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Journal of Cellular Biochemistry 116:299–309 (2015)

Journal of Cellular Biochemistry

Cancer Specificity of Promoters of the Genes Controlling Cell Proliferation

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ABSTRACT

Violation of proliferation control is a common feature of cancer cells. We put forward the hypothesis that promoters of genes involved in the control of cell proliferation should possess intrinsic cancer specific activity. We cloned promoter regions of *CDC6, POLD1, CKS1B, MCM2,* and *PLK1* genes into pGL3 reporter vector and studied their ability to drive heterologous gene expression in transfected cancer cells of different origin and in normal human fibroblasts. Each promoter was cloned in short (335–800 bp) and long (up to 2.3 kb) variants to cover probable location of core and whole promoter regulatory elements. Cloned promoters were significantly more active in cancer cells than in normal fibroblasts that may indicate their cancer specificity. Both versions of *CDC6* promoters were shown to be most active while the activities of others were close to that of *BIRC5* gene (survivin) gene promoter. Long and short variants of each cloned promoter demonstrated very similar cancer specificity with the exception of *PLK1*-long promoter that was substantially more specific than its short variant and other promoters under study. The data indicate that most of the important *cis*-regulatory transcription elements responsible for intrinsic cancer specificity are located in short variants of the promoters under study. *CDC6* short promoter may serve as a promising candidate for transcription targeted cancer gene therapy. J. Cell. Biochem. 116: 299–309, 2015.

KEY WORDS: PROMOTER; CANCER SPECIFIC; CELL PROLIFERATION; CANCER GENE THERAPY

Transcription regulation is carried out as a result of complex interactions of numerous factors such as *cis*-regulatory elements in DNA, factors of transcription, the organization and remodeling of chromatin and DNA modification, interactions of transcriptional machinery with nuclear periphery and others [Segal and Widom, 2009; Lenhard et al., 2012; Spitz and Furlong, 2012]. Recent full-genomic research of the sequences involved in regulation of transcription, in particular, the study of transcriptomes in comparison with genomic sequences performed by ENCODE consortium [Djebali et al., 2012; Gerstein et al., 2012] have provided massive information concerning structural elements regulating transcription—enhancers, promoters, insulators, etc., their location in the genome, their interaction with transcriptional factors, and the chromatin structure in regions of their location. Nevertheless, information about interaction of these elements with each other still lags from the data on their structure and localization [Gerstein et al., 2012]. In particular, the relative contribution of various regulatory elements in tissue specificity of expression remains unknown [Heintzman et al., 2009]. The generally accepted point of view attributes this role mainly to transcription enhancers [Heintzman and Ren, 2009; Visel et al., 2009] leaving to promoters passive role of assembling of transcription initiating complex.

Abbreviations: TSS, transcription start site; CDC6, cell division cycle 6; POLD1, polymerase (DNA directed), delta 1, catalytic subunit; CKS1B, CDC28 protein kinase regulatory subunit 1B; MCM2, minichromosome maintenance complex component 2; PLK1, polo-like kinase 1; BIRC5, baculoviral inhibitor of apoptosis repeat containing 5; TERT, telomerase reverse transcriptase; CMV, cytomegalovirus; LT, lung tumor; LN, normal lung; SEM, standard error of the mean.

Conflict of interest: The authors declare that they have no conflict of interest.

Grant sponsor: Russian Academy of Sciences; Grant sponsor: Ministry of the Industry and Trade of the Russian Federation; Grant number: 11411.1008700.13.084; Grant sponsor: RFBR; Grant number: 13-04-40170-H comfi.

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Manuscript Received: 29 January 2014; Manuscript Accepted: 29 August 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 4 September 2014

DOI 10.1002/jcb.24968 • © 2014 Wiley Periodicals, Inc.

However, it was demonstrated in a series of studies (i.e., [Landolin et al., 2010]) that relatively short promoter DNA sequences (~1,000 bp length) inserted in plasmids with a reporter gene may ensure its tissue specific expression, indicating the promoter intrinsic ability to function in tissue specific manner. Wide research of properties of promoters isolated from other elements of the genome in comparison to their endogenous tissue specificity could form the basis for an estimation of relative contributions of various regulatory elements of the genome in regulation of tissue specificity of genes expression. Of particular but important case from both fundamental and practical standpoints is the problem of emergence of cancer specificity of gene regulation during cancer initiation and evolution within organism.

There is also more applied interest to identification of cancer specific promoters that are independent from distant regulatory elements. These promoters could be used as a part of gene-therapeutic constructions ensuring so-called transcriptional targeting—cancer specific expression of therapeutic genes. Several such promoters are known. However, they have two important drawbacks. First, human promoters as a rule are considerably less active as compared with widely used and strong CMV immediately early genes promoter [Yew et al., 1997]. Second, most of described human cancer specific promoters are generally highly active only in particular types of cancers or in limited set of cancers [Dorer and Nettelbeck, 2009]. Even relatively strong tumor-specific promoters with rather wide spectrum of activity, such as the promoter of the apoptosis inhibitor *BIRC5* (baculoviral IAP repeat containing five, or survivin) gene [Chen et al., 2004; Zhu et al., 2004] or *TERT* (telomerase reverse transcriptase) gene promoter [Gu and Fang, 2003] still reveal highly uneven activity profiles among various cancer cell types. For instance, *BIRC5* promoter activity in adenovirus vector varies from 0.07% to 28% of the CMV promoter activity in different tissues in vivo [Zhu et al., 2004]. Even combination of *TERT* and *BIRC5* promoters hardly allows to rise effectiveness of transgene transcription over single promoter [Alekseenko et al., 2012]. Meanwhile, non-specific activity of viral promoters such as CMV promoter makes one to use other means to ensure specificity of transgene expression [Dorer and Nettelbeck, 2009]. So discovery of new cancer specific promoters with enhanced activity and specificity would be highly desirable.

We have put forward the hypothesis that promoters of genes controlling DNA replication and cell proliferation can possess intrinsic tumor specific activity that could be suppressed in normal cells. To check this hypothesis we cloned promoters of *CDC6, POLD1, CKS1B, MCM2,* and *PLK1* genes known to be involved in various aspects of control of DNA synthesis and cell proliferation.

