Title: PAX5 activates the transcription of the human telomerase reverse transcriptase gene in B cells.
Authors: Bougel S, Renaud S, Braunschweig R, Loukinov D, Morse HC 3rd, Bosman FT, Lobanenkov V, Benhattar J
Journal: The Journal of pathology
Year: 2010 Jan
Volume: 220
Issue: 1
Pages: 87-96
DOI: 10.1002/path.2620

Published in final edited form as:
J Pathol. 2010 January ; 220(1): 87–96. doi:10.1002/path.2620.

PAX5 activates the transcription of the human telomerase reverse transcriptase gene in B cells

Stéphanie Bougel[1], **Stéphanie Renaud**[2], **Richard Braunschweig**[1], **Dmitri Loukinov**[2], **Herbert C Morse III**[2], **Fred T. Bosman**[1], **Victor Lobanenkov**[2], and **Jean Benhattar**[1,*]

[1]Institute of Pathology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, CH-1011 Lausanne, Switzerland [2]Laboratory of Immunopathology, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health, Rockville, MD 20852, USA

Abstract

Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA. Its activity is not detectable in most somatic cells but it is reactivated during tumorigenesis. In most cancers, the combination of *hTERT* hypermethylation and hypomethylation of a short promoter region is permissive for low-level *hTERT* transcription. Activated and malignant lymphocytes express high telomerase activity, through a mechanism that seems methylationin-dependent. The aim of this study was to determine which mechanism is involved in the enhanced expression of *hTERT* in lymphoid cells. Our data confirm that in B cells, some T cell lymphomas and non-neoplastic lymph nodes, the *hTERT* promoter is unmethylated. Binding sites for the B cell-specific transcription factor PAX5 were identified downstream of the ATG translational start site through EMSA and ChIP experiments. ChIP assays indicated that the transcriptional activation of *hTERT* by PAX5 does not involve repression of CTCF binding. In a B cell lymphoma cell line, siRNA-induced knockdown of PAX5 expression repressed *hTERT* transcription. Moreover, ectopic expression of PAX5 in a telomerase-negative normal fibroblast cell line was found to be sufficient to activate *hTERT* expression. These data show that activation of *hTERT* in telomerase-positive B cells is due to a methylation-independent mechanism in which PAX5 plays an important role.

Keywords

hTERT; PAX5; B cells; chromatin immunoprecipitation; CTCF; telomerase; DNA methylation

Introduction

The telomerase enzyme allows germ cells, stems cells and cancer cells to divide indefinitely [1]. Human telomerase possesses a highly regulated subunit called telomerase reverse transcriptase (hTERT), which is the limiting factor for its activity [2,3]. The hTERT

*Correspondence to: Jean Benhattar, Institute of Pathology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Bugnon, 25, CH-1011 Lausanne, Switzerland. Jean.Benhattar@chuv.ch.

No conflicts of interest were declared.

SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Table S1. Primer sequences for RT–PCR

Table S2. Primer sequences for ChIP experiments

Table S3. Primer sequences for quantitative RT–PCR

expression is nearly imperceptible in the majority of differentiated somatic cells, which lead to inevitable telomeric attrition and subsequently cellular senescence. High levels of hTERT are detected in proliferative somatic cells such as endometrial tissues or activated lymphocytes, but also in most immortalized and cancer cells.

hTERT transcription has been shown to be influenced by numerous activators and inhibitors, such as c-Myc, Sp1, Hif-1, Mbi-1, USF1/2, oestrogen response element, p53, Mad1, myeloid-specific zinc finger protein 2 (MZF-2), TGFβ, Wilms' tumour 1 (WT1) and CTCF [4–6]. In addition, a possible role of DNA methylation in *hTERT* transcription regulation can be expected, as the *hTERT* promoter is situated within a dense CG-rich CpG island. In normal somatic cells the *hTERT* promoter is unmethylated, although the transcription of the gene is repressed. However, in most cancer cells, hypermethylation of this region correlates with expression of the gene and with perceptible telomerase activity [7–10]. This apparent contradiction with the classical mechanism of transcriptional repression by DNA methylation was recently clarified. DNA methylation exhibits a dual role in hTERT transcriptional regulation by interfering with the binding of inhibitors, such as the CTCF transcription factor, and by partial hypomethylation of the core promoter, which allows the *hTERT* gene to be permissive for transcription [11]. Furthermore, the absence of methylation in association with active chromatin marks around the transcription start site of *hTERT* indicates that expression and DNA methylation patterns of the *hTERT* promoter are not in contradiction to the general model of gene silencing mediated by DNA methylation [12].

In a small subset of telomerase-positive tumours, *hTERT* expression appears to be regulated by a methylation-independent mechanism [12–14]. For example, the *hTERT* promoter is methylated in only 30% of ovarian cancers, almost all of which are telomerase-positive [13]. Cells of the lymphoid system also seem to escape methylation-dependent mechanism of *hTERT* regulation. Leukaemias and lymphomas, including B cell chronic lymphocytic leukaemia (CLL), express high levels of telomerase but exhibit low levels of *hTERT* promoter methylation [14]. More recently, acute myeloid leukaemia (HL-60) and Burkitt's lymphoma (Raji) cell lines, as well as normal lymphocytes, were found to have hypomethylated *hTERT* promoters [12].

B cells exhibit notably longer telomeres than any other blood cell population, such as T cells, natural killer cells and monocytes [15]. B cells in the germinal centre of tonsils have longer telomeres compared to naïve and memory B cells [16]. As expected, low levels of telomerase activity are observed in naïve and memory B cells, in contrast to germinal centre B cells that exhibit high telomerase activity [16,17].

Paired box (PAX) proteins include nine members that are important regulators in early development for tissue specificity [18]. Once bound to DNA, PAX proteins can play the role of transcriptional activators or repressors [19–21]. Deregulation of *PAX* genes has been associated with a variety of cancers, including astrocytoma, medulloblastoma, lymphoma and Wilm's tumour [22,23]. Moreover, PAX expression has been suggested to be essential for survival of cancer cells. Recently, PAX8 has been implicated in the activation of *hTERT* and *hTR* promoters, which in turn activate telomerase in glioma [24]. PAX2, PAX5 and PAX8 belong to the same subgroup and thus could impact on *hTERT* regulation in a tissue-specific manner. During B cell development, the *PAX5* gene is expressed in early B cell precursors (pro-B cells) and continues to be expressed up to mature B cells, but not in terminally differentiated plasma cells [25,26]. As a consequence, PAX5 expression is used as a lineage-specific marker in B cells neoplasms [27,28]. PAX5 has been shown to promote the expression of target genes encoding crucial components of the (pre)BCR signalling cascade, such as the receptor signalling chain Igα, also called CD79a and mb-1 [29,30], the

costimulatory receptor CD19 [21,31] and the central adaptor protein BLNK [32]. PAX5 also facilitates the V_H–DJ_H recombination step and can activate other transcription factor genes [33].

Our working hypothesis for the experiments reported in this paper was that hTERT regulation in B cells is methylation-independent. To confirm our hypothesis, methylation status of the hTERT promoter was investigated in normal and malignant lymphoid tissues. Then, the B cell-specific factor PAX5 was considered for its participation in the induction of hTERT expression in telomerase-positive B cells.

Experimental procedures

Cell culture

The Burkitt's lymphoma cell lines Daudi and Ramos and the pre-B cell leukaemia line Nalm6 were kindly provided by Dr Benedicte Baisse (CHUV, Lausanne, Switzerland). The Burkitt line, Raji, was kindly provided by Apoxis (Lausanne, Switzerland). HeLa (cervical adenocarcinoma), PC-3 (prostate adenocarcinoma) and BJ (normal fibroblasts) cells were obtained from ATCC (Manassas, VA, USA). Cell lines were cultured in the medium recommended by ATCC.

Tissue samples

The lymphomas and normal tissues came from the files of the Institute of Pathology of Lausanne. The samples included: four histologically non-neoplastic lymphoid tissues (two lymph node biopsies and two tonsil biopsies); six B cell non-Hodgkin lymphomas (NHLs), comprising three high-grade and three low-grade NHLs; and six T cell NHLs. All diagnoses were confirmed by a pathologist (RB). The lymphoma cases were selected to ensure that sufficient populations of T or B cells were present. The use of human tissues for this study was done according to the guidelines of the local ethics committee.

