PAX8 Regulates Telomerase Reverse Transcriptase and Telomerase RNA Component in Glioma

Yu-Jen Chen,[1] Hamish G. Campbell,[2] Anna K. Wiles,[1] Michael R. Eccles,[1] Roger R. Reddel,[2] Antony W. Braithwaite,[1,2] and Janice A. Royds[1]

[1]Department of Pathology, Dunedin School of Medicine, University of Otago, Dunedin, New Zealand and [2]Children's Medical Research Institute, Westmead, and University of Sydney, New South Wales, Australia

Abstract

Paired box (*PAX*) developmental genes are frequently expressed in cancers and confer survival advantages on cancer cells. We have previously found that *PAX* genes are deregulated in glioma. We have now investigated the expression of *PAX* genes in glioma and their role in telomere maintenance. The mRNA level of *PAX8* showed a positive correlation with telomerase activity in glioma biopsies (r^2 = 0.75, P < 0.001) and in established glioma cell lines (r^2 = 0.97, P = 0.0025). We found that PAX8 is able to coordinately transactivate the promoter for both the telomerase catalytic subunit (*hTERT*) and the telomerase RNA component (*hTR*) genes. By electrophoretic mobility shift assay, quantitative PCR, and a telomerase activity assay, we show that PAX8 binds directly to the *hTERT* and *hTR* promoters, up-regulating *hTERT* and *hTR* mRNA, as well as telomerase activity. Additionally, *PAX8* small interfering RNA down-regulated *hTERT* and *hTR*. Collectively, these results show that PAX8 may have a role in telomerase regulation. [Cancer Res 2008; 68(14):5724–32]

Introduction

The termini of linear eukaryotic chromosomes are comprised of telomeric repetitive, noncoding DNA, made up of (TTAGGG)*n* hexanucleotide repeats and associated proteins (1). Telomere ends cannot be fully replicated by the conventional DNA replication machinery, resulting in telomere shortening at each cell division (2). Telomere loss to a critical length can lead to decreased cell viability (3), and increased end-to-end chromosome fusions, which promote genomic instability (4). Thus, loss of terminal nucleotides with each cell division limits the replicative capacity of eukaryotic cells. Therefore, it is believed that cancer cells activate telomere maintenance mechanisms (TMM) to solve this problem and are thus able to replicate indefinitely (5). The most thoroughly studied TMM is telomerase, which is a reverse transcriptase that adds telomeric repeats to chromosomal ends (6). Telomere length may also be maintained in cancer cells by telomerase-independent mechanisms referred to as alternative lengthening of telomeres (7).

Telomerase is a multicomponent complex; the main catalytic holoenzyme consists of human telomerase RNA (*hTR*), human telomerase reverse transcriptase (*hTERT*), and dyskerin (8). Because telomerase can be activated by exogenous TERT expression in cells, TERT has been regarded as a limiting factor in telomerase activation (9). Several lines of evidence, however, indicate that *hTR* may be limiting (10, 11). Both *hTERT* and *hTR* are required for a functional telomerase holoenzyme. Telomerase function is stringently regulated at multiple levels, including transcriptional and translational regulation, posttranslational modification, and protein subcellular localization (12). The deregulation of *hTERT* in cancer cells can be achieved by gene amplification (13). However, most studies have focused on the transcriptional regulation of *hTERT* expression. Several transcription factors have been shown to transcriptionally regulate *hTERT*, including c-Myc and Sp1 (14). In addition, multiple tumor suppressor pathways, such as the p53 and transforming growth factor-β pathways, negatively regulate telomerase (15). The *hTERT* promoter activity is also regulated by hormones and growth factors such as estrogen, basic fibroblast growth factor, and epidermal growth factor (16).

Paired domain–containing transcription factors (PAX) are encoded by a developmental gene family, classified into four subgroups according to the presence or absence of an octapeptide region and the presence, absence, or truncation of a homeodomain (17). *PAX* genes of the same subgroup are also expressed in similar patterns during development (17). *PAX2, PAX5*, and *PAX8* belong to the same subgroup containing an octapeptide domain and a truncated homeodomain. They are expressed in the midbrain-hindbrain junction during brain development (18). The deregulation of *PAX* genes has been linked with many types of cancer, such as astrocytoma, medulloblastoma, lymphoma, and Wilms' tumor (19, 20). In another study, we examined *PAX2, PAX5*, and *PAX8* expression in a panel of 54 gliomas. We found *PAX8* to be overexpressed in the majority of these gliomas, whereas *PAX2* and *PAX5* were overexpressed to a lesser degree.[3] Because we previously found TMM to be of prognostic value in glioma (21), we considered the hypothesis that *PAX* genes, in particular *PAX8*, might be associated with telomere maintenance, by possibly facilitating survival and immortalization of cells.

In this article, we show that PAX8 activates the *hTERT* and *hTR* promoters, which in turn activate telomerase, supporting the hypothesis that PAX8 may be an important regulator of telomerase activity and of cell survival in some gliomas.

Materials and Methods

Tissue samples and cell culture. We obtained tissue samples from patients recruited by Neurosurgical Units at Dunedin and Christchurch

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Janice A. Royds, Department of Pathology, Dunedin School of Medicine, University of Otago, P.O. Box 913, Dunedin, New Zealand. Phone: 64-3479-7471; Fax: 64-3479-7136; E-mail: Janice.royds@stonebow.otago.ac.nz.

©2008 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-08-0058

[3] Y-J. Chen et al., unpublished data.

Downloaded from cancerres.aacrjournals.org on May 28, 2017. © 2008 American Association for Cancer Research.

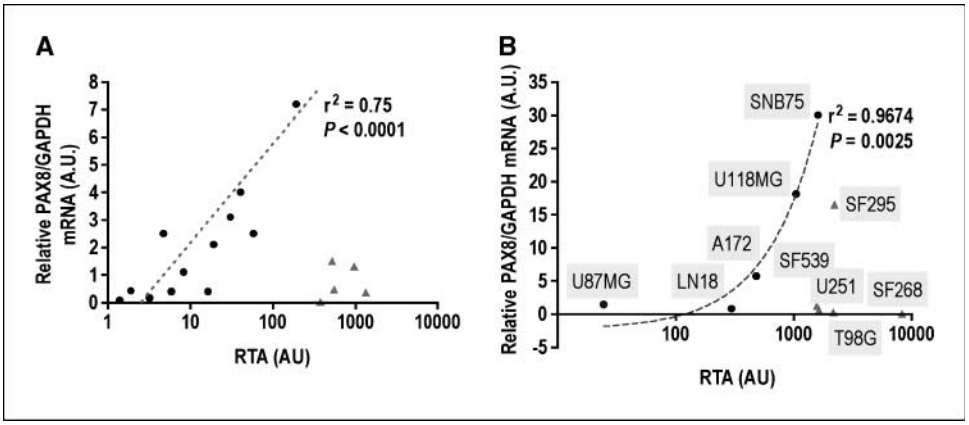

Figure 1. Correlation of relative *PAX8* mRNA level with RTA in glioma specimens (*A*) and in established glioma cell lines (*B*). Glioma samples with low to moderate telomerase activity (●), glioma samples with very high telomerase activity (▲). A regression of samples with low to moderate telomerase activities (*dotted line*). Samples with high telomerase activities were not included in the regression. The names of the established cell lines are designated. *AU,* arbitrary units (log scale).

Hospitals (2002–2005). The study was approved by the institutional ethics committees and all patients provided informed consent. The inclusion and exclusion criteria have been previously published (21). Histologic diagnoses were made by consultant neuropathologists.