CDC6 gene encodes Cell division cycle six protein. The best-characterized function of CDC6 is the assembly of prereplicative complexes at origins of replication during the G1 phase of the cell division cycle. Besides, CDC6 controls S-phase checkpoint and manifests oncogenic potential being overexpressed [Borlado and Mendez, 2008]. *POLD1* gene encodes a catalytic subunit of DNA polymerase delta with both polymerase and proofreading exonuclease activity which is involved in DNA replication and repair. Exonuclease deficiency of POLD1 is associated with tumorigenesis while knockout of *POLD1* gene causes embryonic lethality in mice [Lange et al., 2011]. MCM2 (the homolog of S. cerevisiae minichromosome maintenance two protein) is a subunit of MCM2–7 replicative helicase that both unwinds duplex DNA and powers fork progression during DNA replication [Bochman and Schwacha, 2009]. The product of *CKS1B* gene is a component of CDC28 protein kinase which interacts with many cell cycle regulators including SKP2—cyclin E-p27^{KIP} complex. Overexpression of *CKS1B* in different malignancies is associated with poor prognosis [Krishnan et al., 2010]. *PLK1* gene encodes Polo-like kinase 1 that plays a critical roles in the cell division including centrosome maturation, mitotic spindle assembly, and regulation of mitotic exit and cytokinesis [Song et al., 2012].

The genes promoters of which were chosen for cloning are known to have elevated expression in wide range of human tumors [Zhang et al., 2007]. Here we report the ability of cloned promoters to direct higher levels of reporter gene expression in tumor cells of different origin than in normal cells. We used primary human fibroblasts from two sources as normal controls. The activities of the promoters were collated with endogenous expression of corresponding genes in the same cancer and normal cells that were used in transfection experiments as well as in small series of lung tumors and morphologically normal lung biopsies from lung cancer patients. We compared the promoters under study with known cancer specific promoter of *BIRC5* gene [Mityaev et al., 2010] and estimated perspectives of use of newly cloned promoters in cancer gene therapy.

MATERIALS AND METHODS

PROMOTERS

Promoters were amplified from genomic DNA that was isolated from normal human brain using Taq or Tersus DNA polymerase (Evrogen, Russia). Primers were synthesed using ABI 3900 synthesizer (Applied Biosystems, Inc.). Coordinates of promoters and primers used for amplification are presented in Table I. All ATG sites with extended reading frames that may interfere with ATG codon of *luc* gene of

TABLE I. Primers Used for Amplification of Promoters

Promoters	Primers (5′–>3′)
CDC6 (−1539;+238), 1777 bp[a]	GCTAGCGATCATGGCACGGCACTCA
Short var. (−565;+238), 803 bp	GCTAGCCTAGGCTCTTCACTGTCACCA
Reverse primer	GCTAGCTCAGACCTCCAGCGAGCTCA
POLD1 (−1338;+66), 1404 bp	GGTACCTGAATACAATCCAGCCCGGAG
Short var. (−502;+66), 568 bp	GGTACCCAAGTGTCCCTATCATGCGTTG
Reverse primer	GGTACCCCTCTACTCACCCGCTTCAAAC
CKS1B (−910;+106), 1016 bp	GGTACCGGTCCCACAAAGATAAAGCTCC
Short var. (−226;+106), 334 bp	GGTACCGGTCCCTCCTTCACGCTTC
Reverse primer	GGTACCTATGATCGCTCGGTTTGCTAG
MCM2 (−1948; +57) 2005 bp	ATCCGAGGTGCATCCTTCAC
Short var. (−310; +57), 367 bp	CCGGCCTCTGTTGTCTTGT
Reverse primer	AGCAGTACCACGATCCTCTCC
PLK1 (−2338; +35), 2373 bp	GCAAGACTCCATCTCAACAACA
Short var. (−404; +35), 439 bp	CTTTGCGGTTCTAACAAGCTCTC
Reverse primer	CAGACCTCGATCCGAGCAG

[a]Promoter coordinates relative to transcription start site (TSS) of the gene. Two forward and one reverse primer were used for each promoter. Additional restriction sites are underlined.

pGL3 vector were controlled and avoided. Each promoter was amplified using one reverse and two different forward primers to produce long and short variants. Amplified promoters were cloned in pAL-TA vector (Evrogen, Russia) and recloned in pGL3 basic vector (Promega, Madison, WI) in front of gene *luc*. *POLD1* and *CKS1B* promoters were recloned by KpnI site, *CDC6* promoter by NheI site, *MCM2*-short promoter by NcoI/SacI site. EagI-fragment of promoter *PLK1*-long, EagI-fragment of promoter *PLK1*-short and EcoRI-fragment of promoter *MCM2*-long were treated with Klenow fragment of DNA polymerase I and recloned in pGL3 that was cut by NcoI and treated with Klenow fragment. Plasmid clones with promoters in necessary orientation were selected and verified by restriction analysis and sequencing. All resulting clones exactly matched to corresponding NCBI GeneBank sequences with the exception of long variants of *POLD1* and *MCM2* promoters which contained one and two nucleotides substitutions correspondingly.

CELL LINES AND TISSUES

Cell lines and tissues used in present work are listed in Table II. The cells were grown in DMEM/F12 (1:1) medium containing 10% fetal calf serum, 10 U/ml penicillin, 10 μg/ml streptomycin, and 0.25 μg/ml amphotericin (Invitrogen, USA) at 37 °C and 5% CO_2. Fibroblasts IVL-7NS were prepared from normal lung tissue adjacent to tumor according to a standard protocol [Adams, 1980; Kopantzev et al., 2010]. Tumor associated fibroblasts IVP-9TS were prepared from pancreas adenocarcinoma as described earlier [Kopantzev et al., 2010]. LLC (Lewis lung carcinoma) and S37 (murine sarcoma) cell cultures were established from mice transplanted tumors via culturing of collagenase-digested tumor tissue. LLC cells were maintained in DMEM/F12, 1:1 medium containing 10% fetal calf serum, 2 mM L-glutamine, 10 U/ml penicillin, and 10 μg/ml streptomycin. S-37 cells were maintained in RPMI 1640 medium containing 12,5% fetal calf serum, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 10 U/ml penicillin, and 10 μg/ml streptomycin. Tissue specimens were obtained from lung tumor surgery patients at the NN Blokhin Cancer Research Center of Russian Academy of Medical Science, Moscow, Russia, as described in an earlier report [Kopantzev et al., 2010].