RT–PCR

Total RNA of frozen tissues and cultured cells was extracted using Trizol-LS (Invitrogen, Basel, Switzerland) according to the manufacturer's protocol. The extraction protocol for fixed tissues was described previously [34]. RT–PCRs were performed using Super- Script One-Step RT–PCR or Quantitative RT–PCR ThermoScript™ One-Step System (Invitrogen) (for primers and RT–PCR for each individual gene, see Supporting information, Table S1).

DNA methylation analysis

DNA was extracted from frozen and fixed tissues and cultured cells using the DNeasy tissue kit (Qiagen). DNA (2 μg) was modified with sodium bisulphite and used to amplify a 224 bp fragment of the *hTERT* promoter, as previously described [35]. PCR products were analysed by a methylation-sensitive dotblot assay (MS-DBA) [35] and confirmed by direct sequencing [9] and methylation-sensitive single-strand conformation analysis (MS-SSCA) [36,37].

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides of the *hTERT* exonic region and the *CD79A* promoter region (hTERT, sense, 5′-GCTGGTGCAGCGCGGGGACCCGGCGGCTTT-3′; CD79A, sense 5′-AGCGAGGGCCACTGGAGCCCATCTCCGGGG-3′) were labelled with the DIG-Oligonucleotide 3′-End Labelling Kit (Roche). Gel shift reactions were performed using the DIG Gel Shift Kit (Roche) with 0.5 pmol DIG-labelled oligonucleotide and 5 μg Nalm6 or

Raji cell extracts. A supershift assay was performed with a PAX5 rabbit antibody (Active Motif, Carlsbad, USA) on Raji cell extracts, according to the manufacturer's protocol.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using EZ ChIP (Upstate Biotechnology, Lake Placid, NY, USA), following the manufacturer's instructions with some modifications. After crosslinking with 1% formaldehyde and sonication to shear DNA, lysates from 2×10^6 cells (PAX5) or 4×10^6 cells (CTCF) were diluted in ChIP dilution buffer for immunoprecipitation or stored at 4 °C to be directly uncrosslinked and purified (DNA input fraction). Magnetic beads (40 µl, Dynabeads Protein G, Invitrogen) were incubated for 1 h at room temperature in 60 µl of the blocking solution with either 2 µg goat polyclonal anti-PAX5 antibody (Santa Cruz, CA, USA), 10 µg mouse polyclonal anti-CTCF antibody (Rockville, MD, USA) or without antibody. After washing and incubation overnight at 4 °C with the chromatin solution, the beads were washed twice with the following solutions: low-salt, high-salt, LiCl and finally Tris-EDTA (TE). The eluate was then resuspended in 200 µl 5% Chelex solution and incubated for 10 min at 100 °C to reverse the protein–DNA crosslinks. After purification, the immunoprecipitated DNA was analysed by quantitative real-time PCR with specific primers (see Supporting information, Table S2). The human CTCF-binding site N, a *MYC* insulator site (MYC-N), and H19 were used as positive controls and a CTCF non-binding site, G of *MYC* (MYC-G) was used as a negative control [38,39]. For chromatin immunoprecipitation of PAX5, *CD19*, which is a well-known target of PAX5, was used as a positive control, whereas *KRAS*, which does not contain PAX5 binding sites, was used as a negative control.

Immunohistochemistry (IHC)

Antigen retrieval was performed using a pressure cooker for 2 min in 10 mM sodium citrate buffer, pH 6. The slides were incubated overnight at 4 °C with the anti-TERT antibody (1 : 50, EST21-A; Alpha Diagnostic International, San Antonio, TX, USA), the anti-PAX5 monoclonal antibody (1 : 50, BD Biosciences Pharmingen, San Jose, CA, USA) or the CD3 monoclonal antibody (1 : 1, Novocastra, Newcastle upon Tyne, UK). After washing, the EnVision$^+$ System – HRP AEC (TERT) or the EnVision$^+$ Peroxidase rabbit followed by DAB staining (PAX5 and CD3) were used according to the manufacturer's instructions (Dako, Glostrup, Denmark). The slides were then counterstained with haematoxylin.

Transient transfection

Jet PEI transfection reagent (2 µl) (Polyplus-transfection, Illkirch, France) was used to transfect, in 10^5 normal fibroblast BJ cells, 2 µg PAX5 expression plasmid (phPAX5, a kind gift from Professor M Busslinger, Research Institute of Molecular Pathology, Vienna, Austria) [40]. Cells treated the same way but without plasmid were used as a transfection control. Dnase extraction and total RNA extraction were performed 48 h after transfection.

Transfection of siRNA

A double-stranded annealed Stealth RNAi oligonucleotide targeting *PAX5* was designed by Invitrogen software (sense, 5′-GAGGAUAGUGGAACUUGCUCAUCAA-3′). A non-specific fluorescent siRNA (Invitrogen) was used as a control. Transfection of 130 pM siRNA oligonucleotides in 4×10^6 Raji cells was performed with Amaxa Nucleofector (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's protocol. The efficiency of RNA silencing was checked by western blot with PAX5 antibody (BD Biosciences, Erembodegem, Belgium) and confirmed by quantitative RT–PCR.

Quantitative RT–PCRs were performed on a Rotorgene 6000 cycler (Corbett Research, Sydney, Australia). *hTERT* and *PAX5* mRNAs were amplified using the Quantitative RT–PCR Thermoscript One-Step System (Invitrogen) (the primers and probes are described in the Supporting information, Table S3). *CD19* and β-*actin* were amplified by the same enzymes, but with 1.25 μM SYTO 9 fluorescent dye (Invitrogen) instead of the labelled probes. The relative level of each mRNA was calculated on the basis of the two standard curve relative quantification method. Gene expressions were normalized to β-*actin* and to the cells transfected with the non-coding siRNA. At least two independent determinations of fold differences were used to calculate the average fold difference values and associated standard deviations (SDs).

Results

In lymphoid cells, a hypomethylated *hTERT* promoter allows *hTERT* expression

To define the methylation status of the *hTERT* promoter in lymphoid tissues, we analysed six primary B cell lymphomas, six primary T cell lymphomas and four non-malignant lymphoid tissues. Four human lymphoid tumour cell lines were also investigated. RT–PCR analysis confirmed that *hTERT* transcripts were present in all the lymphoma tissues and cell lines as well as in the non-neoplastic lymphoid tissues (Figure 1A). The β-*actin* gene was simultaneously amplified as a control.

Using MS-DBA, we next explored the methylation status of the *hTERT* promoter. In B cell lymphomas and non-neoplastic lymphoid tissues, the *hTERT* promoter was unmethylated, while it was hypermethylated in half of the T cell lymphomas (Figure 1B). The *hTERT* promoter was methylated in Daudi cells but unmethylated in the other three cell lines (Raji, Ramos and Nalm6). Direct sequencing and MS-SSCA confirmed the results obtained by MS-DBA (data not shown). To summarize, in some T cell lymphomas *hTERT* expression goes along with *hTERT* promoter methylation, as is the case for most solid tumours. In transformed B cells and non-neoplastic lymphocytes, however, *hTERT* is expressed in the presence of a hypomethylated promoter. In this situation, hTERT expression must be regulated by a methylation-independent mechanism.

Putative PAX5 binding sites are present in the *hTERT* gene

To determine whether transcription factors specific to lymphoid cells might be involved in *hTERT* regulation, we searched for new transcription factor binding sites using MatInspector software (http://www.genomatix.de/matinspector.html). MatInspector revealed two potential binding sites for PAX5, also known as B cell-specific activator protein (BSAP), a transcription factor involved in B cell differentiation and function [25,26], from +110 to +137 bp and +489 to +516 bp downstream of the ATG translational start site (Figure 2A). PAX5 binding sites match the consensus sequence at 9 and 11 out of 15 positions, for exon 1 and exon 2, respectively (Figure 2B). This suggested that PAX5 might be involved in the regulation of *hTERT* transcription in lymphoid cells.