A panel of human glioma cell lines, SF268 (from NCI-60), T98G, A172, LN-18, and U87MG (from American Type Culture Collection) were grown at 37°C and 10% CO_2 in humidified atmosphere, in DMEM (Life Technologies) supplemented with 10% FCS, 2 mmol/L of glutamine, and 100 units/mL of penicillin/streptomycin.

Telomerase activity assay. Telomerase activity was quantified using TeloTAGGG Telomerase PCR ELISA^{PLUS} Kit (Roche Applied Science) as previously described (21).

Real-time PCR. The expression of *PAX8, PAX2, hTERT*, and *hTR* mRNA in established glioma cell lines, glioma specimens, small interfering RNA (siRNA)–treated glioma cell lines, and glioma cells overexpressing *PAX8* were detected using real-time PCR. Briefly, total RNA was DNase-treated and then subjected to reverse transcription using SuperScript III and random primers (Invitrogen). For quantitative PCR, first-strand cDNA synthesized from 50 ng of RNA was used for each real-time PCR reaction. Relative quantitation of mRNA by real-time PCR using SYBR-green detection and analyzed using the Relative Expression Software Tool 2005 (REST).[4] The primer sequences and PCR conditions are listed in Supplementary Table S1.

Investigation of binding sites in the *hTERT* and *hTR* promoters. Possible binding sites for PAX2/5/8 in the *hTERT* and *hTR* promoters were determined using ConSite (22). They were also analyzed by aligning the PAX2, PAX5, and PAX8 consensus-binding sequence (forward, 5′-RNGM-ANTSANGCNKRAC-3′; reverse, GTYMNGCNTSANTKCNY; R = A/G, M = C/A, S = G/C, K = T/G, Y = T/C; ref. 23) and the PAX8 consensus sequence (forward, 5′-GGGCATCAGAGCATGGA; reverse, TCCATGCTCTGATGCCC; ref. 24) with the *hTERT* promoter and the *hTR* promoter.

Plasmid transfection and reporter gene assays. Cells (5×10^4 cells/well) were seeded into six-well plates, cultured overnight, and transfected with the *hTERT* or *hTR* promoter-luciferase plasmids (1 μg per well of hTERT3915 or hTR2.1kb and the same copy number of hTERT255 or hTR306 with pGL2 to normalize the total amount of DNA transfected; from J. Carl Barrett; ref. 25) using FuGENE6 transfection reagent (Roche Applied Science). Cells were also transfected with either pCMV5.PAX8, pCMVPAX2b, pCMVPAX2c, or control pcDNA3 plasmids. Preparation of cell lysates and measurement of luciferase activity (Promega) have been previously described (26). Assays were performed in duplicate and repeated at least twice.

siRNA transfection. Cells (1×10^5 cells/well) were seeded into six-well plates, cultured overnight and transfected with 10 nmol/L of *PAX8, PAX2*, or non–target siRNA using RNAiMAX transfection reagent (Invitrogen). Twenty-four hours posttransfection, RNA was isolated and real-time PCR was carried out as above. *PAX8* and matched control siRNA oligos were obtained from Ambion (*PAX8*, 5′-UCUUUAUUUAUUACAUGAA-3′; non–target 5′-UUCUCCGAACGUGUCAGGU-3′). *PAX2* and corresponding control siRNAs were obtained from Dharmacon (siGenome On-Target plus SMART-pool accession number, NM_003989; Dharmacon).

Electrophoretic mobility shift assay. Sense and antisense oligonucleotides (Supplementary Table S2) were annealed and labeled with second-generation DIG Oligonucleotide 3′-End Labeling Kit (Roche Applied Science). After transfection with pCMV5.PAX8 for 2 days, nuclear extracts were prepared following the published protocol (27). Nuclear extract (1 μg) was incubated with 300 ng of poly(dI-dC) in the presence or absence of unlabeled competitors for 15 min, in a 20 μL reaction containing 20 mmol/L of Tris (pH 8.0), 60 mmol/L of NaCl, 5 mmol/L of $MgCl_2$, 4% glycerol, and 100 μg of bovine serum albumin. DIG-labeled probes were then added and incubated for 15 min. The reaction mixture was then resolved on a 5% nondenaturing polyacrylamide gel in 0.5× Tris-Glycine-EDTA buffer. DNA was transferred to a positively charged nylon membrane, Hybond-N$^+$ membrane (Amersham Pharmacia Biotech, Inc.) at 15 V overnight. The membrane was dried and then UV cross-linked for 15 min. The signal was detected using anti–Digoxigenin-AP antibody (1:10,000 dilution; Roche Applied Science) and CSPD-Star chemiluminescent substrate (Roche Applied Science). The membrane was exposed to X-ray film (Kodak) for 5 s to 15 min at room temperature.

Statistical analysis. Statistical analysis was performed using GraphPad InState (version 3.05 for Macintosh, GraphPad Software). The correlation between *PAX8* mRNA expression and relative telomerase activity (RTA) was analyzed using linear regression after dividing the samples into two groups. Glioma biopsies with RTA > 350 were designated as having high telomerase activity.

Results

PAX8 expression is correlated with telomerase activity. Previously, we reported that *PAX8* is expressed in a subset of gliomas (20) and that TMM is of prognostic value for patients with high-grade gliomas (21). We therefore considered the hypothesis that there is a correlation between *PAX8* gene expression and TMM regulation. Results show that RTA is positively correlated with *PAX8* expression level in several glioma biopsies ($n = 14$) that have low to moderate RTA ($P < 0.0001$; $r^2 = 0.75$; Fig. 1*A*). However, no correlation was observed when the RTA was very high (Fig. 1*A*, ▲). This suggests that very high RTA is also controlled by other factors. Likewise, a correlation between the *PAX8* mRNA level and RTA was observed in established glioma cell lines with low to moderate telomerase activity ($n = 5$, $r^2 = 0.97$, $P = 0.0025$; Fig. 1*B*), but not in glioma cell lines with high RTA (Fig. 1*B*, ▲). These data suggest a possible role for PAX8 in transactivating one or more components of the telomerase holoenzyme (8).

[4] http://www.gene-quantification.de/

Downloaded from cancerres.aacrjournals.org on May 28, 2017. © 2008 American Association for Cancer Research.

