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Enhancer Element Potentially Involved in Human Survivin Gene Promoter Regulation in Lung Cancer Cell Lines

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Abstract—We have revealed evolutionarily conserved regions in a 4500-bp DNA sequence 5′-adjacent to the survivin (*BIRC5*) gene. In the transcribed region of the *BIRC5* gene we have detected and characterized in detail a 3′-fragment of the CpG island that stimulated in enhancer-like way the gene promoter activity in normal cells and in a number of cancer, in particular lung cancer, cell lines. When added to the initial 1498-bp survivin promoter region, a transcribed DNA fragment of a CpG island approximately twofold enhanced the promoter activity in cancer cells and in normal lung fibroblasts. The observed effect did not depend upon the orientation of the fragment and distances between the fragment and the transcription initiation site. In the case of a heterologous SV40 virus promoter, the effect was less pronounced. In addition to earlier reports, the results provide new information on the *BIRC5* gene expression regulation and also demonstrate that this gene exon sequences can play a significant role in *BIRC5* gene expression regulation. The data provide another possibility to increase survivin promoter activity without changing its cancer specificity for application in cancer (in particular, lung cancer) gene therapy.

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Key words: regulation of promoter activity, survivin, *BIRC5* gene, enhancer, *cis*-element, conserved sequence

Gene therapy is one of the approaches that are on trial as a strategy to treat cancer, and, in particular, lung cancer, which is the leading cause of cancer deaths worldwide. The success of cancer gene therapy mainly depends on cancer-specific expression of therapeutic genes to destroy tumor cells. Such specific expression can be directed by transcription promoters that are active in tumors but not in normal cells. A number of such promoters have been described in recent reviews [1-4], among them a promoter regulating transcription of the *BIRC5* gene that encodes survivin. Survivin is a member of the inhibitor of apoptosis proteins (IAP) family and one of the central players in the tumor formation process [5]. This promoter meets almost all properties of ideal therapeutic promoters: it is highly tumor-specific and it is active in the great majority (80-85%) of tumors [6-8], thus opening a possibility for its use in the development of new anticancer gene therapy drugs [9]. However, similar to most tumor-specific promoters [9-11], it has lower activity in comparison with such promoters as the constitutive CMV and SV40 promoters with their enhancers. According to different authors, the survivin promoter activity in different cell lines varies from 0.3 to 16% of the CMV promoter activity [10, 12-14]. As a result of this, the suitability of the survivin promoter for cancer gene therapy is questioned by a number of authors [11, 13], although the majority are of an opposite opinion [9, 10, 14-16]. Therefore, it is highly desirable to reconstruct the promoter to enhance its activity while remaining strictly cancer-specific. It might be achieved knowing the promoter elements essential for regulation of its activity.

It was supposed that the promoter region of the *BIRC5* gene extends from ~1400 bp upstream to about 40 bp downstream of the gene transcription start site [17]. This DNA stretch contains many sites involved in interaction with various transcriptional regulators [18], such as CDE (cell cycle dependent element), probably CHR (cell cycle gene homology region) [19], as well as different transcription factor binding sites predominantly located in a non-translated region −250 ... +70 relative to the transcription start point. The *BIRC5* gene promoter is repressed by pRb and p53 tumor suppressors [20, 21], as well as by the Egr1 transcription factor [22]. In contrast,

Abbreviations: *BIRC5*, survivin gene; CDE, cell cycle-dependent element; NSCLC, non-small cell lung cancer; SEM, standard error of the mean.

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pro-oncogenic factors c-myc [23], TCF4 (complexed with β-catenin), and Stat3 and the transcription factors of the E2F subfamily activate expression of the *BIRC5* gene [5]. The transcription factors Sp1 [17, 24], KLF5 [25], DEC1 [26], and HIF-1α [27] are also known to take part in the activation of the survivin gene promoter. Of the transcription factors listed above, Sp1, KLF5, c-myc, HIF-1α, TCF4, Stat3, pRb, p53, and Egr1 have functional binding sites in the *BIRC5* promoter region, which suggests their possible implication in direct control of *BIRC5* expression. Although the available data contain extensive information on the *BIRC5* gene transcription regulation, there is still much to be done to gain a comprehensive understanding of the mechanisms conferring tumor specificity on the survivin gene expression, which is necessary to construct modified promoter with improved properties for its applicability for cancer (in particular lung cancer) gene therapy.