PAX5 binds the *hTERT* CpG island *in vitro* and *in vivo*

To determine whether the predicted PAX5 binding sites in the *hTERT* exon were authentic, we first performed EMSAs using extracts from Raji cells. A specific band for PAX5 was obtained with the *CD79A* oligonucleotide (Figure 3A, lane 1), which served as a positive control [21,41]. A similar band was obtained with the *hTERT* probe (lane 2). To check the specificity of the band, cold competitor oligonucleotides were added to the labelled *hTERT* probe. A 100-fold molar excess of *CD79A* and *hTERT* competitors resulted in almost complete inhibition of PAX5 binding (lanes 4 and 6). The same results were obtained with Nalm6 extracts (data not shown). A 5–150-fold increase in the amount of cold *CD79A* probe

also resulted in a progressive inhibition of binding (Figure 3B). Competitive EMSA, with an oligonucleotide in which four specific bases were mutated, did not eliminate PAX5 binding (Figure 3A, lane 5), indicating that PAX5 binding was specific. Pre-incubation of Raji cell extracts with a PAX5-specific antibody resulted in a supershifted band (Figure 3A, lane 7), confirming that PAX5 does bind to the predicted target sequence in the first exon of *hTERT*.

To determine whether PAX5 bound to the *hTERT* gene under physiological conditions, chromatin immunoprecipitation (ChIP) experiments were performed, using Nalm6 and Raji cells. DNA samples isolated from the input, the anti-PAX5-bound and the no-antibody fractions were analysed by quantitative real-time PCR. *CD19* is a well-known target of PAX5 [31] and was used as positive control. The results indicated an approximately 10-fold enrichment of *hTERT* exon 1 when normalized with the negative control *KRAS* gene, and around eight-fold enrichment of *hTERT* exon 2 (Figure 3D). Enrichment of the *CD19* gene was about three to four times greater than that for *hTERT*, which was not surprising, as the binding of PAX5 to the *CD19* target sequences is very strong. In the no-antibody fraction, no enrichment was detected with either *hTERT* or *CD19* (data not shown). Thus, PAX5 is bound *in vivo* to the first and the second exons of *hTERT*, providing strong evidence that PAX5 could be involved in the transcriptional regulation of the gene in B cells.

PAX5 does not inhibit binding of CTCF to *hTERT in vivo*

A possible explanation for the effect of PAX5 on hTERT expression could be that it interferes with CTCF-binding to the *hTERT* promoter. The CTCF transcription factor was found to be essential for repression of *hTERT* transcription in a variety of normal somatic cells [6]. As PAX5 binding sites lie downstream CTCF target sequences (Figure 2), we therefore performed ChIP analysis to analyse CTCF binding. In Raji cells, *hTERT* exon 1 was enriched approximately four-fold compared to the negative control, which is in the same range as in the two positive controls, *MYC-N* and *H19* (Figure 4). After transfection with a *PAX5* siRNA, a strong reduction in the binding of PAX5 was observed on *hTERT* and *CD19* (Figure 4B), whereas chromatin immunoprecipitation of CTCF did not reveal any significant change in the binding of CTCF to *hTERT* (Figure 4A). Thus, PAX5 binding to the *hTERT* exonic region does not block CTCF binding.

hTERT and PAX5 have similar patterns of expression in B cell lymphomas and the B cell areas of non-neoplastic lymphoid tissues

PAX5 is a specific marker for all stages of B cell differentiation except for plasma cells [42]. In our series, *PAX5* mRNA was detected by RT–PCR in all B and T cell NHLs, as well as in non-neoplastic lymphoid tissues and cell lines. The presence of *PAX5* mRNA in the T cell lymphomas could be due to the presence of normal B cells in the tumour tissues.

By IHC of consecutive sections, hTERT and PAX5 were both detected in the same regions of the six B cell lymphomas, suggesting that they were present in the same tumour cells (Figure 5A, B). The T cells were identified by CD3 expression. In B cell lymphomas, the normal T cells did not appear to express either PAX5 or hTERT (Figure 5A, C). In the four non-neoplastic lymphoid tissues, both PAX5 and hTERT were expressed in germinal centre B cells and B cells of the mantle zone (Figure 5G, H), whereas $CD3^+$ T cells were PAX5- and hTERT-negative (Figure 5G–I). The expression of hTERT observed in germinal centre and mantle zone was concordant with the published data on telomerase activity [16]. In the six investigated T cell lymphomas, hTERT was expressed in the $CD3^+$ neoplastic T cells (Figure 5D, F) while PAX5 was not (Figure 5E, F). As expected, in T cell lymphomas, PAX5 was only expressed in normal B cells and therefore PAX5 had no role in activating *hTERT* expression in tumour T cells. In summary, PAX5 and hTERT co-localize in normal

and malignant B cells, supporting the suggestion that PAX5 might be involved in hTERT activation in these cells.

Suppression of PAX5 by siRNA represses *hTERT* transcription in telomerase-positive cells

To determine whether a reduction in PAX5 expression would be associated with a change in *hTERT* expression, we transfected Raji cells with a PAX5 siRNA. After transfection, the down-regulation of PAX5 protein was confirmed by western blot, while the levels of *PAX5, hTERT* and *CD19* transcripts were monitored by quantitative RT–PCR. Cells transfected with a scrambled siRNA were used as a control. Twentyfour hours after transfection, *PAX5* transcript levels were reduced ~50% in association with significant reductions in the levels of *CD19* and *hTERT* expression (~30%) (Figure 6A). After 48 h, *PAX5* transcripts were reduced by 77% in association with reductions of 57% and 64% in transcripts for *CD19* and *hTERT*, respectively. These studies showed that inhibition of PAX5 leads to a strong down-regulation of *hTERT* expression, indicating that PAX5 is essential for *hTERT* expression in B cells.

PAX5 activates *hTERT* transcription in normal telomerase-negative cells

To determine whether ectopic expression of PAX5 could activate *hTERT* expression in normal telomerase-negative cells, we transfected normal BJ fibroblasts with a *PAX5* expression plasmid. RT–PCR and western blot analyses of PAX5 expression 40 h post-transfection confirmed the siRNA silencing efficiency (Figure 6A). RT–PCR analyses of transcripts for *CD19*, an established target of PAX5, showed substantial expression in the transfected cells. These data are in agreement with previous studies demonstrating that ectopic expression of PAX5 led the up-regulation to *CD19* and other PAX5-target genes [21,43]. Interestingly, the transfected cells expressed *hTERT* transcripts at similar levels as *CD19*. The level of expression of *hTERT* and *CD19* was apparently lower in BJ than in Raji cells, this could be explained by the absence in transfected BJ cells of specific transcription factors necessary for a high level of expression of these genes. Non-transfected and mock-transfected BJ cells did not express transcripts of *PAX5, CD19* or *hTERT*. This experiment showed that ectopic expression of PAX5 is sufficient to activate *hTERT* transcription in normal somatic cells.

Discussion

In the present study, we showed that *hTERT* is transcribed in association with the unmethylated 5′ region in B cells, B cell lymphomas and B cell lymphoma cell lines, defining a novel methylation-independent mode of *hTERT* regulation. EMSA and ChIP assays identified two binding sites in *hTERT* for the B cell-specific transcription factor PAX5. These sites lie downstream of the ATG translational start site and are located in the first exon and at the beginning of the second exon of *hTERT*. Moreover, in B cells, decreasing PAX5 expression resulted in a significant reduction in *hTERT* expression. Importantly, we showed that ectopic expression of PAX5 in telomerase-negative normal cell lines is sufficient to activate *hTERT* expression. Taken together, these data strongly support a role for PAX5 in the transcriptional activation of *hTERT* in B cells. Validation of our observations by functional studies will be important.

hTERT is a new PAX5 target, which has no direct link to B cell differentiation, in contrast to the well-known PAX5 target genes. Among the principal targets of PAX5, three genes, *CD79A, CD19* and *PDCD1* (*PD-1*), code for cell surface molecules involved in signal transduction, while the products of two other target genes, *MYCN* (*N-Myc*) and *LEF1*, are nuclear transcription factors [31,40]. About 170 PAX5-activated genes have been identified [44]. These genes mediate diverse biological functions in B cells, such as adhesion,

migration, signalling and germinal-centre B cell formation, and demonstrate the pleiotropic role of PAX5 in control of the B-lineage commitment.