TRANSFECTION

Transient transfections were performed in 24-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to recommendation of the manufacturer, in duplicates. Cotransfection with plasmid pRL-TK (Promega, Madison, WI) was used in all experiments as the internal control to minimize errors caused by the differences in transfection efficiencies in independent replicates. Parallel transfection of cells with pGL3 Basic Vector (pGL3-BV), pGL3 Promoter Vector (pGL3-PV) (Promega, Madison, WI), pGL3-CMV Pr/Enh Vector (pGL3-pCMV, containing AseI/BglII fragment of cytomegalovirus immediately early genes promoter from pEGFP-N1 plasmid (Clontech Laboratories, Inc) and *BIRC5* promoter (1,500 bp) cloned in pGL3 [Mityaev et al., 2010] were performed in each experiment. Forty-eight hours after transfection, cells were lysed and the activities of Renilla and firefly luciferases were measured using Dual-Luciferase Reporter Assay System (Promega, Madison, WI) using luminometer GENios Pro (Tecan, Switzerland). The obtained values of the luminescence for the firefly luciferase were normalized to the values for the Renilla luciferase, and data from duplicated wells were averaged. For each cell line under study, at least three independent transfections were performed, the data were corrected for background luminescence (pGL3-BV), normalized by SV40 promoter activity (pGL3-PV) and averaged. Resulting relative luminescence index was considered as measure of the promoter activity in given cell line. To estimate cancer specificity of each promoter, its activity in human cancer cells was additionally normalized by its activity in normal fibroblasts IVL-7NS.

ENDOGENOUS MRNA CONTENT ANALYSIS

Total RNA was purified from cells and tissues by standard procedure using guanidine isothiocyanate/phenol [Chomczynski and Sacchi, 1987]. Finally, RNA was purified with RNeasy Mini RNA Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendation. All RNA preparations were treated with DNAse I (Promega, Madison, WI) as described by the manufacturer. Purity and quality of RNA were tested by electrophoresis in 1% agarose gels with ethidium bromide. RNA was quantitated spectrophotometrically. The first cDNA strands were synthesized according to standard protocol using random

TABLE II. Cells and Tumors Used for Expression Analysis by qPCR

Cell line, tumor	Characteristics	Source
A375	Malignant melanoma	ATCC
A431	Epidermoid carcinoma of the skin	ATCC
A549	Lung carcinoma	ATCC
Calu-1	Lung epidermoid carcinoma	ECACC
Hep G2	Hepatocellular carcinoma	ATCC
HT-1080	Fibrosarcoma	ATCC
PANC-1	Epithelioid pancreatic carcinoma	ATCC
IVL-7NS	Primary normal lung fibroblasts	See text
IVP-9TS	Primary pancreas cancer associated fibroblasts	See text
S37	Murine sarcoma cells	See text
LLC	Lewis lung carcinoma	See text
LT_1	Adenosquamous carcinoma, T3N1M0	Biopsy[a]
LT_4	Squamous non-keratinizing lung cancer, T1N0M0	Biopsy
LT_13	Squamous keratinizing lung cancer, T3N0M0	Biopsy
LT_14	Squamous keratinizing lung cancer, T3N2M0	Biopsy
LT_311	Bronchioalveolar carcinoma, T2N1M0	Biopsy

[a]LN_1, LN_4, LN_13, LN_14, LN_311–morphologically normal lung tissue samples from the corresponding patients.

hexamer primer (Promega, Madison, WI) and PowerScript reverse transcriptase (Clontech Laboratories, Inc.). Quantitative real-time PCR was carried out with Roche LightCycler® 480 and with primers listed in Table III. All primer pairs were tested for cDNA specificity by determining length of amplification products and by direct sequencing of the products. PCR products accumulation was detected with SybrGreen® I using qPCRmix-HS SYBR reagent kit (Evrogen, Russia) and by end-point electrophoresis in 1% agarose gel. Each PCR was performed in triple repeats in 25 μL final volume containing 10 ng cDNA, 1xPCR buffer and enzyme mix and 5 pmol of each primer. The following thermal cycling conditions were used: a denaturation program (95°C for 5 min), an amplification program repeated 45 times (95 °C for 30 sec, 66 °C for 30 sec, and 72 °C for 60 sec), and a melting-curve program (65–97 °C with a warming rate of 0.11 °C per sec). Negative controls (no revertase samples and deionized water) were included in each run. Data analysis (relative quantification) and normalization were performed by Roche Light-Cycler® 480 Software taking into account real amplification effectiveness that was determined by LinRegPCR program [Ramakers et al., 2003]. 18s RNA, *GAPDH*, *ACTB,* and *CUSB* genes were used as references for mRNA content normalization.

STATISTICAL ANALYSIS

Statistical analysis of the data was performed using Prism 5 (GraphPad Software, Inc.), Statistica 8.0 (StatSoft, Inc.) and Excel 2010 (Microsoft, Redmond, WA) programs.

RESULTS

CLONING OF THE PROMOTERS

Promoter regions of five genes (*PLK1, CKS1B, POLD1, MCM2,* and *CDC6*) were cloned from human genome DNA as described in Materials and Methods section. The promoter of each gene was cloned in short (335–800 bp) and long (up to 2.3 kb) variants to cover probable location of core and whole promoter regulatory elements. Selected transcription factor binding sites known from literature data are shown on Figure 1.

TABLE III. Primers Used for Expression Analysis by qPCR

Gene	Sequence	Product length, bp
18s RNA	CGCGGTTCTATTTTGTTGGT ATGCCAGAGTCTCGTTCGTT	521
GAPDH	TTAGCACCCCTGGCCAAGG CTTACTCCTTGGAGGCCATG	541
ACTB	GAGCGGGAAATCGTGCGTGACATT GATGGAGTTGAAGGTAGTTTCGTG	234
GUSB	CTATTTCCACGGTGTCAACAAGC GATCACATCCACATACGGAGC	469
CDC6	CAAGAAGGCACTTGCTACCAG CCAGTTGATCCATCTCGTCCA	414
POLD1	CTGGTCCACCTTCATCCGTA GTTCACCAGCACCATGAGC	454
CKS1B	ACGAGGAGTTTGAGTATCGACA TCCTCCATCTGCCAAGTGTG	483
MCM2	CAGAACTACCAGCGTATCCGA TCACCACGTACCTTGTGCTTG	437
PLK1	CAGCACGTCGTAGGATTCCA TGGTTTGCCCACTAACAAGGT	417
BIRC5	GCGCCATTAACCGCCAGAT CAAGTCTGGCTCGTTCTCAGT	221