With the purpose of creation of a highly active tumor-specific promoter for non-small cell lung carcinoma (NSCLC) gene therapy, we aimed to identify functional regulatory sequences within the promoter of *BIRC5* that might be responsible for enhanced expression levels of this gene in tumor cells. An *in silico* comparative analysis of the genomes of human, chimpanzee, mouse, and dog revealed three conserved regions in the 5′ non-transcribed region of the *BIRC5* gene. These regions were characterized and shown to affect the *BIRC5* promoter activity. A region within the CpG island was found to possess enhancer activity. Using a DNA fragment containing this region, we constructed a modified survivin promoter with enhanced promoter activity in most lung cancer cell lines under study, which are characterized by low level or absence of p53 protein function.

MATERIALS AND METHODS

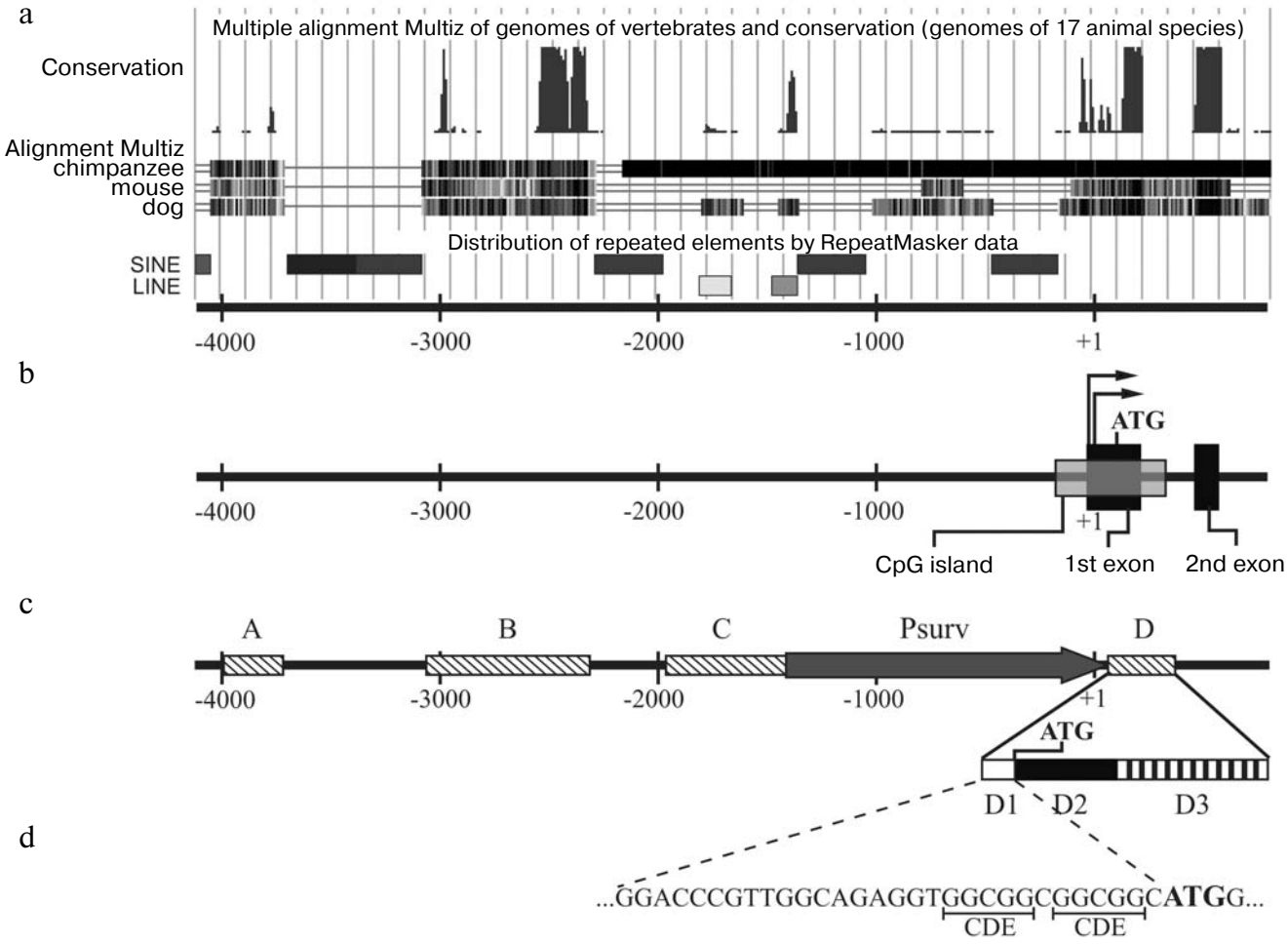
Cell cultures. The following cancer cell lines were used: A549 (lung carcinoma, LC), Calu1 (epidermoid lung carcinoma, ELC), NCI-H23 (lung adenocarcinoma, LAC), NCI-H358 (bronchoalveolar lung carcinoma, BLC), and HeLa (cervical adenocarcinoma, CAC). Fibroblasts IVL-11NS (NLF) were obtained according to a standard protocol [28] from normal lung tissue adjacent to tumor (the specimen was obtained from a lung tumor surgery patient at the Vishnevsky Surgery Institute). The cells were grown in DMEM/F12 (1 : 1) medium containing 10% fetal calf serum, 60 μg/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (Invitrogen, USA) at 37°C and 5% CO_2.