PAX8, which belongs to the same subgroup of PAX proteins as PAX5, has been implicated in the activation of *hTERT* in glioma [24]. PAX8 failed to activate the *hTERT* promoter in telomerase-negative primary cell lines, and other factors seem to be necessary for the expression of hTERT. In contrast, activation of PAX5 was sufficient to initiate the transcription of *hTERT* in telomerase-negative primary cell lines. Apparently, the action of PAX5 on *hTERT* is very different from that of PAX8. PAX8 mainly seems to act on the formation of the transcription complex, whereas the major role of PAX5 in transcriptional activation does not seem to be to recruit basal transcription machinery, but is likely to modulate the structure of local chromatin, allowing other sequence-specific factors to activate transcription. Indeed, PAX5 can activate transcription through association with chromatin effector enzymes such as DAXX, CREB-binding protein (CBP) and GCN5, which possess histone acetyltransferase (HAT) activity. PAX5 can also interact with BRG1, a catalytic component of the Swi/Snf chromatin remodelling complexes [45]. On the other hand, CTCF directly binds to SIN3A, which condenses chromatin and prevents transcription by recruitment of histone deacetylase (HDAC) activity [46]. Therefore, the simultaneous binding of CTCF and PAX5 on *hTERT* exons might produce opposing effects on chromatin: the recruitment of histone modification and nucleosome remodelling activities by PAX5 might antagonize chromatin-mediated transcriptional repression by CTCF. Additional studies need to be performed to more accurately understand how CTCF and PAX5 interact in regulating *hTERT* expression.

In summary, we describe a methylation-independent mechanism of *hTERT* regulation that occurs in telomerase-positive B cells. In these cells, *hTERT* is a novel target of PAX5, which is essential for B cell development and function. According to our data, in B cells, PAX5 also participates in cellular mechanisms underlying cell immortality by up-regulating *hTERT* gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the Swiss National Science Foundation (Grants 3100A0-101732 and 3100A0-113505) and in part by the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases.

References

1. Meeker AK, Coffey DS. Telomerase: a promising marker of biological immortality of germ, stem, and cancer cells. A review. Biochemistry (Mosc). 1997; 62:1323–1331. [PubMed: 9467857]
2. Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, et al. *hEST2*, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell. 1997; 90:785–795. [PubMed: 9288757]
3. Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, et al. Telomerase catalytic subunit homologs from fission yeast and human. Science. 1997; 277:955–959. [PubMed: 9252327]
4. Goueli BS, Janknecht R. Regulation of telomerase reverse transcriptase gene activity by upstream stimulatory factor. Oncogene. 2003; 22:8042–8047. [PubMed: 12970752]
5. Poole JC, Andrews LG, Tollefsbol TO. Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). Gene. 2001; 269:1–12. [PubMed: 11376932]

6. Renaud S, Loukinov D, Bosman FT, Lobanenkov V, Benhattar J. CTCF binds the proximal exonic region of hTERT and inhibits its transcription. Nucleic Acids Res. 2005; 33:6850–6860. [PubMed: 16326864]
7. Dessain SK, Yu HY, Reddel RR, Beijersbergen RL, Weinberg RA. Methylation of the human telomerase gene CpG island. Cancer Res. 2000; 60:537–541. [PubMed: 10676632]
8. Devereux TR, Horikawa I, Anna CH, Annab LA, Afshari CA, Barrett JC. DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (*hTERT*) gene. Cancer Res. 1999; 59:6087–6090. [PubMed: 10626795]
9. Guilleret I, Yan P, Grange F, Braunschweig R, Bosman FT, Benhattar J. Hypermethylation of the human telomerase catalytic subunit (*hTERT*) gene correlates with telomerase activity. Int J Cancer. 2002; 101:335–341. [PubMed: 12209957]
10. Nomoto K, Maekawa M, Sugano K, Ushiama M, Fukayama N, Fujita S, et al. Methylation status and expression of human telomerase reverse transcriptase mRNA in relation to hypermethylation of the *p16* gene in colorectal cancers as analyzed by bisulfite PCR–SSCP. Jpn J Clin Oncol. 2002; 32:3–8. [PubMed: 11932355]
11. Renaud S, Loukinov D, Abdullaev Z, Guilleret I, Bosman FT, Lobanenkov V, et al. Dual role of DNA methylation inside and outside of CTCF-binding regions in the transcriptional regulation of the telomerase *hTERT* gene. Nucleic Acids Res. 2007; 35:1245–1256. [PubMed: 17267411]
12. Zinn RL, Pruitt K, Eguchi S, Baylin SB, Herman JG. hTERT is expressed in cancer cell lines despite promoter DNA methylation by preservation of unmethylated DNA and active chromatin around the transcription start site. Cancer Res. 2007; 67:194–201. [PubMed: 17210699]
13. Widschwendter A, Muller HM, Hubalek MM, Wiedemair A, Fiegl H, Goebel G, et al. Methylation status and expression of human telomerase reverse transcriptase in ovarian and cervical cancer. Gynecol Oncol. 2004; 93:407–416. [PubMed: 15099954]
14. Bechter OE, Eisterer W, Dlaska M, Kuhr T, Thaler J. CpG island methylation of the hTERT promoter is associated with lower telomerase activity in B-cell lymphocytic leukemia. Exp Hematol. 2002; 30:26–33. [PubMed: 11823034]
15. Martens UM, Brass V, Sedlacek L, Pantic M, Exner C, Guo Y, et al. Telomere maintenance in human B lymphocytes. Br J Haematol. 2002; 119:810–818. [PubMed: 12437664]
16. Weng NP, Granger L, Hodes RJ. Telomere lengthening and telomerase activation during human B cell differentiation. Proc Natl Acad Sci USA. 1997; 94:10827–10832. [PubMed: 9380719]
17. Hu BT, Lee SC, Marin E, Ryan DH, Insel RA. Telomerase is upregulated in human germinal center B cells *in vivo* and can be re-expressed in memory B cells activated *in vitro*. J Immunol. 1997; 159:1068–1071. [PubMed: 9233598]
18. Underhill DA. Genetic and biochemical diversity in the *Pax* gene family. Biochem Cell Biol. 2000; 78:629–638. [PubMed: 11103953]
19. Busslinger M, Klix N, Pfeffer P, Graninger PG, Kozmik Z. Deregulation of PAX-5 by translocation of the Emu enhancer of the IgH locus adjacent to two alternative PAX-5 promoters in a diffuse large-cell lymphoma. Proc Natl Acad Sci USA. 1996; 93:6129–6134. [PubMed: 8650231]
20. Dorfler P, Busslinger M. C-terminal activating and inhibitory domains determine the transactivation potential of *BSAP* (*Pax-5*) *Pax-2* and *Pax-8*. EMBO J. 1996; 15:1971–1982. [PubMed: 8617244]
21. Nutt SL, Morrison AM, Dorfler P, Rolink A, Busslinger M. Identification of *BSAP* (*Pax-5*) target genes in early B-cell development by loss- and gain-of-function experiments. EMBO J. 1998; 17:2319–2333. [PubMed: 9545244]
22. 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; 22:7989–7997. [PubMed: 12970747]
23. Robson EJ, He SJ, Eccles MR. A PANorama of *PAX* genes in cancer and development. Nat Rev Cancer. 2006; 6:52–62. [PubMed: 16397527]
24. Chen YJ, Campbell HG, Wiles AK, Eccles MR, Reddel RR, Braithwaite AW, et al. PAX8 regulates telomerase reverse transcriptase and telomerase RNA component in glioma. Cancer Res. 2008; 68:5724–5732. [PubMed: 18632625]