ACTIVITY OF THE PROMOTERS IN TRANSFECTED CELLS

Seven human and two murine cancer cell lines as well as cultured primary human fibroblasts from morphologically normal lung and from pancreas adenocarcinoma (Table II) were transfected with pGL3-based plasmids bearing cloned promoters and control plasmids. Chemiluminescence of lysates of cells were determined and data were normalized in a way described in Materials and Methods. All cloned promoters were able to drive luciferase expression in all cell lines (Fig. 2, Supp. 1, 2). CMV promoter in pGL3-CMV Promotor/Enhancer Vector that was used as positive control was 100–1,000 times more active than any of the other promoters. Activities of *CDC6* (2–55 fold) and *POLD1* (1.5–16 fold) promoters as short as long variants in most cancer cells were substantially higher than that of SV40 promoter (pGL3-PV) which was used as internal non-specific standard (Fig. 2A; Supp. 1). Both variants of *CDC6* promoters were substantially more active than *BIRC5* and other cloned promoters (Wilcoxon test, $P < 0.01$), while activities of *POLD1, CKS1B, PLK1,* and *MCM2* promoters were comparable to that of *BIRC5* promoter.

In general in vitro profiles of activity of particular long and short versions of promoters among various cell lines appeared to be very similar with the exception of *PLK1*-long whose activity in fibroblasts IVL-7NS was extremely low (Fig. 2A; Supp. 1). That means that short variants of all promoters contain practically all elements necessary for autonomous promoter activity.

In addition to estimate activity of cloned promoters, we aimed to evaluate their possible cancer specificity and to compare the promoters by this feature with recognized cancer specific promoter of *BIRC5* gene. For this purpose we compared activity of the promoters under study in cancer cells with their activity in primary human fibroblasts IVL-7NS and IVP-9TS (Fig. 2; Supp. 1, 2). Activity of all promoters under study with the exception of CMV promoter in cancer cells was significantly higher than that in normal fibroblasts IVL-7NS. It should be noted that activity of *CDC6*-long, *POLD1*, *CKS1B,* and *PLK1* promoters in IVP-9TS fibroblasts was higher than in fibroblasts IVL-7TS, in some cases reaching the levels of their activity in cancer cells (Fig. 2, Supp. 1, 2). Nevertheless, these as well as other promoters under investigation were significantly more active in most cancer cells than in IVP-9TS. We suppose that elevated activity of the promoters in pancreas cancer associated fibroblasts in comparison to fibroblasts form morphologically normal lung may be caused by tumor induced effects. Since fibroblasts isolated from tumor stroma may manifest some unusual features induced by adjacent transformed cells of the tumor, we supposed that fibroblasts IVL-7NS may be more adequate equivalent of normal tissue than IVP-9TS.

So, we estimated cancer specificity of each promoter as ratio of its activity in human only cancer cell lines to its activity in fibroblasts IVL-7NS (Fig. 2B, Supp. 2). The most cancer specific promoters were *PLK1*-long, *PLK1*-short, and *CKS1B*-short. These promoters were found to be significantly more specific than *BIRC5* promoter

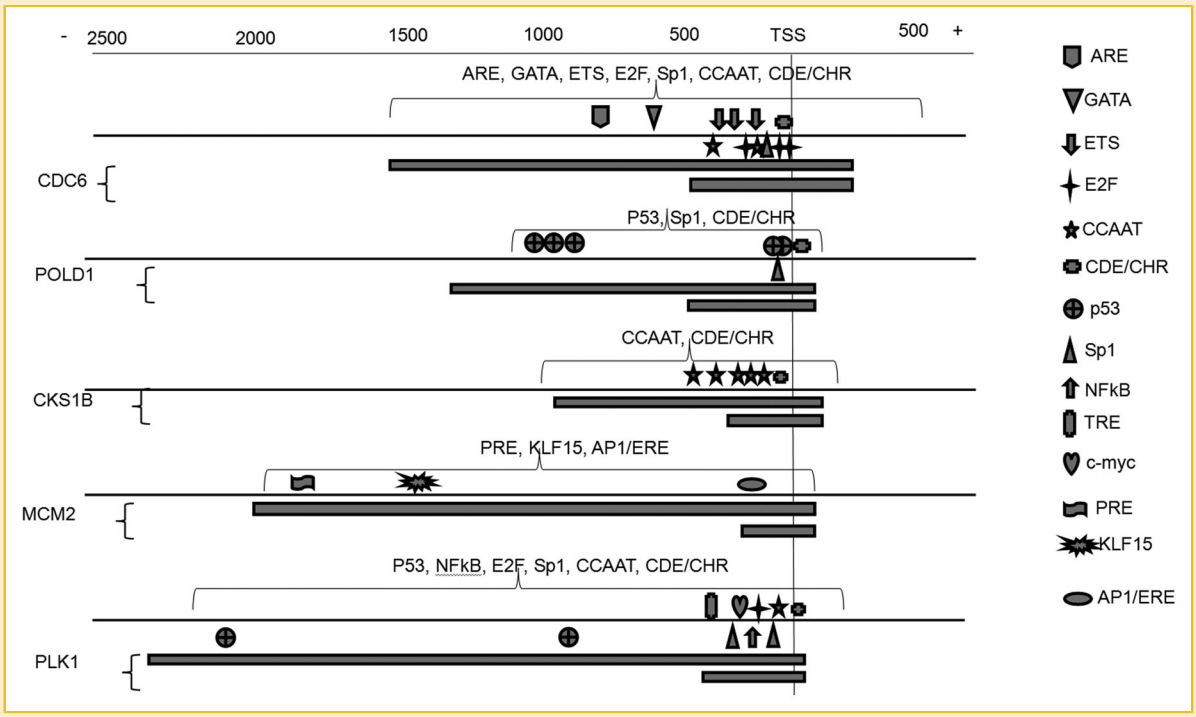

Fig. 1. Schemes and selected factor binding sites according to literature data for *CDC6* [Hateboer et al., 1998; Jin and Fondell, 2009], *POLD1* [Li and Lee, 2001], *CKS1B* [Rother et al., 2007], *PLK1* [Uchiumi et al., 1997; Zhu et al., 2002; McKenzie et al., 2010; Lin et al., 2011], and *MCM2* [Ray and Pollard, 2012] promoter regions. Long boxes correspond to promoter fragments cloned. Positions of known factor binding sites are indicated by symbols shown on the right of the figure.