Isolation of genomic DNA. Genomic DNA from the specimen of tumor tissue was isolated using a Wizard Genomic DNA Purification Kit (Promega, USA) as described previously [18].

PCR amplification and cloning of upstream and intragenic fragments of the *BIRC5* gene. The survivin promoter from nucleotide −1456 to +42 (the transcription initiation point was taken as +1) was PCR amplified, cloned in pGL3-BV vector, and sequenced as described earlier [18]. The A, B, C, and D fragments (see Scheme 1) were PCR amplified using isolated genomic DNA as a template. The D1′, D2, and D3 fragments were PCR amplified using plasmid pGL3-Psurv-D as a template. Since the D1 fragment was too short (only 30 bp), we amplified the D1′ fragment composed of D1 and the survivin promoter fragment used for preparing plasmid pGL3-Psurv-D1 (see below). The primers used for amplification, primer annealing temperatures (T_{an}), and sizes of the amplified fragments are listed in the table. To substitute the ATG initiation codon for TTG in the amplified D fragment, the SurvD-R primer contained an A→T substitution. The PCR reaction mixture contained 25 ng of genomic DNA or 5 ng of plasmid DNA in 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 2.5 mM $MgCl_2$, 0.25 mM each of dNTPs, 0.2 μM each of primers, and 1 U Taq DNA-polymerase (State Research Institute of Genetics and Selection of Industrial Microorganisms, Russia). DNA in the reaction mixture was pre-denatured at 95°C for 2 min and then PCR amplified for 30 cycles of DNA denaturation at 95°C for 45 sec, primer annealing at T_{an} temperature (see table) for 30 sec, and elongation at 72°C for 60 sec. The final elongation step was at 72°C for 5 min. After completion of the reaction, the amplification products were separated electrophoretically in 1% agarose gels and then eluted using a Wizard SV Gel and PCR Clean-Up System (Promega) and cloned in the pGEM-T vector (Promega). Plasmids with inserts of the amplified fragments were isolated from *Escherichia coli* clones using a Wizard Plus SV Minipreps kit (Promega). The structure of the amplified fragments within plasmids pGEM-T/Psurv, pGEM-T/A, pGEM-T/B, pGEM-T/C, pGEM-T/D, pGEM-T/D1′, pGEM-T/D2, and pGEM-T/D3 was confirmed by sequencing.

Plasmid preparation. For gene regulation studies, the survivin promoter was cloned into a promoterless reporter pGL-BV vector. To this end, plasmid pGEM-T/Psurv with the insert of the 1498-bp survivin promoter was used. This plasmid was digested with *Bgl*II and *Hin*dIII restriction enzymes (Fermentas, Lithuania), the digestion products obtained were electrophoretically separated in a 1% agarose gel, and the required DNA fragments were eluted from the gel and ligated to a pGL3-BV vector (Promega) pre-digested with the same restriction enzymes. As a result, the pGL3-Psurv plasmid was obtained and used for preparing derivative constructs.