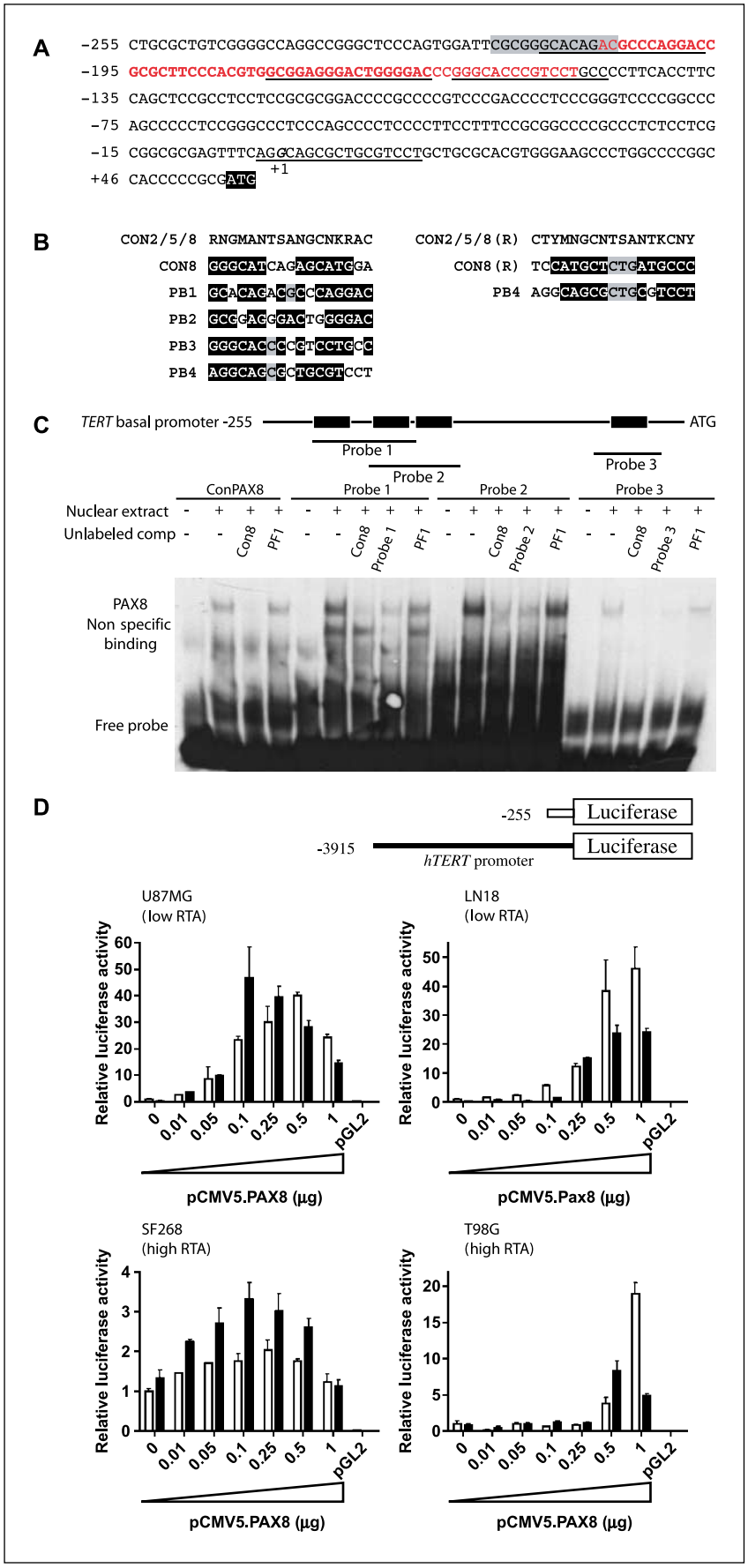

Figure 2. *hTERT* is a target for PAX transcription factors. *A,* potential PAX2/5/8 binding sites in the promoter region of the *hTERT* promoter. The major transcription initiation site (+1; in *italics* and *boldface*) and the translation initiation codon (ATG; highlighted in *black*). The region responsible for full promoter activity (highlighted in *boldface* and *red*), potential PAX2/5/8 binding sites from position −255 to the translational initiation codon (*underlined*), and the Smad3 binding site (highlighted in *gray*). *B,* alignment of the PAX2, PAX5, and PAX8 potential binding sites (*PB1–PB4*; the first potential binding site from the distal end of the promoter is defined as PB1) in the *hTERT* promoter with the PAX2/5/8 consensus binding sequence and the PAX8 consensus binding sequence. Nucleotides which are the same as the PAX2/5/8 consensus sequence (highlighted in *black*), and nucleotides which are the same as the PAX8 consensus sequence (highlighted in *gray*). *C,* an EMSA was used to analyze the binding of PAX8 to digoxigenin-labeled oligonucleotide probes containing the predicted PAX binding sites found in the minimal *hTERT* promoter. The labeled probes were incubated with nuclear extract from U87MG cells transfected with 1 μg of pCMV5.PAX8 for 2 d. To confirm the specificity of binding, the addition of 15-fold excess cold probe, consensus PAX8 binding sequence (*ConPAX8*), or irrelevant oligonucleotide (*PF1*) was undertaken as indicated. Comp: competitor. *D,* transactivation of the *hTERT* promoters by overexpression of PAX8 in glioma cell lines. Glioma cell lines with different telomerase activity were transfected with the *hTERT* luciferase constructs and varying amounts of a PAX8 expression vector. The effect of PAX8 on the short (*white columns*) and the long (*black columns*) promoters of *hTERT* was tested. Luciferase activities were measured 48 h after transfection. Relative luciferase activities were normalized to the luciferase value of the minimal promoter of *hTERT* in each cell line (*columns,* mean; *bars,* SE).

Downloaded from cancerres.aacrjournals.org on May 28, 2017. © 2008 American Association for Cancer Research.

The *hTERT* promoter contains potential PAX-binding sites. To explore the potential transcriptional regulation of *hTERT* by PAX8, possible binding sites for PAX2/5/8 in the *hTERT* promoter were sought using the regulatory element prediction software, Consite (22). Four potential PAX2/5/8 binding sites (PB1–PB4) were found in the *hTERT* promoter (Fig. 2*A* and *B*), one of which is 40 bp upstream of the start codon (28). Three of the putative sites (PB1–PB3) are located in the minimal region responsible for full promoter activity (Fig. 2*A*; ref. 28). One of the potential PAX2/5/8 binding sites (PB1) overlaps with a Smad3 binding site (Fig. 2*A*; ref. 29), which is similar to the PAX8 binding site in the sodium/iodide symporter gene promoter (30). The identification of these potential binding sites suggests that PAX8 could transactivate *hTERT*.

PAX8 binds to the *hTERT* promoter *in vitro*. To address whether PAX8 can bind these potential PAX2/5/8 binding sites in the *hTERT* promoter, we performed electrophoretic mobility shift assays (EMSA) using three probes spanning the four predicted binding sites (Fig. 2*A*). DNA-protein complexes were detected with all three *hTERT* probes, which could be competed out with an excess of unlabeled probe and unlabeled PAX8 consensus sequence (ConPAX8), but not by irrelevant oligonucleotides containing activator protein-1–like binding sites (PF1, see Supplementary Data; ref. 31). Probe 3 showed a weaker signal than probes 1 and 2 (Fig. 2*C*), as probe 3 contains only one possible binding site, whereas probes 1 and 2 contain two possible binding sites (Fig. 2*C*). These results were also confirmed using *in vitro*–translated PAX8 protein (data not shown).

Overexpression of PAX8 activates the *hTERT* promoter. We next tested whether PAX8 activates the *hTERT* promoter. pCMV5.PAX8 was cotransfected with *hTERT* promoter-luciferase constructs into glioma cell lines. Two *hTERT* luciferase reporter constructs were used. One luciferase reporter contains 255 bp of the *hTERT* promoter, and the other construct contains 3,915 bp of the *hTERT* promoter.

Because we had shown in Fig. 1 that cells with high telomerase activity might not be regulated by PAX8, we chose cell lines with high (T98G and SF268) and low (U87MG and LN18) RTA. Reporter assays were carried out 48 hours after transfection. Results showed that PAX8 expression markedly induced luciferase activity of both the short and the long *hTERT* promoters in a dose-dependent manner (Fig. 2*D*). In accordance with Fig. 1, the effect was higher in glioma cell lines with low RTA (Fig. 2*D*). Overexpression of PAX8 in these two cell lines activated the *hTERT* promoter ~50-fold (Fig. 2*D*). In contrast, the two glioma cell lines with constitutively high RTA had lower levels of activation at ~3-fold and 18-fold in SF268 and T98G, respectively (Fig. 2*D*). Hence, PAX8 is not only able to bind, it is also capable of activating the *hTERT* promoter *in vitro*.