(Wilcoxon test, $P < 0.05$) while other cloned promoters were comparable to BIRC5 promoter in their specificity. CMV promoter as one could expect was not specific at all, it expressed luciferase stronger in several cancer cell lines, and weaker in other lines than in fibroblasts.

Thus, all five promoters under study revealed cancer specific activity higher than or comparable to that of *BIRC5* promoter. These data also indicate that short promoter variants contain regulatory elements determining cancer specificity of transcription. Long promoter versions may contain extra regulatory elements that slightly reinforce (*PLK1*) or weaken (*CKS1B*) cancer specificity of the promoters.

ENDOGENOUS EXPRESSION OF CDC6, POLD1, CKS1B, PLK1 AND MCM2 GENES IN CELL LINES AND TISSUES

We examined endogenous expression of the genes in human cultured cells as well as in lung tumors and matching morphologically normal lung tissues (Fig. 3). Expression of cancer specific *BIRC5* gene was used for comparison. In general, expression levels of all genes in tissue samples were about tenfold lower than those in cancer cells. Genes *CDC6, CKS1B, MCM2, PLK1* as well as *BIRC5* in all seven cancer cell lines, and *POLD1* in six cancer cell lines (with the exception of PANC-1) expressed higher than in normal fibroblasts from both sources (Fig.3A). These results are consistent with data received in transfection experiments. Meanwhile only *CDC6* and *PLK1* as well as *BIRC5* genes expressed significantly higher in all lung tumors than in matching normal lung samples (Fig.3B). *MCM2* and *POLD1* genes demonstrated elevated expression in four and three tumors correspondingly. *CKS1B* mRNA level was elevated in two tumors only. So, the most cancer specific expression was found for *CDC6* and *PLK1* genes. *CKS1B, MCM2,* and *POLD1* genes showed high specificity of expression in cancer cell lines but lower specificity in lung tumors.

The data demonstrate that properties of a promoter may differ in vivo and in vitro and that there may exist additional genomic sequences in genome involved in the regulation of the promoters.

DISCUSSION

In spite of obvious complexity of structure and regulation of promoters in higher eucaryotes, a core promoter usually occupies no more than 100 bp upstream of the transcription start site (TSS), while most necessary transcription factor binding sites ensuring not only gene expression but regulation also may stretch not farther than ~500 bp from TSS [Suzuki et al., 2004; Taylor et al., 2006]. Especially this may concern evolutionary conserved promoters of genes involved in basic cell functions such as control of the DNA replication and the cell cycle.

In this research we have checked our hypothesis that promoters of the genes involved in cell proliferation control should be more active in cancer cells than in normal ones. For this purpose we cloned

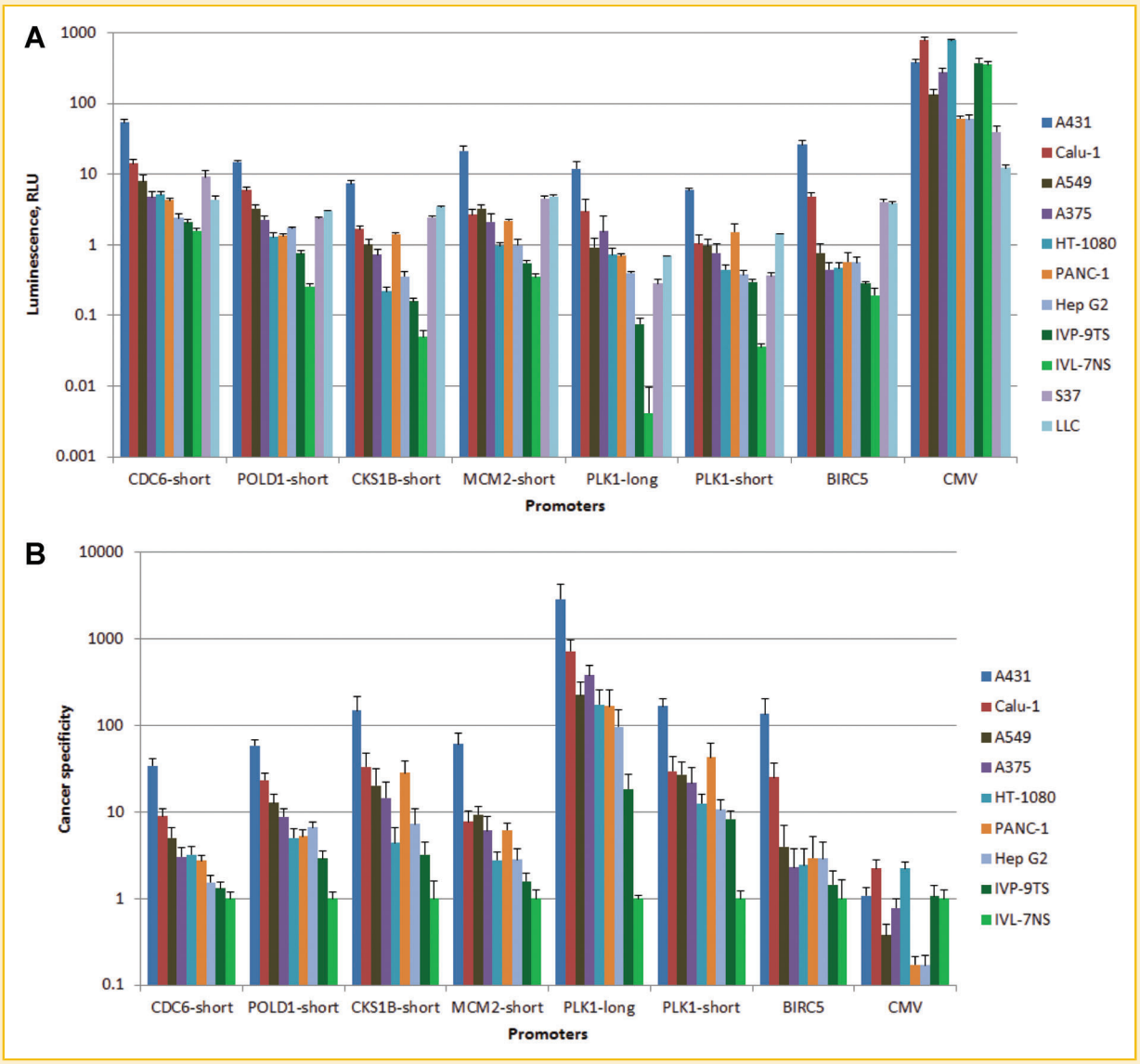

Fig. 2. Activity and cancer specificity of cloned promoters. A: Normalized chemiluminescence of lysates of human and murine cells transfected by plasmids with the corresponding promoters. The mean values and standard error of the means (SEM) are presented in logarithmic scale. B: Cancer specificity of the promoters expressed as ratios of the chemiluminescence levels of the lysates of human cancer cells and fibroblasts IVL-7NS for each promoter. With the exception of *PLK1* only results concerning short promoters are shown. Other promoters are presented in the Supplementary Material (Supp.1, 2).