To study the effect of A, B, C, and D putative regulatory sequences on the survivin promoter activity, they were cloned into the pGL3-Psurv plasmid. The orientation and relative positions of the cloned fragments and promoter were the same as in human genomic DNA. To obtain plasmid pGL3-Psurv-D (Scheme 2), the D fragment was excised from plasmid pGEM-T/D with *Nco*I



Structure of the human survivin (*BIRC5*) gene promoter region. a) Distribution of conserved sequences and repeat elements in the human survivin gene promoter region. The height and density of the vertical bar clusters in the "Conservation" field reflect the conservatism of DNA sequences. Horizontal bars in the "Alignment Multiz" field illustrate a multiple alignment of human genomic DNA sequences with those of chimpanzee, mouse, and dog. Matches are marked with short vertical bars that form together hatched rectangles corresponding to homologous regions. Below is shown the distribution of SINE and LINE repeat elements. b) Location of the CpG island relative to the two known transcription initiation sites marked with bent arrows. c) Location of cloned potential regulatory sequences (A, B, C, and D). D1 is a non-translated region of the first exon, D2 is a translated part of the first exon, and D3 is a part of the first intron. Psurv is a *BIRC5* gene promoter. d) The nucleotide sequence of the D1 fragment (bordered by dotted lines). Two putative CDE regulatory elements are marked. The ATG translation initiation codon of *BIRC5* is marked in bold. The coordinates are relative to the first transcription initiation point

Scheme 1

and *Sma*I (Fermentas) restriction enzymes, its sticky ends were filled in with Klenow fragment, and the D fragment was blunt cloned into pGL3-Psurv pre-digested with *Hin*dIII and treated with Klenow fragment, adjacent to the 3′ end of the survivin promoter. To prepare plasmids pGL3-C-Psurv-D, pGL3-BC-Psurv-D, and pGL3-ABC-Psurv-D, the C, B, and A fragments, taken from plasmids pGEM-T/C, pGEM-T/B, and pGEM-T/A, respectively, were inserted into pGL3-Psurv-D. The C fragment was inserted between the *Bgl*II and *Nhe*I sites, the B fragment – between the *Nhe*I and *Sac*I sites, whereas the A fragment was inserted into the *Kpn*I site. The plasmids with inserts, oriented relative to the Psurv promoter as in genomic DNA, were selected.

To study the enhancer activity of the D fragment, four plasmids (pGL3-Psurv-DFor, pGL3-Psurv-DRev, pGL3-PV-DFor, and pGL3-PV-DRev) were prepared. pGL3-Psurv-DFor and pGL3-Psurv-DRev were prepared by cloning the D fragment into plasmid pGL3-Psurv with the survivin promoter, while for pGL3-PV-DFor and pGL3-PV-DRev this fragment was cloned into pGL3-PV (Promega) containing the SV40 early promoter (see Scheme 2). To this end, pGL3-Psurv and pGL3-PV plasmids were digested with *Sal*I (Fermentas), treated with Klenow fragment, and ligated to the D fragment excised from the pGEM-T/D plasmid. In this instance, the recombinant plasmids with inserts in the direct and reverse orientation with respect to the luciferase gene were selected.

Primers used for amplification of analyzed fragments

Amplified fragment	Primer name	Primer sequence 5′ to 3′	T_{an}, °C*	PCR product size, bp
A	SurvA-F SurvA-R	GGTACCGATCTGTTCGCCTGACATCCTG GGTACCAGGCAGAAAGGCAGAAGAGTC	62	281
B	SurvB-F SurvB-R	GAGCTCCTATGTTCGTTCTCTCACAGCC GCTAGCCCTAACTCCTTTTCACTTCTGC	62	761
C	SurvC-F SurvC-R	GCTAGCGTTCCTTTCCTCCCTCCTGA AGATCTACCCTTCACCCAGATTTTCTG	62	540
D	SurvD-F SurvD-R	CCCGGGACCCGTTGGCAGAGGTGGCG GCGGCGGCTTGGG** TAAGCTTCCTCGATGGGGACAAAGCAG	70	326
D1'	SurvD1'-F SurvD1'-R	TAGCAATGGCACAATCTCAGCTC ATAAGCTTAAGCCGCCGCCGCCACCTC	60	539
D2	SurvD2-F SurvD2-R	ATCCCGGGCTTGGGTGCCCCGACGTTG TTAAGCTTCGCTCCGGGGTGCAGGCGC	60	127
D3	SurvD3-F SurvD-R	TACCCGGGAGACTGCCCGGCCTCCTGG	60	198

* Annealing temperature used in PCR is shown.
** Mutated ATG codon is underlined.