Activation of the *hTERT* promoters by PAX8 is independent of *TP53* status. It has been shown that p53 inhibits *hTERT* expression and telomerase activity (32, 33). Also, PAX2, PAX5, and PAX8 have been reported to repress the *TP53* promoter (34). To determine whether the activation of the *hTERT* promoter by PAX8 is partly due to its inhibitory effect on p53, we analyzed the activation of the *hTERT* promoters by PAX8 in the isogenic colorectal cell lines, HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ (35). We found that PAX8 transactivated both *hTERT* promoters in these cell lines to a similar extent (Fig. 3). This suggests that the suppression of p53 is unlikely to play a key role in the activation of *hTERT* by PAX8.

The *hTR* promoter contains potential PAX-binding sites. As well as *hTERT, hTR* may also be regulated by PAX8. We therefore investigated whether PAX protein can regulate the *hTR* promoter (36). Analysis of the promoter, indicated that six possible PAX2/5/8 binding sites (PB1–PB6) are present in the short *hTR* promoter (−306 bp; Fig. 4*A*). Of these, the sequence of PB1 has the highest similarity to the consensus binding site of PAX2/5/8 and PB5 had the lowest similarity (Fig. 4*B*). These data suggest that PAX8 may also transactivate *hTR*.

PAX8 binds to the *hTR* promoter *in vitro*. To investigate whether PAX8 binds to the potential PAX2/5/8 binding sites in the *hTR* promoter, five probes spanning the possible binding sites in the short promoter of *hTR* were used for EMSA (Fig. 4*C*). Probes 1, 3, 4, and 5 contain one possible PAX2/5/8 binding site each, whereas probe 2 spans the region of *PB2* and *PB3*. Specific binding was observed with probes 1, 2, and 4, as the binding signals could be competed with excess unlabeled *ConPAX8* probe, but not with the irrelevant *PF1* probe (Fig. 4*C*). By contrast, only nonspecific binding was observed with probe 3, which contains *PB4*, as excess unlabeled *ConPAX8* did not compete out the signal (Fig. 4*C*). Similarly, nonspecific binding was observed with probe 5, which contains *PB6*, as an excess of unlabeled *PF1* probe competed out

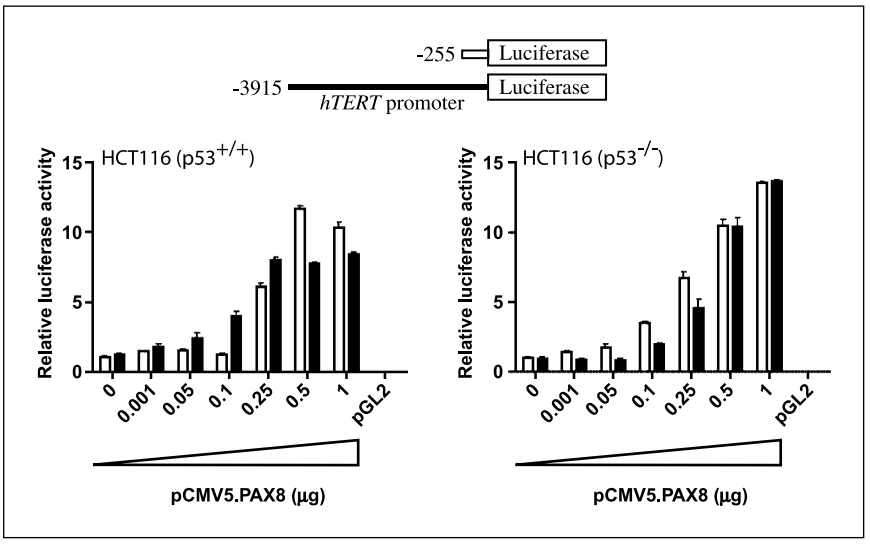

Figure 3. Activation of the *hTERT* promoter by PAX8 is independent of *TP53* status. The activation of the *hTERT* promoter by PAX8 was analyzed in HCT116 ($p53^{+/+}$) or HCT116 ($p53^{-/-}$) isogenic cell lines 48 h after transfection, as in Fig. 2.

Downloaded from cancerres.aacrjournals.org on May 28, 2017. © 2008 American Association for Cancer Research.