promoters of *CDC6*, *POLD1*, *CKS1B*, *MCM2*, and *PLK1* genes in pGL3 vector so that the luciferase gene was expressed under control of the cloned promoters. Promoter of each gene was cloned in short and long variants so that core promoter and more distal trancription regulation elements were included. We transfected cancer and normal cells of different origin with constructed vectors and measured activity of luciferase synthesised under control of the cloned promoters. Preliminary information on cancer specificity of the long forms of these promoters was published by us earlier [Kashkin et al., 2013]. In the present work we compared long and short forms of the promoters in extended series of cell lines including murine cells. The data on the activity of the promoters were collated with the levels of the expression of corresponding genes in cell lines and in tissue specimens. Also we planned to estimate the perspectives of the use of the cloned promoters for transgene expression in cancer gene therapy.

The schematic presentation of the promoters used and known literature data concerning selected transcription factor binding sites are shown in Figure 1. As it shown in Figure 2 (Supp. 1, 2), all cloned promoters were able to produce functional luciferase as in human as in murine cells, that conforms to multiple data on evolutionary conservativeness of basic proliferation mechanisms. This observation let us hope that artificial genetic constructs with the promoters under study may be tested on animal models at least for safety,

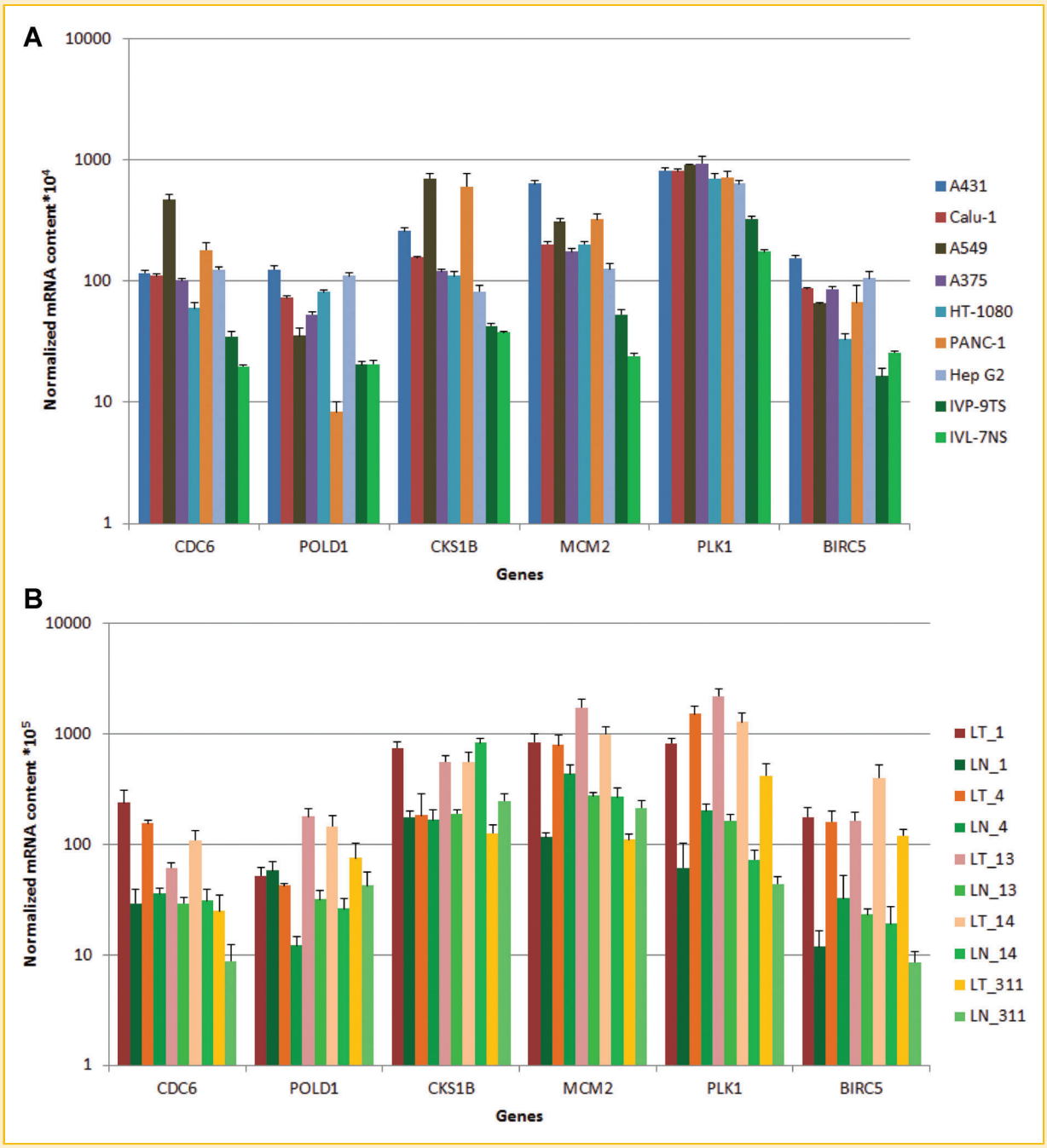

Fig. 3. Expression levels of eight genes in cultured cells (A) and in lung tumors (B), logarithmic scale. LT, tumor; LN, morphologically normal lung tissue of the same patient. Mean values and standard errors of the mean (SEM) are presented. mRNA content was normalized by expression of four housekeeping genes (see Materials and Methods).

though behavior of such constructs in animals and in humans may differ in details.