25. Barberis A, Widenhorn K, Vitelli L, Busslinger M. A novel B-cell lineage-specific transcription factor present at early but not late stages of differentiation. Genes Dev. 1990; 4:849–859. [PubMed: 2116362]
26. Nutt SL, Urbanek P, Rolink A, Busslinger M. Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. Genes Dev. 1997; 11:476–491. [PubMed: 9042861]
27. Mhawech-Fauceglia P, Saxena R, Zhang SZ, Terracciano L, Sauter G, Chadhuri A, et al. Pax-5 immunoexpression in various types of benign and malignant tumours: a high-throughput tissue microarray analysis. J Clin Pathol. 2007; 60:709–714. [PubMed: 16837628]
28. Torlakovic E, Torlakovic G, Nguyen PL, Brunning RD, Delabie J. The value of anti-pax-5 immunostaining in routinely fixed and paraffin-embedded sections: a novel pan pre-B and B-cell marker. Am J Surg Pathol. 2002; 26:1343–1350. [PubMed: 12360049]
29. Fitzsimmons D, Hodsdon W, Wheat W, Maira SM, Wasylyk B, Hagman J. Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. Genes Dev. 1996; 10:2198–2211. [PubMed: 8804314]
30. Nutt SL, Urbanek P, Rolink A, Busslinger M. Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. Genes Dev. 1997; 11:476–491. [PubMed: 9042861]
31. Kozmik Z, Wang S, Dorfler P, Adams B, Busslinger M. The promoter of the *Cd19* gene is a target for the B-cell-specific transcription factor BSAP. Mol Cell Biol. 1992; 12:2662–2672. [PubMed: 1375324]
32. Schebesta M, Pfeffer PL, Busslinger M. Control of pre-BCR signaling by Pax5-dependent activation of the *BLNK* gene. Immunity. 2002; 17:473–485. [PubMed: 12387741]
33. Fuxa M, Skok J, Souabni A, Salvagiotto G, Roldan E, Busslinger M. Pax5 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain gene. Genes Dev. 2004; 18:411–422. [PubMed: 15004008]
34. Guillou L, Coindre JM, Gallagher G, Terrier P, Gebhard S, Somerhausen ND, et al. Detection of the synovial sarcoma translocation t(X; 18) (SYT; SSX) in paraffin-embedded tissues using reverse transcriptase-polymerase chain reaction. A reliable and powerful diagnostic tool for pathologists — a molecular analysis of 221 mesenchymal tumors fixed in different fixatives. Hum Pathol. 2001; 32:105–112. [PubMed: 11172303]
35. Clement G, Benhattar J. A methylation sensitive dot blot assay (MS-DBA) for the quantitative analysis of DNA methylation in clinical samples. J Clin Pathol. 2005; 58:155–158. [PubMed: 15677535]
36. Bian YS, Yan P, Osterheld MC, Fontolliet C, Benhattar J. Promoter methylation analysis on micro-dissected paraffin-embedded tissues using bisulfite treatment and PCR-SSCP. Biotechniques. 2001; 30:66–72. [PubMed: 11196322]
37. Bianco T, Hussey D, Dobrovic A. Methylation-sensitive, single-strand conformation analysis (MS-SSCA): a rapid method to screen for and analyze methylation. Hum Mutat. 1999; 14:289–293. [PubMed: 10502775]
38. Pugacheva EM, Tiwari VK, Abdullaev Z, Vostrov AA, Flanagan PT, Quitschke WW, et al. Familial cases of point mutations in the XIST promoter reveal a correlation between CTCF binding and pre-emptive choices of X chromosome inactivation. Hum Mol Genet. 2005; 14:953–965. [PubMed: 15731119]
39. Renaud S, Pugacheva EM, Delgado MD, Braunschweig R, Abdullaev Z, Loukinov D, et al. Expression of the CTCF-paralogous cancer-testis gene, brother of the regulator of imprinted sites (BORIS), is regulated by three alternative promoters modulated by CpG methylation and by CTCF and p53 transcription factors. Nucleic Acids Res. 2007; 35:7372–7388. [PubMed: 17962299]
40. Nutt SL, Morrison AM, Dorfler P, Rolink A, Busslinger M. Identification of *BSAP* (*Pax-5*) target genes in early B-cell development by loss- and gain-of-function experiments. EMBO J. 1998; 17:2319–2333. [PubMed: 9545244]
41. Wheat W, Fitzsimmons D, Lennox H, Krautkramer SR, Gentile LN, McIntosh LP, et al. The highly conserved β-hairpin of the paired DNA-binding domain is required for assembly of Pax–Ets ternary complexes. Mol Cell Biol. 1999; 19:2231–2241. [PubMed: 10022910]

42. Jensen KC, Higgins JPT, Montgomery K, Kaygusuz G, van de Rijn M, Natkunam Y. The utility of PAX5 immunohistochemistry in the diagnosis of undifferentiated malignant neoplasms. Mod Pathol. 2007; 20:871–877. [PubMed: 17529924]
43. Cozma D, Yu D, Hodawadekar S, Azvolinsky A, Grande S, Tobias JW, et al. B cell activator PAX5 promotes lymphomagenesis through stimulation of B cell receptor signaling. J Clin Invest. 2007; 117:2602–2610. [PubMed: 17717600]
44. Schebesta A, McManus S, Salvagiotto G, Delogu A, Busslinger GA, Busslinger M. Transcription factor Pax5 activates the chromatin of key genes involved in B cell signaling, adhesion, migration, and immune function. Immunity. 2007; 27:49–63. [PubMed: 17658281]
45. Gregory PD, Wagner K, Horz W. Histone acetylation and chromatin remodeling. Exp Cell Res. 2001; 265:195–202. [PubMed: 11302684]
46. Lutz M, Burke LJ, Barreto G, Goeman F, Greb H, Arnold R, et al. Transcriptional repression by the insulator protein CTCF involves histone deacetylases. Nucleic Acids Res. 2000; 28:1707–1713. [PubMed: 10734189]
47. Czerny T, Busslinger M. DNA-binding and transactivation properties of Pax-6: three amino acids in the paired domain are responsible for the different sequence recognition of Pax-6 and BSAP (Pax-5). Mol Cell Biol. 1995; 15:2858–2871. [PubMed: 7739566]

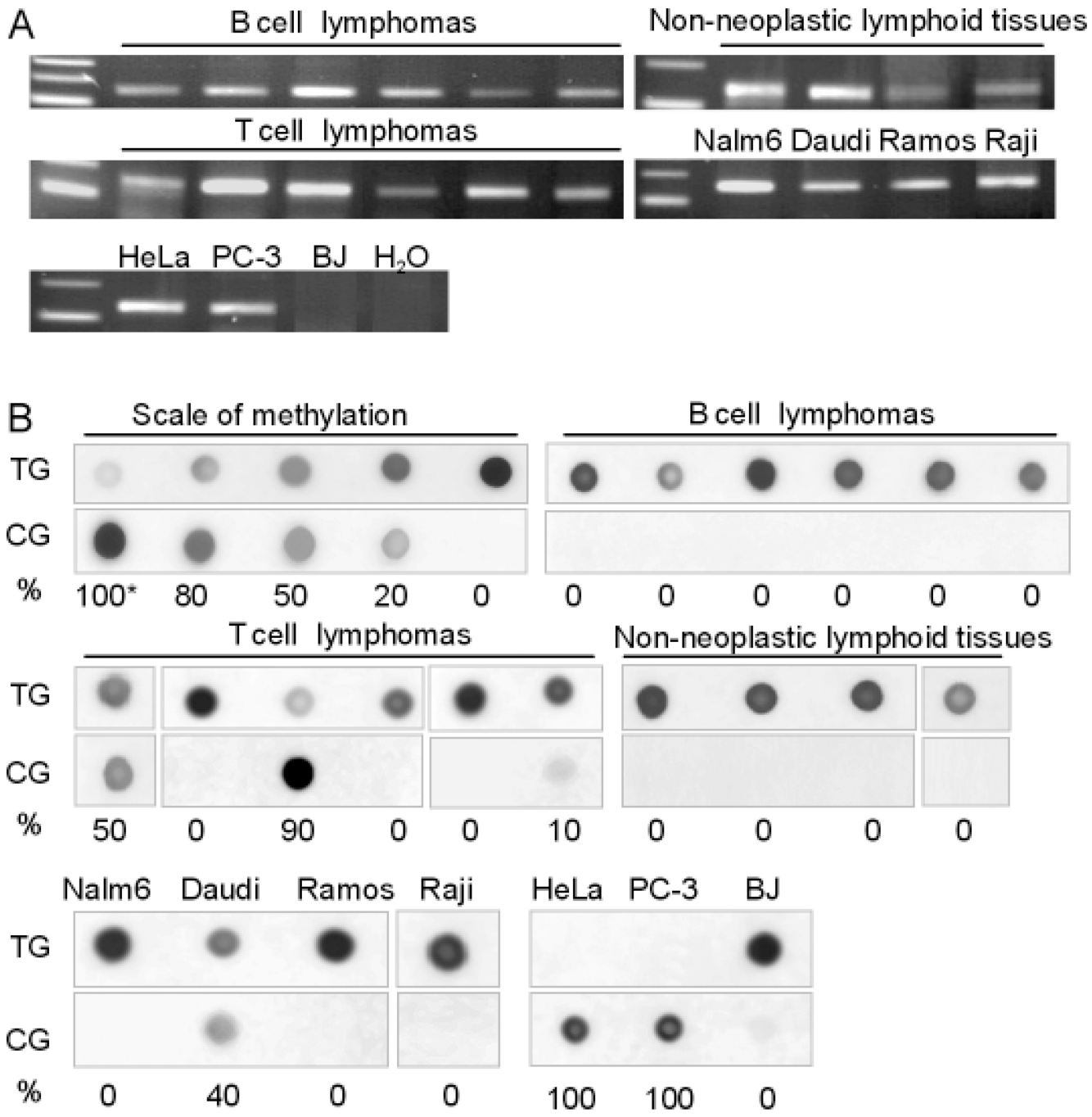


Figure 1.

