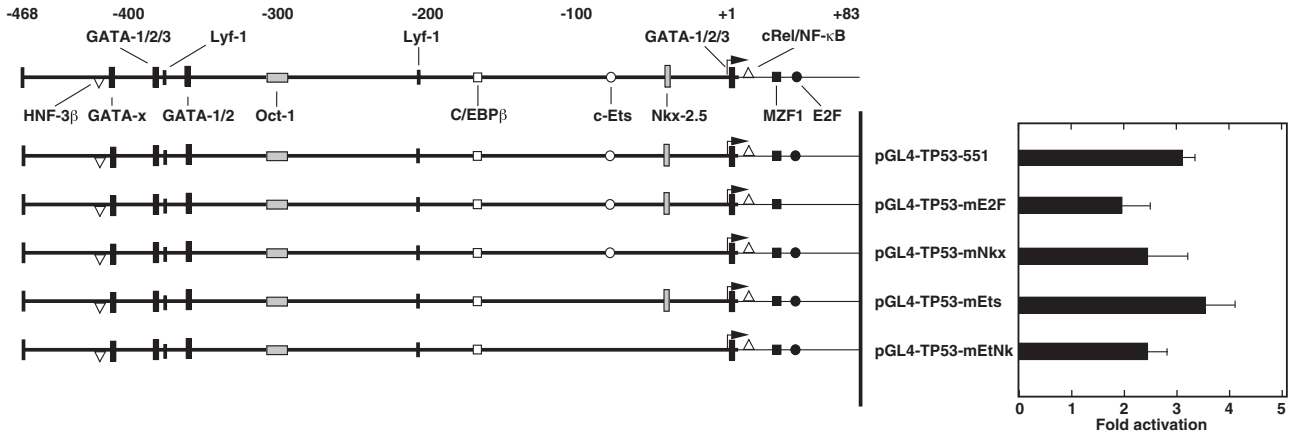

Fig. 5 Mutation analysis on c-Ets, Nkx-2.5 and E2F binding elements in the 551-bp human *TP53* promoter region. Similar experiment as described under the legend to Fig. 4 was carried out with pGL4-TP53-551, pGL4-TP53-mE2F, pGL4-TP53-mNkx, pGL4-TP53-mEts and pGL4-TP53-mEtNk. Histograms show fold induction of Luc activities by Rsv (20 μM) for 24 h treatment. Results are shown as means ± SD from four independent experiments.

widely known that p53 controls the expression of genes that encode DNA-damage response factors and cell cycle/apoptosis regulating factors (*1*, *55*). Moreover, in the presence and even absence of stress, p53 protein localizes in the mitochondria as well as the nuclei (*9*, *56*), suggesting that it does not only control transcription in nuclei, but also that it affects metabolic reactions in mitochondria (*9*). It is noteworthy that p53 regulates mitochondrial respiration, inducing expression of the *SCO2* gene, which encodes the synthesis of cytochrome c oxidase 2 (*57*). The p53 is also reported to affect metabolic stress and aging (*58*). Furthermore, p53 regulates malic enzymes in human and mouse cells to induce cellular senescence without causing apoptosis (*59*). The functions of p53 as a key regulator for glycolysis/oxidative phosphorylation balance in a cell have been reviewed (*60*).

A recent study showed that telomere dysfunction induces the expression of the *Tp53* gene to suppress the function of Pgc-1α (*7*), implying that telomeres exert signals to mitochondria. Greater damage to chromosomes will affect mitochondrial proteins, including Bcl-2, Bcl-X_L, BAX and cytochrome c, to induce cell death (*61*). Our previous study showed that the promoter activities of *TERT*, *WRN* and several shelterin-encoding genes are induced by Rsv in HeLa S3 cells (*14*, *21*). Besides GC-boxes and Sp1 binding elements, the duplicated GGAA (TTCC) motif is a common sequence in the 5'-upstream regions of the *TERT* and *WRN* genes (*13*, *33*). Furthermore, recent studies in cancer genomes revealed that mutations on the GGAA (TTCC) motifs or the creation of Ets binding elements in the *TERT* promoter are frequently found in human melanoma (*62*, *63*). Thus, co-induction of the *TP53* and *TERT* genes through the duplicated GGAA motifs may account for keeping telomeres to their appropriate length. Variety of clinical trials showed that health-promoting responses, including reduction in generation of reactive oxygen species (ROS) and induction of insulin sensitivity, are caused by Rsv (*64*). Further investigations are required to elucidate the mechanisms by which Rsv-induced signals regulate the cellular senescence, lifespan and longevity of organisms.

Acknowledgements

The authors are grateful to S. Larsen for critical reading of the manuscript and discussion.

Funding

This work was supported in part by a Grant-in Aid from the Ministry of Education, Culture, Sports, Science and Technology Japan (No. 24510270) and a Research Fellowship from the Research Center for RNA Science, RIST, Tokyo University of Science.

Conflict of Interest

None declared.

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