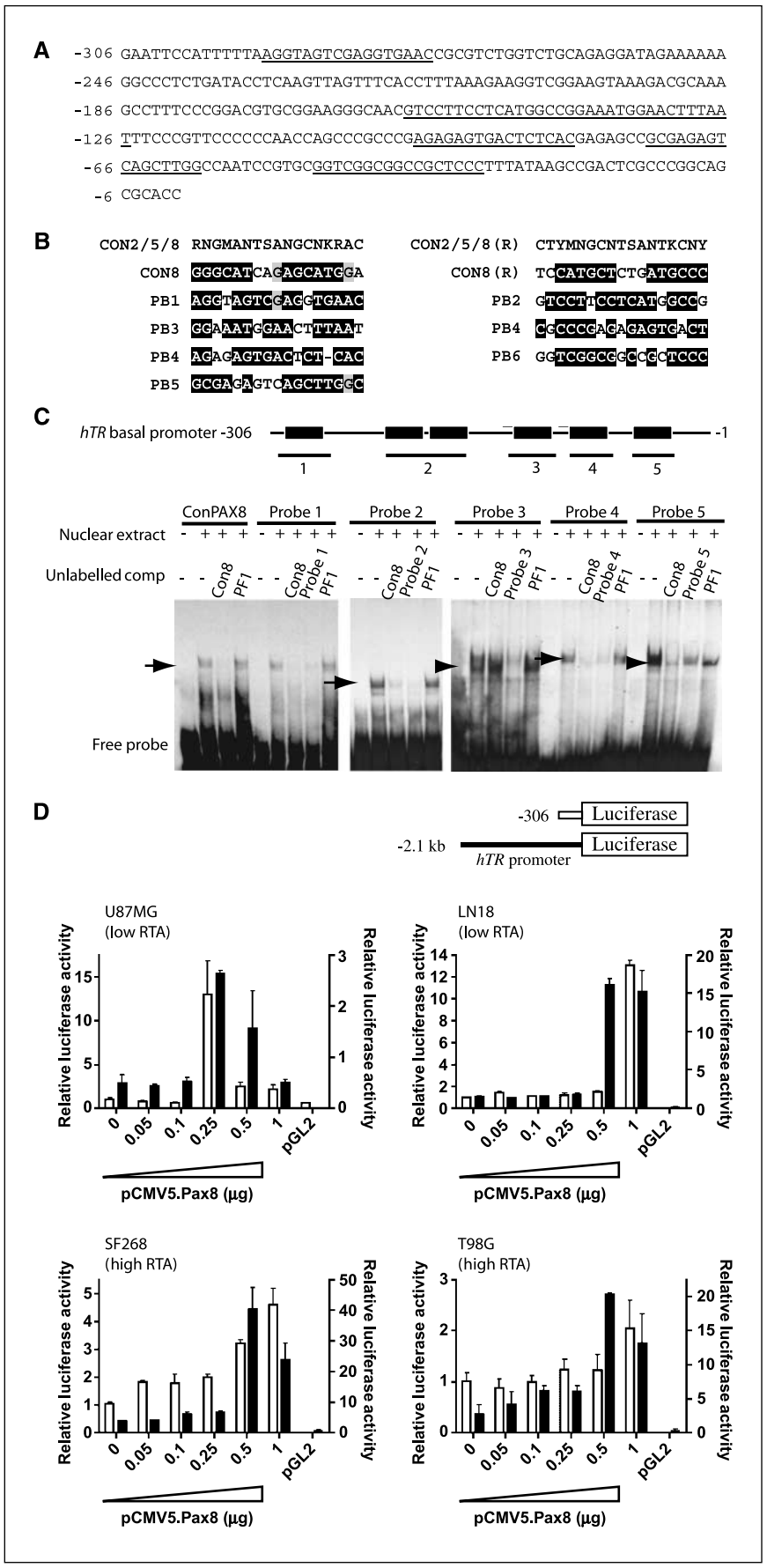

Figure 4. *hTR* is a target of the PAX transcription factor family. *A,* potential PAX2/5/8 binding sites in the minimal promoter region of the *hTR* promoter. Potential PAX2/5/8 binding sites from position −306 to the transcriptional start site (*underlined*). *B,* alignment of the PAX2, PAX5, and PAX8 potential binding sites (*PB1–PB6*) in the *hTR* promoter with the PAX2/5/8 consensus binding sequence and the PAX8 consensus binding sequence. *C,* EMSA was used to analyze the binding of PAX8 to digoxigenin-labeled oligonucleotide probes containing predicted PAX binding sites found in the *hTR* promoter. *Arrows,* PAX8 binding; *arrowheads,* nonspecific binding. *D,* transactivation of the *hTR* promoter by overexpression of PAX8 in glioma cell lines. Relative luciferase activities were normalized to the luciferase value of the short promoter (−306) of *hTR* in each cell line (*columns,* mean; *bars,* SE). *Left Y-axis,* the transactivation level of the short *hTR* promoter (*white columns*); *right Y-axis,* the transactivation level of the long *hTR* promoter (*black columns*; refer to details in Fig. 2).

Downloaded from cancerres.aacrjournals.org on May 28, 2017. © 2008 American Association for Cancer Research.

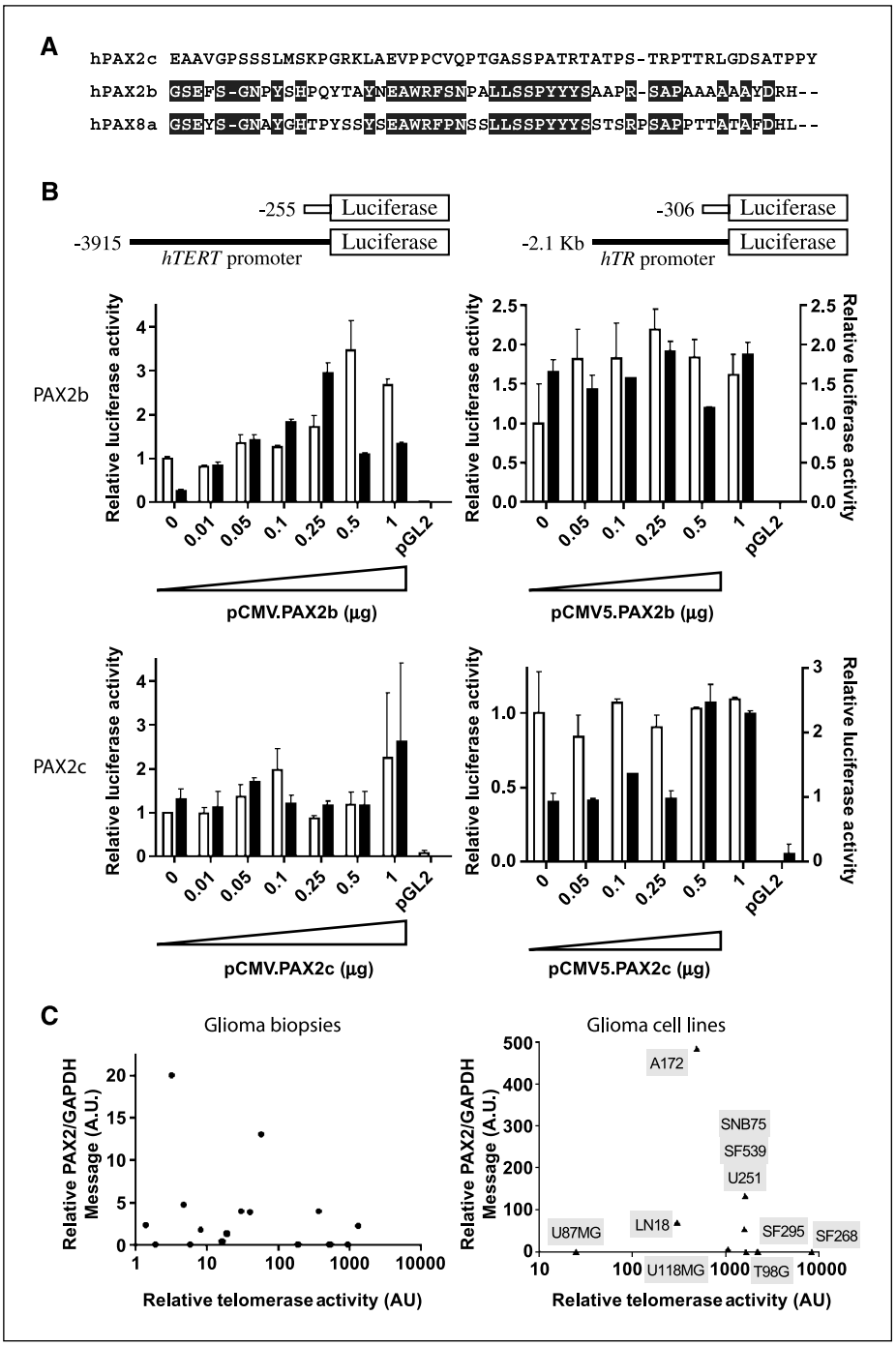

Figure 5. The effect of PAX2b or PAX2c overexpression on *hTERT* and *hTR* promoters in U87MG cell lines. *A,* alignment of the COOH-terminal amino acid sequences of PAX2b and PAX2c with PAX8. *Highlighted regions,* similarities between PAX2b and PAX8. *B,* the effect of PAX2b and PAX2c on *hTERT* and *hTR* promoters was analyzed by a luciferase assay. pGL2-Ctrl plasmids were transfected for comparison. Relative luciferase activities were normalized to the luciferase value of the short promoter of *hTERT* or *hTR* in each cell line (*columns,* mean; *bars,* SE). For the *hTR* promoters, the transactivation level of the short *hTR* promoter (*left Y-axis*), and the transactivation level of the long *hTR* promoter (*right Y-axis*). *C,* correlation of relative *PAX2* mRNA level with RTA in glioma tumor biopsies and established glioma cell lines. The names of the established cell lines are designated. *AU,* arbitrary unit (log scale).

the signal (Fig. 4*C*). These results suggest that PAX8 could regulate the *hTR* promoter.

Overexpression of PAX8 activates the *hTR* promoter. We next tested whether PAX8 is able to activate the *hTR* promoter using two *hTR* luciferase reporter constructs. One luciferase reporter contains a 306-bp fragment of the *hTR* promoter, and the other construct contains a 2.1 kb *hTR* promoter fragment (Fig. 4*D*). Luciferase assays showed that PAX8 activated both of the *hTR* promoters in glioma cell lines (Fig. 4*D*). In most cases, PAX8 had a stronger activation effect on the long-*hTR* promoter (−2.1 kb) than on the short-*hTR* promoter (−306 bp), which suggests there are possible PAX8-responsive elements in the long-*hTR* promoter (−2.1 kb) as well (Fig. 4*D*). The activation of the short *hTR* promoter was higher in cells with low RTA at ~15-fold compared with those with high RTA at ~5-fold (Fig. 4*D*).