hTERT mRNA expression and *hTERT* methylation in lymphoid tissues and cell lines. (A) Detection of *hTERT* expression by RT–PCR in six B cell lymphomas, six T cell lymphomas, four non-neoplastic lymphoid tissues and four lymphoid cell lines. HeLa and PC-3 cells were used as positive controls. Normal BJ fibroblasts were used as a negative control. (B) Methylation analysis of the *hTERT* promoter by methyl-sensitive dot-blot assay (MS-DBA). Hybridization with a TG probe revealed the non-methyl part of the PCR and hybridization with a CG probe detected the methyl part of the PCR. A scale of methylation (100%, 80%, 50%, 20% and 0%) was used to quantify the percentage of methylation found in the different samples. 100* indicates that the 100% methylation possesses a residual

background with the non-methyl probe. As control, HeLa and PC-3 harboured an *hTERT* methylated promoter, whereas *hTERT* was unmethylated in BJ. The same samples used for RT–PCR were tested for methylation

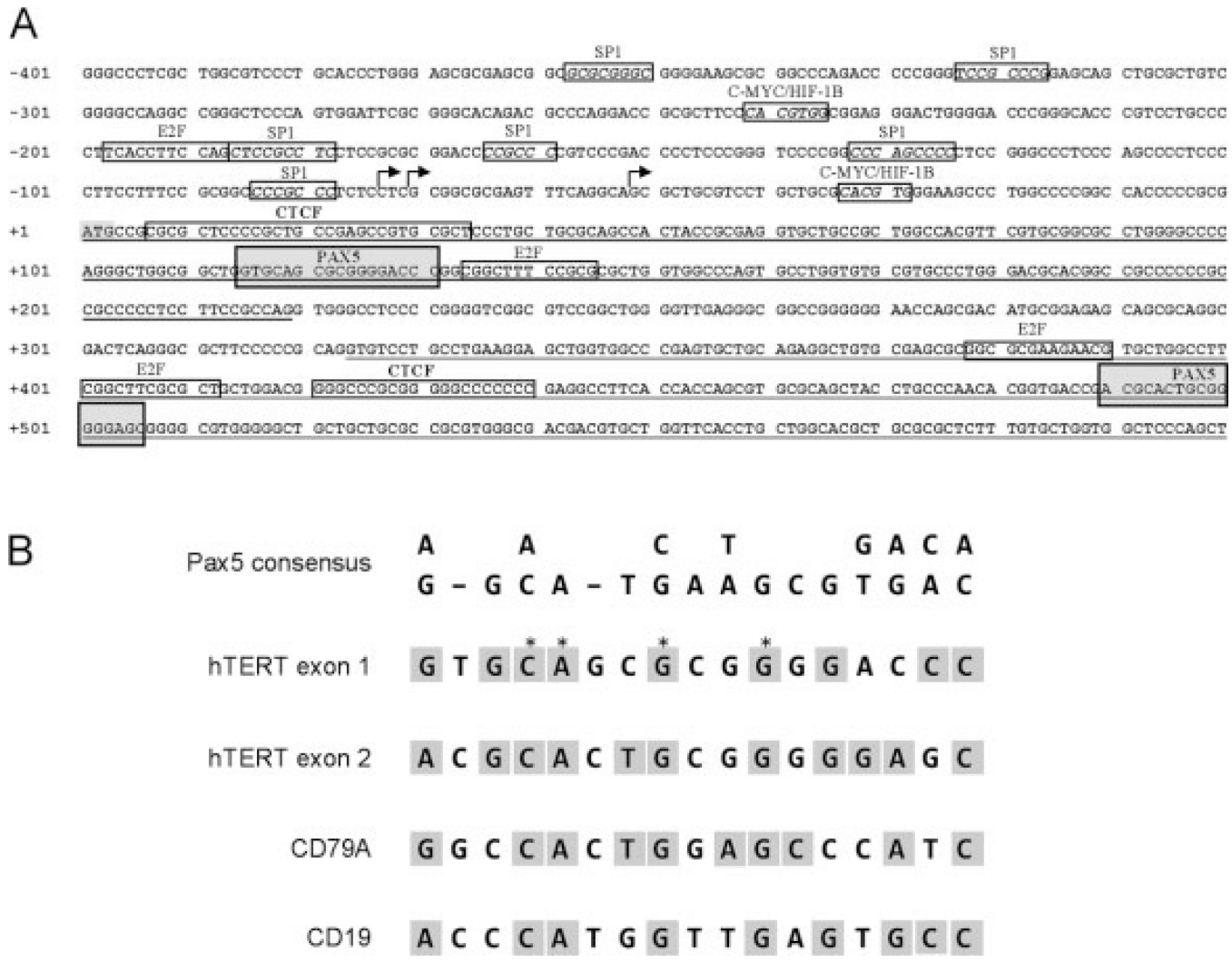


Figure 2.

PAX5 binding sites in the *hTERT* gene. (A) Localization of putative transcription factor binding sites on *hTERT* sequences from −401 to +600 bp flanking the ATG (+1). The main transcriptional start sites are indicated by arrows. The ATG translational start site is highlighted in grey. The exonic regions are underlined. Known binding sites of SP1, c-MYC, E2F and CTCF are shown in small boxes. Potential binding sites of PAX5 predicted by the MatInspector program are shown in large grey boxes. (B) Alignment of the *hTERT, CD79A* and *CD19* sequences with the PAX5 consensus recognition motif [47]. Consensus nucleotides are shown in grey overlay. *Point mutations