To estimate the regulatory effect of the D1, D2, and D3 fragments on the survivin promoter activity, pGL3-Psurv-D1, pGL3-Psurv-D2, and pGL3-Psurv-D3 plasmids were prepared, each containing the insert ligated to the 3′ end of the *BIRC5* gene promoter.

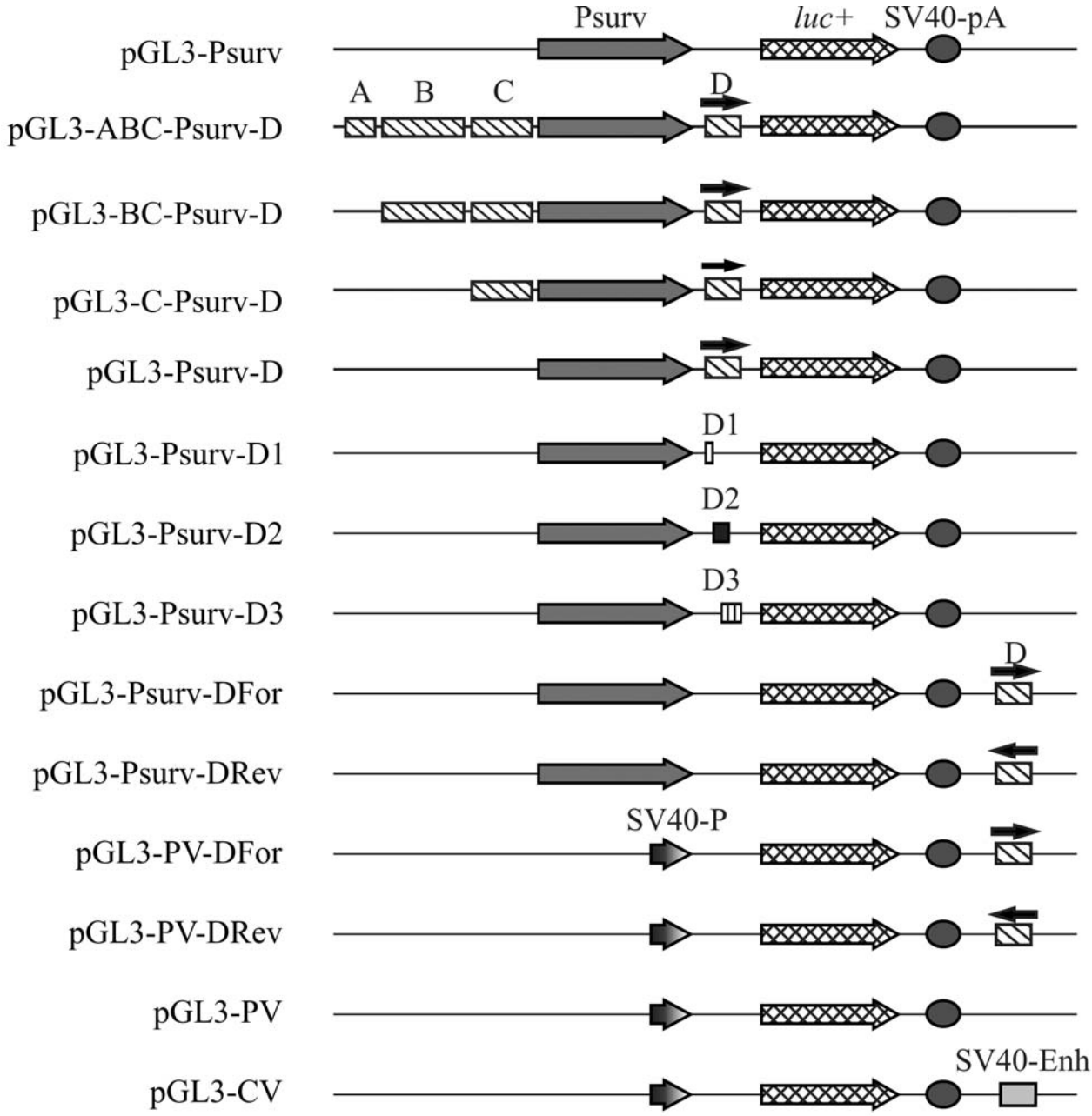
The plasmid pGL3-Psurv-D1 was prepared as follows: a 504-bp fragment excised from pGEM-T/D1′ with *Bst*API (Sibenzyme, Russia) and *Hin*dIII restriction enzymes was ligated to the *Bst*API and *Hin*dIII sites of the 5829-bp fragment excised with the same enzymes from pGL3-Psurv.