Effect of PAX2 isoforms on the *hTERT* and *hTR* promoters. PAX proteins of the same subgroup often recognize similar binding sites (17). It is not known whether PAX2 carries out the same function as PAX8 in glioma cells. Several alternatively spliced forms of *PAX* genes have been reported (23, 37, 38). Alternatively spliced *PAX* transcripts with the same DNA binding domain, but different COOH-terminals, are able to recognize identical binding sites in DNA, albeit with different transactivation capabilities (23). Hence, we also tested whether different isoforms of PAX2 act on the *hTERT*

Downloaded from cancerres.aacrjournals.org on May 28, 2017. © 2008 American Association for Cancer Research.

and the *hTR* promoters differently. Two of the common isoforms were chosen: PAX2b and PAX2c. *PAX2b* is different from *PAX2c* in that it does not contain exon 10 and thus has a COOH-terminal similar to *PAX8* (Fig. 5*A*). Promoter-reporter results show (Fig. 5*A* and *B*) that PAX2b can transactivate the *hTERT* promoters 3-fold to 6-fold with increasing amounts of transfected plasmids, but had no effect on *hTR*. In contrast, PAX2c had relatively little effect on either promoter in two different glioma cells (Fig. 5*B*; data not shown). Thus, it seems unlikely that PAX2 regulates telomerase. In agreement with this, *PAX2* mRNA expression and the RTA levels showed no correlation in glioma biopsies and glioma cell lines (Fig. 5*C*).

PAX8 increases the mRNA of *hTERT* and *hTR*, as well as telomerase activity. To assess whether PAX8 also increases *hTERT* and *hTR* mRNA, we quantified the mRNA level of *hTERT* and *hTR* using real-time PCR after cells were transfected with 0.1 to 0.5 μg of *pCMV5.PAX8* for 2 days. Two-fold to 4-fold increases in *hTERT* and *hTR* mRNA expression were observed in LN18, SF268, and U87MG cells (Fig. 6*A*). A low activation was also observed in A172 cells, but was not included because of low transfection efficiency (data not shown).

To determine the effect of endogenous *PAX8* and *PAX2* on *hTR* and *hTERT*, T98G cells were transfected with either *PAX8* or *PAX2* siRNA and the mRNA levels of *hTERT* and *hTR* were determined by real-time PCR 24 hours posttransfection. Results show that *PAX8* silencing reduces both the *hTR* and *hTERT* expression levels by two-thirds compared with the control (Fig. 6*B*). Whereas *PAX2* silencing had no effect on *hTR* mRNA levels with a small but insignificant reduction in *hTERT* mRNA levels ($P = 0.5$). These results are consistent with the previous overexpression results showing that PAX8 can transactivate both *hTR* and *hTERT* promoters, yet PAX2 has a minimal effect on either promoter. Similar results were obtained in A172 and SNB75 cells (data not shown).

We next measured RTA after transfection of cells with increasing amounts of *pCMV5.PAX8*. Results show that PAX8 increased RTA by up to 50% ($P < 0.001$) with 0.1 μg of *pCMV5.PAX8* (Fig. 6*C*), but higher amounts of *pCMV5.PAX8* reduced RTA ($P < 0.001$; Fig. 6*C*). Collectively, these data provide support for the hypothesis that PAX8 is capable of regulating telomerase in glioma cells.

Discussion

In the present study, we have found a correlation between *PAX8* mRNA expression and RTA in glioma biopsies and cell lines (Fig. 1). Both *hTERT* and *hTR* have potential PAX2/5/8 binding sites in their promoters, which were validated by EMSA (Figs. 2*C* and 4*C*). We also showed that PAX8 transactivates both the *hTERT* and *hTR* promoters (Figs. 2*D* and 4*D*), increases *hTERT* and *hTR* mRNA, and increases RTA (Fig. 6*A* and *C*). Specific knockdown of *PAX8* also reduces *hTERT* and *hTR* mRNA (Fig. 6*B*). Thus, PAX8 may be an important regulator of telomerase in some gliomas.

The correlation between *PAX8* mRNA levels and RTA was only found in glioma specimens and in established glioma cell lines that had low to moderate RTA (Fig. 1). Furthermore, transactivation of the *hTERT* and the *hTR* promoters by PAX8 was much more efficient in cells with low RTA (Fig. 2*D*). Such data suggest that, in these gliomas, RTA is more sensitive to PAX8 levels than in others. By contrast, in gliomas with high RTA, there must be other factors controlling the activity. The logarithmic nature of the relationship between *PAX8* mRNA level and RTA is consistent with other factors contributing to telomerase activity (Fig. 1*A*).