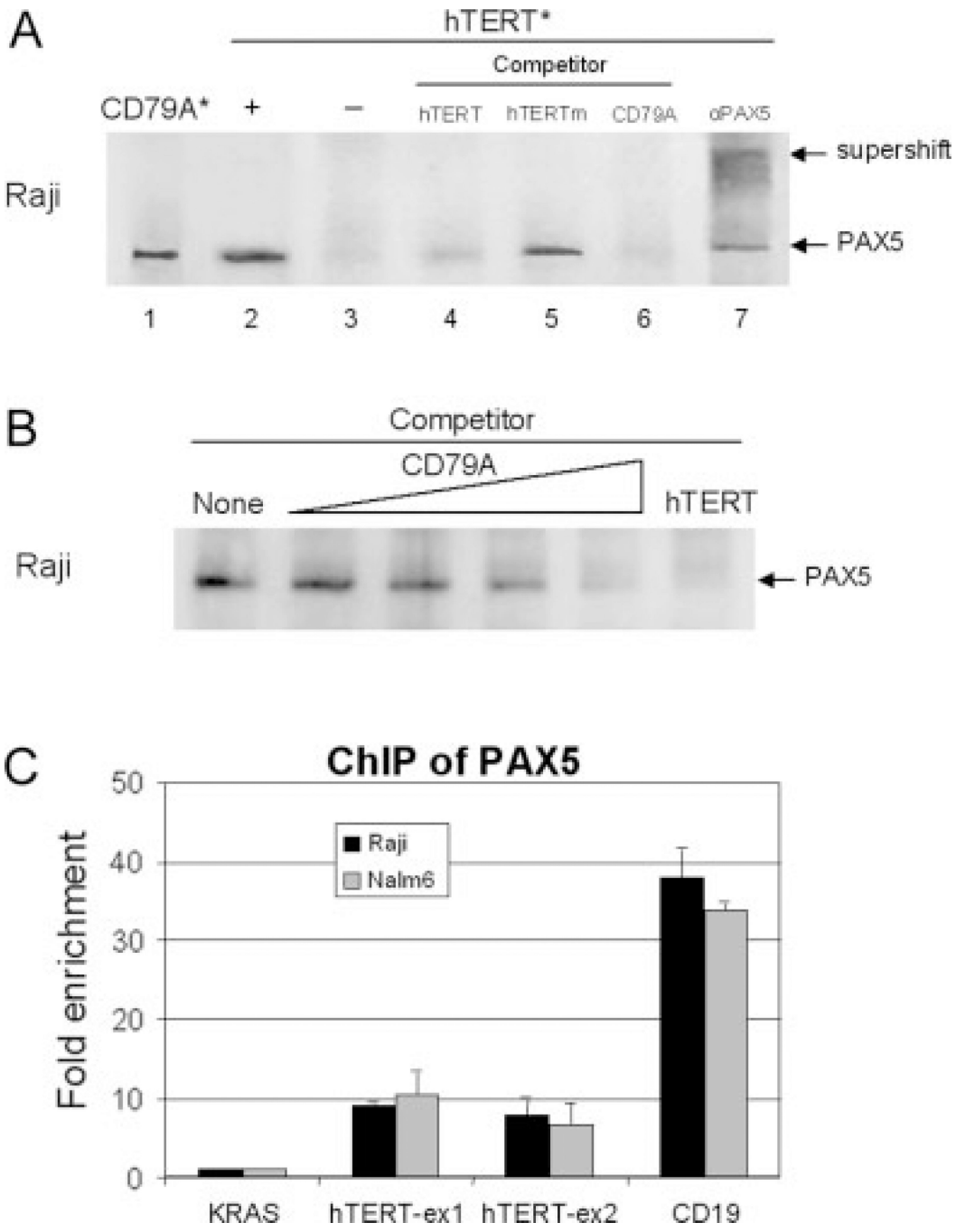
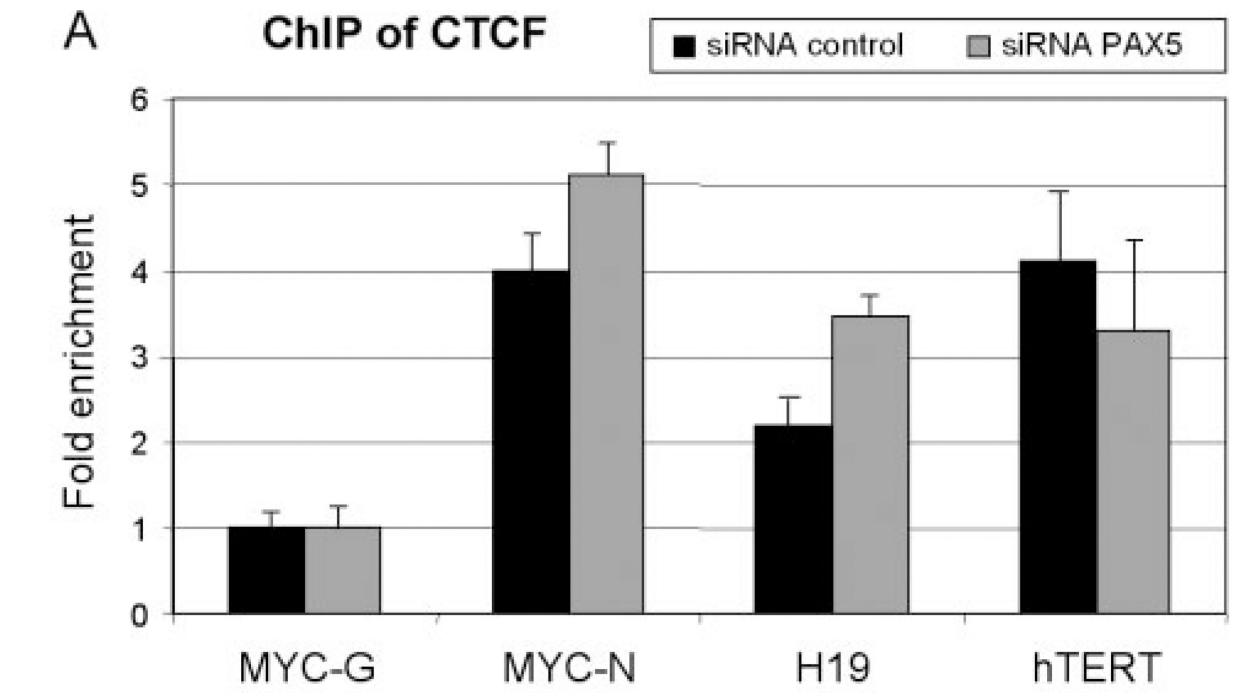


Figure 3.

In vitro and *in vivo* binding of PAX5 in Raji cells. (A) EMSAs were performed with DIG-labelled oligonucleotides representing PAX5 binding sites on *CD79A* as a positive control gene (lane 1) and on the *hTERT* gene (lanes 2–7). A negative control without extract was performed (lane 3). An excess (100×) of unlabelled oligonucleotide (hTERT; CD79A) was used in a competition assay for PAX5 binding to the labelled probes (lanes 4 and 6, respectively). A competition assay was also performed with an hTERT oligonucleotide with four mutations (hTERTm), as described in Figure 2B (lane 5). A supershift was performed with a PAX5 antibody (lane 7, αPAX5). (B) A graduated competition with unlabelled oligonucleotide (CD79A)was performed on Raji cellular extracts, with an excess (5×, 15×,

50× and 150×) of unlabelled *CD79A* oligonucleotide and an excess (150×) of unlabelled *hTERT* oligonucleotide. (*C*) Real-time PCR analysis of *hTERT* DNA fragments precipitated in a chromatin immunoprecipitation (ChIP) assay by a PAX5 antibody in Nalm6 and Raji cells. *CD19* was used to detect specific PAX5 binding and *KRAS* was used to identify non-specific interactions



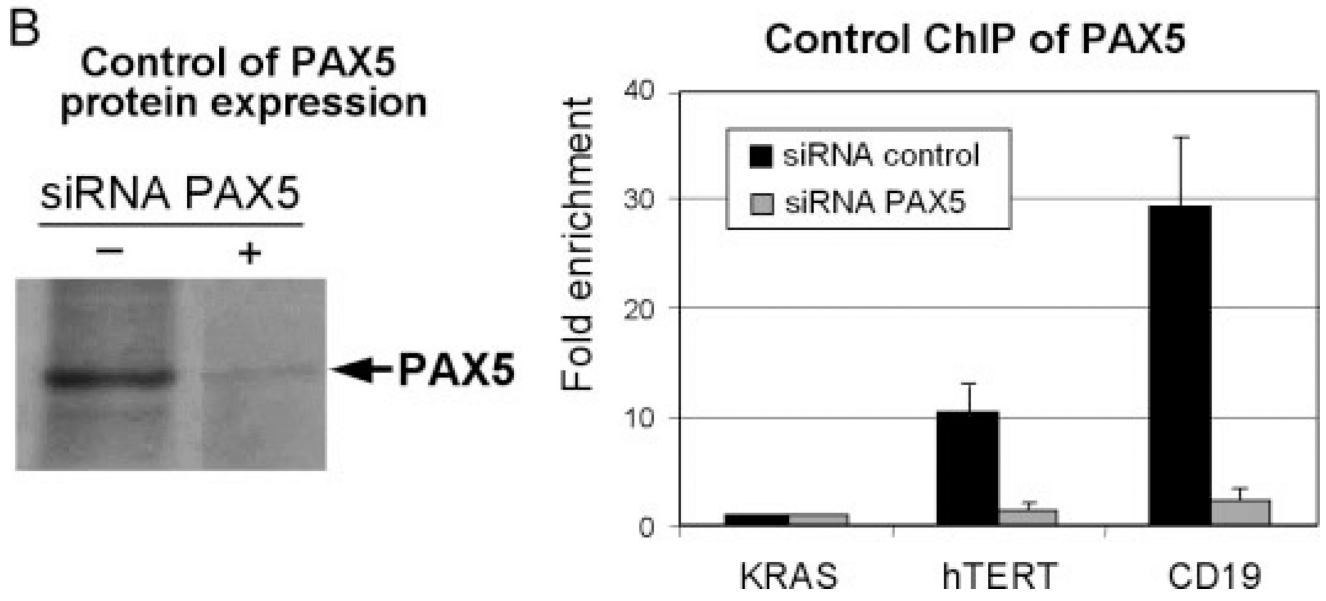


Figure 4.