The D2 and D3 fragments were excised with *Sma*I and *Eco*RI (Promega) from plasmids pGEM-T/D2 and pGEM-T/D3, respectively, and then filled in with Klenow fragment and ligated into pGL3-Psurv linearized with *Hin*dIII and treated with Klenow fragment. The plasmids pGL3-Psurv-D2, and pGL3-Psurv-D3 with inserts, oriented relative to the Psurv promoter as in genomic DNA, were selected.

The prepared plasmids were propagated in *E. coli* and isolated with a yield of 100-150 μg using a QIAGEN Plasmid Midi Kit (Qiagen, Germany).

Cell transfection. Cells were transfected in 24-well plates with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's recommendations. Plasmid DNA (0.88 μg per well) containing a reporter plasmid with inserted firefly luciferase gene and a normalization plasmid pRL-TK (Promega) in the ratio of 10 : 1 was used for transfection. Cells were grown in antibiotic-free DMEM/F12 (1 : 1) medium containing 10% fetal calf serum for 48 h, and activities of firefly and *Renilla reniformis* luciferases were measured in cell extracts using the Dual-Luciferase Reporter Assay System (Promega) on a GENios Pro luminometer (Tecan, Switzerland). In parallel experiments, the cells were transfected with the promoterless plasmid pGL3-BV and plasmid pGL3-PV (positive control) containing the firefly luciferase gene under the control of the SV40 virus early promoter. The plasmid pRL-TK that provided a constitutive expression of *R. reniformis* luciferase was used as an internal control for reducing the error due to different efficiency of transfection in a series of independent experiments. The firefly luciferase activity values were normalized to the values of *R. reniformis* luciferase activity. At least three independent transfections were performed for each experimental construct.

Bioinformational analysis. Nucleotide sequences were analyzed through means available at http://genome.ucsc.edu. Conserved DNA sequences were searched using calculated evolutionary conservation scores in 17 vertebrates and the phastCons program [29] based on a phylogenetic hidden Markov model. DNA sequences of the human, chimpanzee, mouse, and dog genomes were aligned using the Vertebrate Multiz alignment [30]. CpG islands were searched based on standard criteria [31]. The arrangement of genomic repeat elements was determined with the RepeatMasker program (http://www.repeatmasker.org) from the UCSC Genome



Plasmid constructs used in this work. All plasmid constructs are derivatives of pGL3-BV. For simplicity, only structures of expression cassettes with the luciferase reporter gene are shown. Arrows show the direction of the sense strand in the cloned fragments. Psurv, *BIRC5* gene promoter (see Scheme 1 for coordinates); *luc*+, firefly luciferase gene; SV40-P, SV40 virus early promoter; SV40-Enh, SV40 enhancer; SV40-pA, SV40 polyadenylation signal; A, B, and C, conserved DNA sequences of the 5′ non-transcribed region of the *BIRC5* gene

Scheme 2

Browser package. Percentage identity (PID) was calculated as described by Doolittle [32]:

$$PID = (A/(B + C)) \times 100\%,$$

where A is identical positions, B is aligned positions, and C is internal gap positions.

Statistical analysis. Mean and standard deviation values were calculated using Excel software (Microsoft, USA). The significance of the difference between values of promoter activity was estimated with Student's t-test assuming that the value distribution is normal. The values were assumed to be statistically different at $p < 0.05$.

RESULTS AND DISCUSSION

Comparative analysis of the *BIRC5* gene nucleotide sequences in various mammals. Earlier experiments on transient transfection of reporter constructs have revealed a ~1500-bp promoter region of the *BIRC5* gene that had the highest promoter activity [17]. We bioinformatically analyzed DNA sequences within the region of −4500 ... +1 (relative to the *BIRC5* gene transcription start point) to identify conserved among various vertebrate species distal and proximal structural elements putatively involved in the *BIRC5* gene transcription regulation. DNA of this region was rich in repeats: 40% of its nucleotide sequence were SINEs (short interspersed nuclear elements) of the Alu family and 10% – LINEs (long interspersed nuclear elements) of the L1 family (Scheme 1a). The similarity stretches were longer between human and chimpanzee: the human promoter contained three extended similarity stretches with approximate coordinates of −4050 ... −3700, −3100 ... −2300, and −2200 ... −1 relative to the transcription start point.