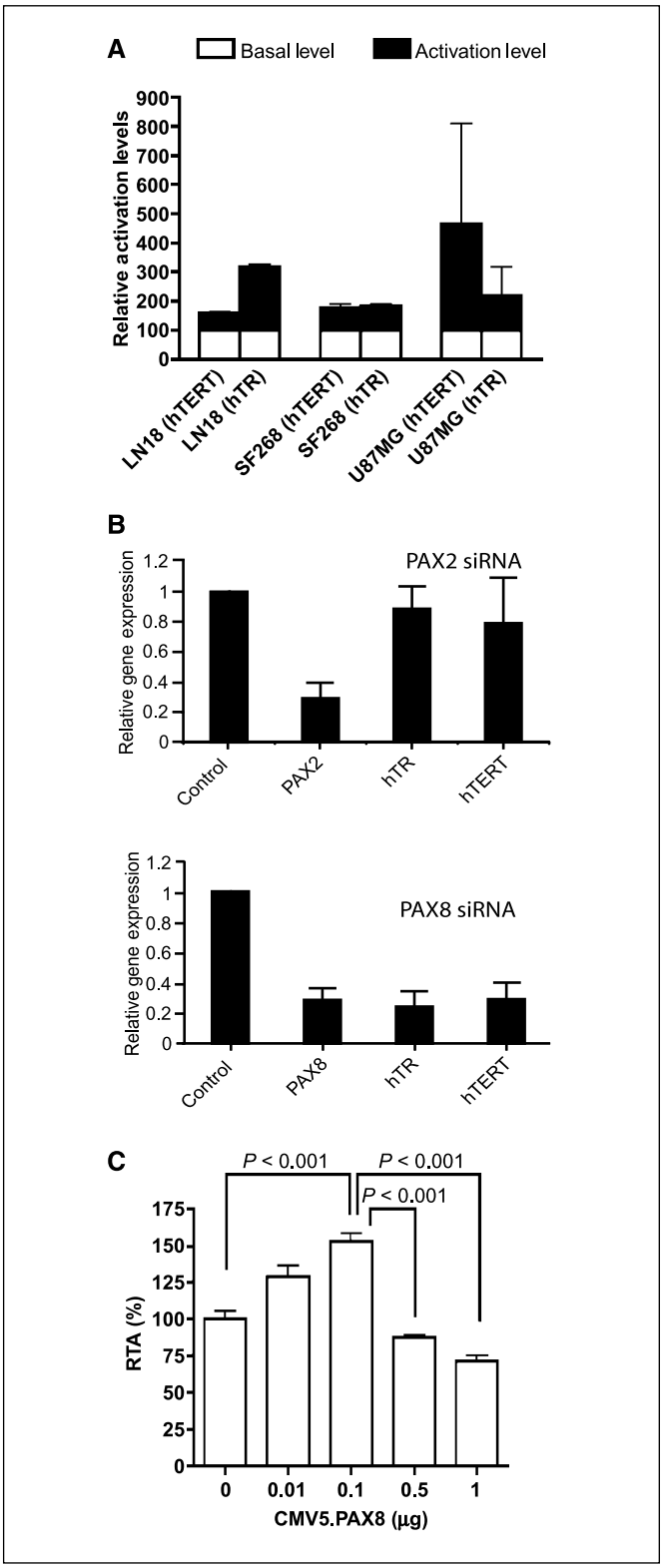

Figure 6. PAX8 increases *hTERT* and *hTR* mRNA and telomerase activity in glioma cells. *A,* mRNA levels of *hTERT* and *hTR* in U87MG cells transfected with 0.1 to 0.5 μg of *pCMV5.PAX8* for 48 h. *B,* relative expression of *hTR* and *hTERT* in T98G cells transfected with 10 nmol/L of *PAX8* or *PAX2* siRNA 24 h posttransfection. mRNA levels were determined by real-time PCR and normalized to the housekeeping gene *B2M* (*control*), expression levels are expressed relative to control transfections ± SE. *C,* RTA of U87MG cells transfected with different amounts of *PAX8* expression vector were measured. *Columns,* mean; *bars,* SE.

Downloaded from cancerres.aacrjournals.org on May 28, 2017. © 2008 American Association for Cancer Research.

Other factors such as transcriptional cofactors and/or chromatin might constrain the effect of PAX8 on RTA, such that very high expression of PAX8 is not able to further increase RTA and even possibly decrease RTA. Results in Figs. 2*D* and 4*D* show that high expression of transfected *PAX8* reduces the level of hTR and hTERT transactivation, and similarly, high levels of *PAX8* decrease RTA (Fig. 6*C*). This could be explained by a limiting cofactor which induces squelching, such that excessive PAX8 reduces the availability of this cofactor to form complete transcription complexes. That other factors are important is evident by the fact that PAX8 failed to activate the *hTERT* promoter in a telomerase-negative primary cell line[5] and in two alternative lengthening of telomere–positive, PAX8-positive fibroblast cell lines (39).[5] Thus, PAX8 is not the only barrier for telomerase activation. In addition, epigenetic modifications, such as methylation, have also been shown to be critical in the control of telomerase activity (33, 40, 41).

Many reports have shown that *PAX* genes have tumor-promoting roles (19), including stimulating cell proliferation, having antiapoptotic function, and inhibiting p53 expression (20, 42, 43).[6] At the same time, accumulating evidence suggests that telomerase has roles in addition to telomere maintenance, such as in the activation of the glycolytic pathway, cell proliferation, and inhibiting apoptosis (44–46). We have shown that PAX8 is able to activate *hTERT* and telomerase activity. Therefore, the antiapoptotic function of *PAX* genes, in addition to activation of the *Bcl-2* gene (47), might also partly act through activating TERT and its antiapoptotic function. Thus, PAX8 and hTERT expression in gliomas might not only facilitate the activation of telomerase activity, but might also increase the survival function for gliomas.

Significance. The results show for the first time that the oncofetal protein, PAX8, has a novel function in telomerase activation in glioma and colorectal cancer cell lines. PAX8 coordinately regulates both *hTERT* and *hTR*. The ability of *PAX* genes to activate telomerase may be specific to only certain *PAX* genes, as it has not been observed with any other *PAX* genes tested thus far (48–50). The activation of *hTERT* expression in glioma cells by PAX8 might explain why patients with telomerase-positive gliomas have a poor prognosis (21) as both PAX8[6] and hTERT confer survival advantages for cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 1/11/2008; revised 4/11/2008; accepted 4/23/2008.

Grant support: Cancer Society of New Zealand and Health Research Council of New Zealand (J.A. Royds); Health Research Council of New Zealand and a program grant from the Cancer Institute New South Wales (A.W. Braithwaite); Health Research Council of New Zealand and the New Zealand Institute for Cancer Research Trust (M.R. Eccles); a program grant from the Cancer Council New South Wales and a fellowship from the National Health and Medical Research Council of Australia (R.R. Reddel); and University of Otago Prestigious Scholarship and University of Otago Postgraduate Publishing Bursary (Y-J. Chen).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank the patients, clinicians, and the Christchurch Tissue Bank, who were involved in the donation of tissue. We also thank Dr. I. Horikawa and Prof. J. Carl Barrett (Center for Cancer Research, National Cancer Institute, Bethesda, Maryland) for providing *hTERT* promoter-luciferase constructs, Prof. Roberto Di Lauro (School of Medicine, University of Naples, Naples, Italy) for *pCMV5.PAX8* expression vector and PAX8 antibody, Associate Prof. Peter Herbison for help with statistical analysis, and Lisa Gallagher for excellent technical help.

[5] Unpublished data.
[6] Y-J. Chen, unpublished results.