The common and well known problem in analysis of promoters is a choice of appropriate controls, especially cell controls. Widely used immortal cell cultures, streakly speaking, may not be taken as normal, while most types of really normal cells from human organism hardly may be cultivated and transfected. So, we tested HUVEC (human endothelial cells from umbilical cord veins), NHK (normal human keratinocytes) and primary human fibroblasts from two sources. IVL-7NS fibroblasts were isolated from morphologically normal lung and IVP-9TS fibroblasts were isolated from panceras cancer stroma. We also introduced a control of cancer specificity of expression using for this purpose a promoter of *BIRC5* (survivin) gene cancer specific activity of which was demonstrated elsewhere [Chen et al., 2004; Zhu et al., 2004; Mityaev et al., 2010]. We revealed that *BIRC5* promoter was as highly active in HUVEC and

NHK cells as in cancer cells (data not presented). Meanwhile activity of *BIRC5* promoter in both types of fibroblasts was significantly lower than in cancer cells (Fig. 2A, Supp. 1). So, only fibroblasts may serve as controls for cancer specificity of the promoters under study. Previously both types of fibroblasts were qualified as normal because they may sustain only limited number of passages in culture, and a mutational analysis did not reveal known mutations of the TP53 and KRAS2 genes in both types of fibroblasts [Kopantzev et al., 2010]. Since fibroblasts IVP-9TS were isolated from tumor stroma and activity of the promoters under investigation in these cells was higher than in IVL-7NS, we supposed that fibroblasts IVL-7NS should be taken as more adequate equivalent of normal tissue than IVP-9TS.

CDC6 gene and its promoter have been studied quite intensively [Zhou and Jong, 1990; Hateboer et al., 1998; Robles et al., 2002; Mallik et al., 2008; Jin and Fondell, 2009]. The expression of CDC6 reaches maximum in G1/S and is downregulated during S and G2/M. *CDC6* gene promoter contains a variety of putative and experimentally proved regulatory sites. Taking into account the literature data, we cloned long variant of *CDC6* promoter as [−1539; + 238] and short variant as [−565; + 238]. In transfection experiments we proved that short *CDC6* promoter is as highly active as the long one and has similar cancer specificity. Both variants of *CDC6* promoters possess significantly higher activity than the promoter of *BIRC5* gene and have comparable cancer specificity with the last one. This may indicate that at least some regulatory elements determining activity and cancer specificity of the promoter in cultured cells are located not farther than −565 base pairs from TSS. The features of *CDC6* promoter conforms well to profiles of *CDC6* gene expression in cells and lung specimens (Fig. 3). Some elements such as GATA or ARE that were not included in short *CDC6* promoter seemed to be not involved in regulation in our experiments but they may be important in other conditions. So, both long and short *CDC6* promoters are promising candidates for therapeutical gene expression in cancer gene therapy.

Promoter of human *POLD1* gene was studied earlier [Zhao and Chang, 1997; Li and Lee, 2001; Song et al., 2009]. Long and short *POLD1* promoters cloned by us demonstrated very similar activity that corresponds to data of Zhao et al [Zhao and Chang, 1997]. Moreover, both promoters were found to be almost equally and highly specific in their ability to express luciferase gene in cancer cells. So we may conclude that most important regulation of the promoter that became apparent in transfection experiments is limited to [−502; + 66] fragment of *POLD1* gene, and that two distant p53 binding sites [Li and Lee, 2001] may not play a role in our conditions.

Though *POLD1* promoters demonstrated higher activity than *BIRC5* promoter in several human cancer cells, the overall difference between these promoters was not significant. Both *POLD1* promoters were close to *BIRC5* promoter by their specificity also. Endogenous expression of *POLD1* gene was elevated in 6/7 human cancer cell lines and in 4/5 of lung tumors, and we consider it as rather high cancer specificity of the expression.

Two variants of *CKS1B* promoters differ from each other by number of CCAAT sites (see [Rother et al., 2007]). Both promoters were approximately as active as *BIRC5* promoter. *CKS1B*-short promoter was found to be more cancer specific than *BIRC5* promoter by Wilcoxon test ($P < 0.05$; Fig. 2B; Supp. 2). At the same time, cancer specificity of *CKS1B*-long promoter was comparable with that of *BIRC5* promoter. The difference between two forms of *CKS1B* promoter in their specificity may arise from the fact that activity of *CKS1B*-short promoter in normal fibroblast IVL-7NS were twice lower than that of the long form, while in cancer cells the two promoter forms worked approximately equally. This indicate that two CCAAT or some other regulatory elements located in [−910;−226] region of *CKS1B* promoter may contribute to enhancing of the transcription of the gene in normal cells but not in cancer cells. Expression of *CKS1B* gene in all human cancer cells was significantly higher than that in normal fibroblasts. Meanwhile, only in two lung tumors the expression of the gene was higher than in the corresponding normal tissue that one may estimate as absence of specificity. So, regulation of *CKS1B* gene in cultured cancer cells may differ from that in tissues.

The data on *MCM2* gene promoter in literature is rather limited, so we cloned two variants of the promoter getting our bearings to studies by Ray and Pollard (2012) [Ray and Pollard, 2012]. *MCM2*-short promoter was more active than the long form in all cells under study ($P < 0.01$, Wilcoxon test), but cancer specificity of the two forms turned out to be almost equal. So one may suppose that regulatory elements determining cancer specificity are concentrated in the short form of *MCM2* promoter, while the long form may contain additional regulatory element(s) that influence promoter activity. Activity and specificity of both *MCM2* promoter forms approximately matched those of *BIRC5* promoter. The results of transfection experiments correspond to elevated expression of *MCM2* gene in all cultured cancer cells and in 4/5 lung tumors.

A number of regulatory elements was discovered in *PLK1* promoter [Uchiumi et al., 1997; Zhu et al., 2002 McKenzie et al., 2010; Lin et al., 2011] (Fig. 1). It was shown that downregulation of *PLK1* expression by p53 occurs directly by transcriptional repression mechanism through two p53 binding sites at [−2067;−2016] and at [−816;−785], and indirectly through CDE/CHR element [McKenzie et al., 2010]. So short variant of *PLK1* promoter cloned by us contained most elements close to TSS of the gene while *PLK1*-long promoter additionally included two p53 binding sites. *PLK1* long and short promoters were similar to each other and to *BIRC5* promoter in their activity. But the long form of *PLK1* promoter exhibited very low activity in IVL-7NS fibroblasts, so cancer specificity of *PLK1*-long promoter was estimated as the highest among the promoters under study. Both *PLK1* promoters were significantly more specific to cancer than *BIRC5* promoter ($P < 0.05$, Wilcoxon test).