ChIP of CTCF in Raji cells 48 h after transfection with either a control siRNA or a siRNA against PAX5. (A) Analysis of *hTERT* DNA fragments precipitated in a CHIP assay by a CTCF-antibody was performed by quantitative real-time PCR. *MYC-N* and *H19* were used to detect specific CTCF binding and *MYC-G* was used to identify non-specific interactions. (B) Controls of PAX5 siRNA treatments. A western blot of PAX5 was performed to control the efficiency of PAX5 siRNA treatment. A real-time PCR analysis of *hTERT* and *CD19* DNA fragments precipitated in a ChIP assay by a PAX5 antibody was used to demonstrate the efficiency of PAX5 siRNA treatment through the failing of PAX5 binding. *KRAS* was used to identify non-specific interactions

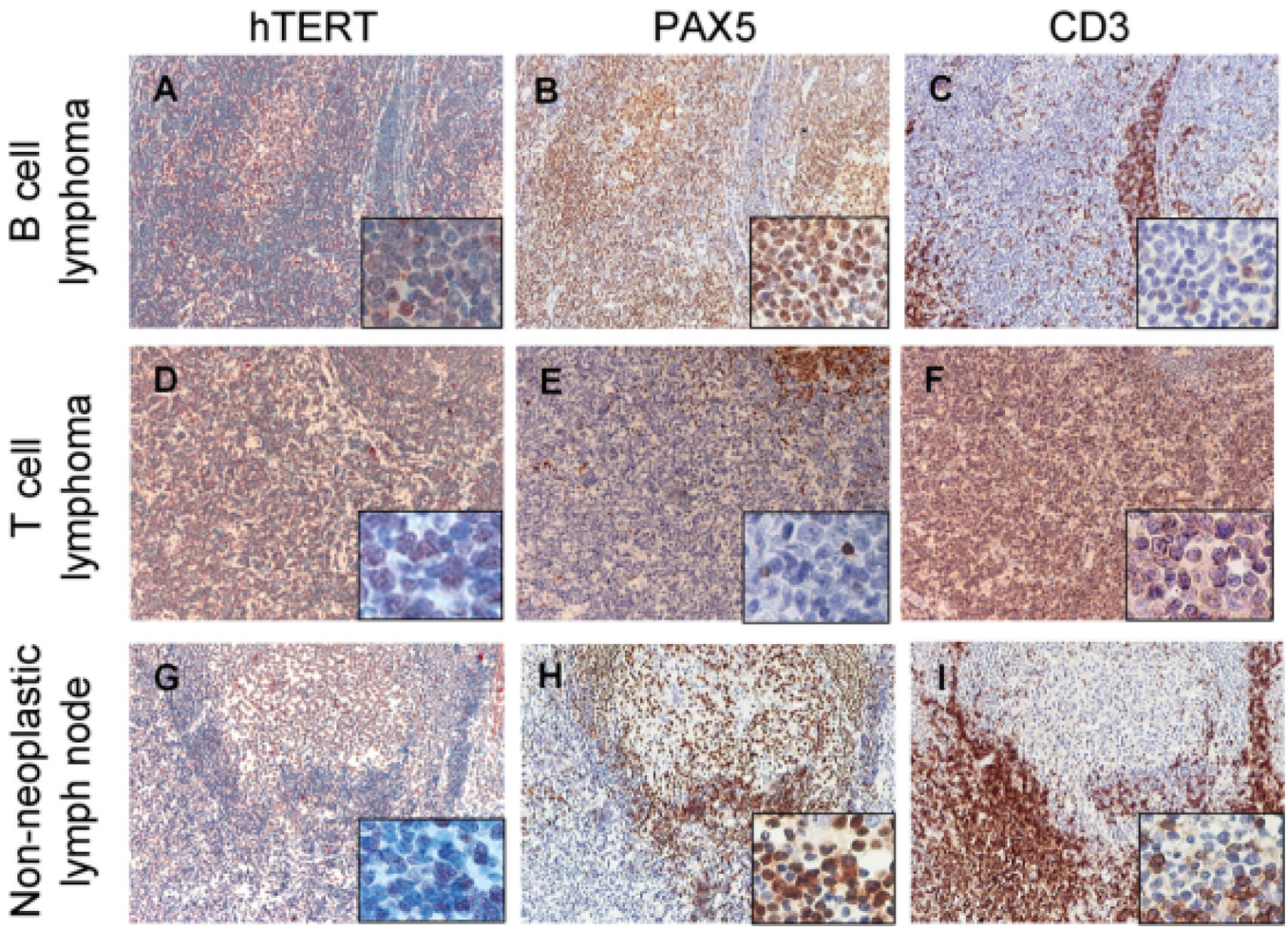


Figure 5.
Immunohistochemistry of hTERT, PAX5 and CD3. Representative images are shown at low magnification (×10) and at high magnification (×40) in the insets. (A–C) B cell lymphoma; (D–F) T cell lymphoma; (G–I) normal lymph node

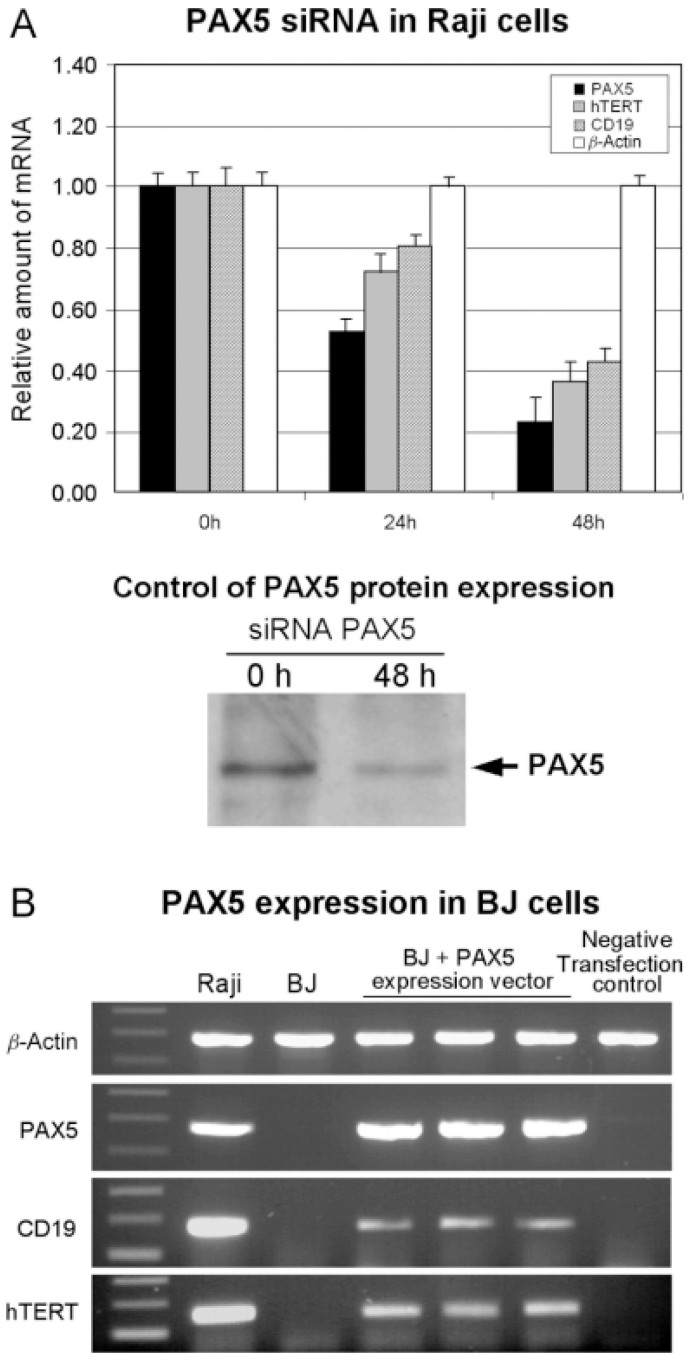


Figure 6.

The effect of activation or inactivation of PAX5. (A) Quantitative RT–PCR of *PAX5, hTERT* and *CD19* after transfection of a *PAX5* siRNA into Raji cells. Quantitations were performed 24 and 48 h after transfection. The relative amounts of each mRNA were normalized to the amounts of mRNA after transfection of a scrambled siRNA compared with β-*actin*. A western blot of PAX5 was performed to control the efficiency of PAX5 siRNA treatment 48 h after transfection. (B) Detection of *PAX5, CD19* and *hTERT* expression by RT–PCR before and after transfection of a *PAX5* expression plasmid into telomerase- and PAX5-negative BJ cells and in Raji-positive control cells. β-*actin* was used as a control for the RT–PCR. Triplicates of the transfection experiments are shown