References

1. Blackburn EH. Structure and function of telomeres. Nature 1991;350:569–73.
2. Levy MZ, Allsopp RC, Futcher AB, Greider CW, Harley CB. Telomere end-replication problem and cell aging. J Mol Biol 1992;225:951–60.
3. Saretzki G, Sitte N, Merkel U, Wurm RE, von Zglinicki T. Telomere shortening triggers a p53-dependent cell cycle arrest via accumulation of G-rich single stranded DNA fragments. Oncogene 1999;18:5148–58.
4. Artandi SE, Chang S, Lee SL, et al. Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature 2000;406:641–5.
5. Colgin LM, Reddel RR. Telomere maintenance mechanisms and cellular immortalization. Curr Opin Genet Dev 1999;9:97–103.
6. Bryan TM, Cech TR. Telomerase and the maintenance of chromosome ends. Curr Opin Cell Biol 1999; 11:318–24.
7. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines [see comment]. Nat Med 1997;3: 1271–4.
8. Cohen SB, Graham ME, Lovrecz GO, Bache N, Robinson PJ, Reddel RR. Protein composition of catalytically active human telomerase from immortal cells. Science 2007;315:1850–3.
9. Bodnar AG, Ouellette M, Frolkis M, et al. Extension of life-span by introduction of telomerase into normal human cells. Science 1998;279:349–52.
10. Wong JM, Collins K. Telomerase RNA level limits telomere maintenance in X-linked dyskeratosis congenita. Genes Dev 2006;20:2848–58.
11. Cristofari G, Lingner J. Telomere length homeostasis requires that telomerase levels are limiting. EMBO J 2006;25:565–74.
12. Weilbaecher RG, Lundblad V. Assembly and regulation of telomerase. Curr Opin Chem Biol 1999;3:573–7.
13. Zhang A, Zheng C, Lindvall C, et al. Frequent amplification of the telomerase reverse transcriptase gene in human tumors. Cancer Res 2000;60:6230–5.
14. Wick M, Zubov D, Hagen G. Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT). Gene 1999;232:97–106.
15. Lin SY, Elledge SJ. Multiple tumor suppressor pathways negatively regulate telomerase. Cell 2003; 113:881–9.
16. Holt SE, Wright WE, Shay JW. Multiple pathways for the regulation of telomerase activity. Eur J Cancer 1997; 33:761–6.
17. Chi N, Epstein JA. Getting your Pax straight: Pax proteins in development and disease. Trends Genet 2002;18:41–7.
18. Schwarz M, Alvarez-Bolado G, Dressler G, Urbanek P, Busslinger M, Gruss P. Pax2/5 and Pax6 subdivide the early neural tube into three domains. Mech Dev 1999;82: 29–39.
19. Robson EJ, He SJ, Eccles MR. A PANorama of PAX genes in cancer and development. Nat Rev Cancer 2006; 6:52–62.
20. Muratovska A, Zhou C, He S, Goodyer P, Eccles MR. Paired-Box genes are frequently expressed in cancer and often required for cancer cell survival. Oncogene 2003; 22:7989–97.
21. Hakin-Smith V, Jellinek DA, Levy D, et al. Alternative lengthening of telomeres and survival in patients with glioblastoma multiforme. Lancet 2003;361:836–8.
22. Lenhard B, Sandelin A, Mendoza L, Engstrom P, Jareborg N, Wasserman WW. Identification of conserved regulatory elements by comparative genome analysis. J Biol 2003;2:13.
23. McConnell MJ, Cunliffe HE, Chua LJ, Ward TA, Eccles MR. Differential regulation of the human Wilms tumour suppressor gene (WT1) promoter by two isoforms of PAX2. Oncogene 1997;14:2689–700.
24. Rossi DL, Acebron A, Santisteban P. Function of the homeo and paired domain proteins TTF-1 and Pax-8 in thyroid cell proliferation. J Biol Chem 1995;270:23139–42.
25. Horikawa I, Cable PL, Mazur SJ, Appella E, Afshari CA, Barrett JC. Downstream E-box-mediated regulation of the human telomerase reverse transcriptase (hTERT) gene transcription: evidence for an endogenous mechanism of transcriptional repression. Mol Biol Cell 2002; 13:2585–97.
26. Royds JA, Hibma M, Dix BR, et al. p53 promotes adenoviral replication and increases late viral gene expression. Oncogene 2006;25:1509–20.
27. Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 1989;17:6419.
28. Horikawa I, Cable PL, Afshari C, Barrett JC. Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. Cancer Res 1999; 59:826–30.
29. Li H, Xu D, Li J, Berndt MC, Liu JP. Transforming growth factor β suppresses human telomerase reverse transcriptase (hTERT) by Smad3 interactions with c-Myc and the hTERT gene. J Biol Chem 2006;281:25588–600.
30. Costamagna E, Garcia B, Santisteban P. The functional interaction between the paired domain transcription factor Pax8 and Smad3 is involved in

Downloaded from cancerres.aacrjournals.org on May 28, 2017. © 2008 American Association for Cancer Research.

transforming growth factor-β repression of the sodium/iodide symporter gene. J Biol Chem 2004;279:3439–46.
31. Ginsberg D, Oren M, Yaniv M, Piette J. Protein-binding elements in the promoter region of the mouse p53 gene. Oncogene 1990;5:1285–90.
32. Shats I, Milyavsky M, Tang X, et al. p53-dependent down-regulation of telomerase is mediated by p21waf1. J Biol Chem 2004;279:50976–85.
33. Xu D, Wang Q, Gruber A, et al. Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells. Oncogene 2000;19:5123–33.
34. Maulbecker CC, Gruss P. The oncogenic potential of Pax genes. EMBO J 1993;12:2361–7.
35. Bunz F, Dutriaux A, Lengauer C, et al. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 1998;282:1497–501.
36. Zhao JQ, Hoare SF, McFarlane R, et al. Cloning and characterization of human and mouse telomerase RNA gene promoter sequences. Oncogene 1998;16:1345–50.
37. Ward TA, Nebel A, Reeve AE, Eccles MR. Alternative messenger RNA forms and open reading frames within an additional conserved region of the human PAX-2 gene. Cell Growth Differ 1994;5:1015–21.
38. Kozmik Z, Czerny T, Busslinger M. Alternatively spliced insertions in the paired domain restrict the DNA sequence specificity of Pax6 and Pax8. EMBO J 1997;16:6793–803.
39. Henson JD, Neumann AA, Yeager TR, Reddel RR. Alternative lengthening of telomeres in mammalian cells. Oncogene 2002;21:598–610.
40. Atkinson SP, Hoare SF, Glasspool RM, Keith WN. Lack of telomerase gene expression in alternative lengthening of telomere cells is associated with chromatin remodeling of the hTR and hTERT gene promoters. Cancer Res 2005;65:7585–90.
41. Colgin LM, Wilkinson C, Englezou A, Kilian A, Robinson MO, Reddel RR. The hTERTα splice variant is a dominant negative inhibitor of telomerase activity. Neoplasia 2000;2:426–32.
42. Bernasconi M, Remppis A, Fredericks WJ, Rauscher FJ III, Schafer BW. Induction of apoptosis in rhabdomyosarcoma cells through down-regulation of PAX proteins. Proc Natl Acad Sci U S A 1996;93:13164–9.
43. Stuart ET, Haffner R, Oren M, Gruss P. Loss of p53 function through PAX-mediated transcriptional repression. EMBO J 1995;14:5638–45.
44. Cao Y, Li H, Deb S, Liu JP. TERT regulates cell survival independent of telomerase enzymatic activity. Oncogene 2002;21:3130–8.
45. Sarin KY, Cheung P, Gilison D, et al. Conditional telomerase induction causes proliferation of hair follicle stem cells. Nature 2005;436:1048–52.
46. Bagheri S, Nosrati M, Li S, et al. Genes and pathways downstream of telomerase in melanoma metastasis. Proc Natl Acad Sci U S A 2006;103:11306–11.
47. Hewitt SM, Hamada S, Monarres A, Kottical LV, Saunders GF, McDonnell TJ. Transcriptional activation of the bcl-2 apoptosis suppressor gene by the paired box transcription factor PAX8. Anticancer Res 1997;17:3211–5.
48. Brun T, Franklin I, St-Onge L, et al. The diabetes-linked transcription factor PAX4 promotes {β}-cell proliferation and survival in rat and human islets. J Cell Biol 2004;167:1123–35.
49. Schaniel C, Gottar M, Roosnek E, Melchers F, Rolink AG. Extensive *in vivo* self-renewal, long-term reconstitution capacity, and hematopoietic multipotency of Pax5-deficient precursor B-cell clones. Blood 2002;99:2760–6.
50. Tang LS, Wlodarczyk BJ, Santillano DR, Miranda RC, Finnell RH. Developmental consequences of abnormal folate transport during murine heart morphogenesis. Birth Defects Res A Clin Mol Teratol 2004;70:449–58.

Downloaded from cancerres.aacrjournals.org on May 28, 2017. © 2008 American Association for Cancer Research.

AACR American Association for Cancer Research

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

PAX8 Regulates Telomerase Reverse Transcriptase and Telomerase RNA Component in Glioma

Yu-Jen Chen, Hamish G. Campbell, Anna K. Wiles, et al.

Cancer Res 2008;68:5724-5732.

Updated version	Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/68/14/5724
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2008/07/09/68.14.5724.DC1

Cited articles	This article cites 50 articles, 19 of which you can access for free at: http://cancerres.aacrjournals.org/content/68/14/5724.full.html#ref-list-1
Citing articles	This article has been cited by 1 HighWire-hosted articles. Access the articles at: /content/68/14/5724.full.html#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

Downloaded from cancerres.aacrjournals.org on May 28, 2017. © 2008 American Association for Cancer Research.