Given that the cells in culture divide much more intense than in most tissues of the living organism, and that the proliferative index of cells in normal tissues with the exceptions of hematopoietic cells and growth plates is very low, we suspect that the activity and specificity of promoters, as defined in our experiments, may be considerably underestimated. Further work with the use of some of the promoters in real gene therapeutic experiments will allow to compare the present in vitro data with in vivo those. However, our results allow to make some preliminary choice of the promoters for

such experiments—these are for example CDC6-short and *PLK1*-long promoters. All the data were received by transient transfection experiments, which is one of the important strategies of gene therapy (see for example database "Gene therapy. Clinical trials worldwide" provided by the Journal of Gene Medicine http://www.wiley.com//legacy/wileychi/genmed/clinical, updated by July 2013). However, from point of view of the deeper insight into mechanisms of the promoter regulation in vivo it is desirable to investigate the promoters further in chromatinized state that is now in progress in our laboratory.

It should be noted that we used cells with different status of p53 gene in transfection experiments. A431 and PANC-1 cells have mutations of the p53 in codon 273, Calu-1 cells have homozygous deletion of the gene, and four other cancer cell lines (A375, A549, Hep G2, HT-1080) as well as normal fibroblasts have wild type p53. The p53 status of tissues was uncertain. It is known that approximately 50% of human cancers bear lesions of p53 gene [Soussi and Wiman, 2007] while another half of tumors keep wild type p53. So, promoters that are not dependent on p53 status but retain cancer specificity seem to be most perspective from the point of view of cancer gene therapy. We have strong indications that all promoters under study, long and short variants, may come under the influence of p53 in some way. Promoters of *POLD1* [Li and Lee, 2001], *PLK1* [McKenzie et al., 2010] and *CKS1B* [Rother et al., 2007] genes were proved to be regulated by p53 directly or indirectly. It is very probable that *MCM2* promoter is repressed by p53 too because it contains p53 binding site(s). Moreover, direct repression by p53 was demonstrated earlier for promoter of *MCM7* gene which encodes one of the MCM2–7 complex proteins [Guida et al., 2005], and proteins acting in one complex are likely regulated synchronously and in the same manner. Direct repression of *CDC6* promoter by p53 was not shown but it is known that p53 regulates many genes involved in cell division control and may act directly or indirectly through interaction with CCAAT- and CDE/CHR-binding factors. In our transfection experiments cloned promoters manifested cancer specificity in cells with mutated or lost p53 (A431, Calu-1 and PANC-1) and in cells with wild type p53, and in cells of different origin. Though the promoters sometimes exhibited higher activity in cells with malfunction of p53 than in cells with wild type p53, this tendency was neither strict nor significant. So, we consider the promoters cloned by us as universal and cancer specific. Nevertheless, we suppose that the influence of p53 on the properties of the promoters under study as well as analysis of the most active and cancer specific length of the promoters need more research.

We demonstrated that both short and long variants of all five promoters exhibited activity and cancer specificity comparable to or higher than those of *BIRC5* gene promoter. Our data testify to the fact that a promoter may manifest different properties in vitro and in vivo. So, the results show the possibility that short (~330–800 bp) proximal promoter may determine specificity of the transcription while more distant or remote genome elements may regulate promoter activity also representing higher levels of regulation.

The cancer specificity of the short promoters involved in the cell proliferation is an interesting phenomenon from an evolutionary point of view. The multicellular organisms gained in the course of evolution the hierarchical regulatory organization where the upper levers (tissues, organs) put strict constraints on the regulation of the lower level (cells, networks, pathways) [Noble, 2012]. One of such constraints may be the suppression of the proliferation capacity to make it harmonized with the proliferation of the surrounding cells. The transition from ordered normal tissue to simplest Metazoa-like tumor [Davies and Lineweaver, 2011] or to chaotic-evolving multitude of heterogenous, competing for resources cancer cells [Merlo et al., 2006; Sharma and Dey, 2011] probably removes these constraints revealing the intrinsic evolutionary conserved proliferation potentials of promoters involved in the cell proliferation.

Intrinsic cancer specificity of core promoters of the genes regulating cell division machinery may be related to one of the main features of a tumor cell in contrast to a normal one—the ability to divide uncontrollably. From practical point of view this feature may be useful for so called transcriptional targeting in cancer gene therapy when a cancer specific promoter restrict expression of a therapeutic gene only within cancer cells thus diminishing side effects due to undesirable expression of the gene in normal tissues. Some promoters described in this study are stronger and more cancer specific than one of the most widely used for this purpose *BIRC5* promoter. This information also defines a field for search of other promoters as means of the expression of therapeutic genes in tumors cells.

CONCLUSION

We proved that all cloned promoters can express heterologous transgene in human and murine cells, and in cancer specific manner. Our data argue for our hypothesis about intrinsic tumor specificity of promoters of genes controlling DNA replication and cell proliferation, and offer prospects for search of new promoters for cancer gene therapy. Though promoters under study were weaker than widely used CMV immediately early genes promoter, two of them (*CDC6, POLD1* promoters) were stronger than SV40 promoter and had similar (*POLD1, CKS1B, MCM2, PLK1* promoters) or even higher (*CDC6* promoters) activity than recognized cancer specific promoter of *BIRC5* (survivin) gene. The most active *CDC6* promoter looks as most promising in our conditions. The *PLK1*-long promoter was superior to other promoters in cancer specificity, but its usability in artificial therapeutical vectors should be tested especially because it may be limited due to the long size of the promoter. *CKS1B*-short promoter also demonstrated higher cancer specificity than *BIRC5* promoter being approximately as active as the last one. We suppose that elevated activity of cloned promoters in tumor associated fibroblasts is caused by tumor-induced effects, so we hope that the promoters may be used not only against cancer cells but against tumor stroma too. Obviously, this assumption should be investigated specially. Besides, properties of isolated promoter in artificial vector system may differ from its behavior in natural genetic and biological environment due to the absence of many regulatory factors governing the promoter in its host cell. Thus, we believe that cloned promoters may be promising candidates for use in cancer gene therapy after optimization and comprehensive study in vivo.

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