A comparison of the orthologous regions in human, chimpanzee, mouse, and dog revealed an evident similarity among all four genomes in only proximal parts of the promoters with coordinates of about −200 ... +1.

When the revealed human–chimpanzee similarity regions were compared to the corresponding regions of the mouse and dog genomes, the regions designated as A, B, and C (Scheme 1c) showed somewhat higher identity level.

For the 281-bp A region (−4008 ... −3728), the human–chimpanzee sequence identity level was 64%, human–mouse – 53%, and human–dog – 50%.

The 761-bp long (−3070 ... −2310) region B with the highest similarity with DNA of 17 genomes of different organisms used for comparison had the most conserved 409 bp part with coordinates (−2719 ... −2310). While the 761-bp B sequences of human and chimpanzee were 68% identical, the identity degree between the whole human B region and its counterpart in the mouse and dog genomes was 55 and 61%, respectively.

Region C of 540-bp length (−1996 ... −1457) contained two LINE elements of the L1 family and had identity degree with the corresponding DNA of chimpanzee 97%. Two stretches of 137 bp (−1824 ... −1638) and 20 bp (−1476 ... −1457) within the C region were 73 and 60%, respectively, identical to the corresponding genomic DNA of dog (Scheme 1a). No high identity was detected with the mouse genome in this region.

As CpG islands might contain important *cis*-elements involved in the *BIRC5* gene regulation, we analyzed also a −196 ... +302 fragment of the CpG island (GC content is about 73%) surrounding the gene transcription start site in the human genome (Scheme 1b). The corresponding CpG islands existed within the *BIRC5* orthologous genes of chimpanzee (497 bp), mouse (518 bp), and dog (1001 bp) with an evident similarity to the human island. The sequence identity degree between the human and chimpanzee *BIRC5* genes was as high as 99.6%. The 326-bp D fragment of the human CpG island (+43 ... +368) adjacent to the survivin promoter studied here included a short 30-bp non-translated region of the first exon (D1), the translated region of this exon (D2), and part of the survivin gene first intron (D3) (Scheme 1, c and d).

Phylogenetic footprinting identifies genomic regions under evolutionary constraints, thus helping in finding putative *cis*-regulatory elements of transcription. It is possibly most helpful in identifying *cis*-regulatory regions in developmental regulatory genes [33]. As the *BIRC5* gene plays an important role in the regulation of developmental processes, we supposed that phylogenetic footprinting might hopefully bring some useful information concerning distant elements involved in its regulation. On the other hand, obtained data about CpG-rich fragments distribution near the transcription initiation site could presumably help to localize proximal *cis*-regulatory elements.

Effect of putative regulatory conserved sequences on *BIRC5* gene promoter activity. The conservation of the A, B, C, and D fragments around the survivin gene promoter Psurv implies their possible significant functional importance. Therefore, we evaluated their impact on the Psurv promoter activity in different cell lines by measuring the expression level of the luciferase reporter gene. To this end, the fragments in question were sequentially cloned into a pGL3-Psurv reporter vector harboring the firefly luciferase gene under the control of the survivin gene basic promoter (Psurv, −1456 ... +42). In all constructs, the order and orientation of the cloned sequences coincided with those in human genomic DNA. Plasmid constructs pGL3-Psurv-D, pGL3-C-Psurv-D, pGL3-BC-Psurv-D, and pGL3-ABC-Psurv-D (Scheme 2) were used to transfect normal fibroblasts (IVL-11NS) and cancerous cells of various origin (A549, Calu1, NCI-H23, NCI-H358, HeLa). The activity of the Psurv promoter and its derivatives was compared with that of the SV40 early promoter within the same pGL3-PV reporter vector.

The data obtained are shown in Fig. 1. In all cell lines analyzed, all hybrid promoters had a detectable activity, and the activity data scattering did not exceed 15%. The generally observed trend in the reconstructed promoter activities in cancer cells was: Psurv-D > Psurv-CD > Psurv-BCD > Psurv-ABCD > SV40 promoter. In most cell lines, the activity of Psurv-D construct exceeded that of the unmodified Psurv. In cells A549, HeLa, and NCI-H358, the activity of Psurv-D was twice as high as that of Psurv (Fig. 1). This effect was observed also for normal fibroblasts (IVL-11NS), demonstrating its non-cancer-specific character. Thus, the modified via the attachment of the D fragment Psurv promoter did not change significantly its cancer specificity (the ratio of

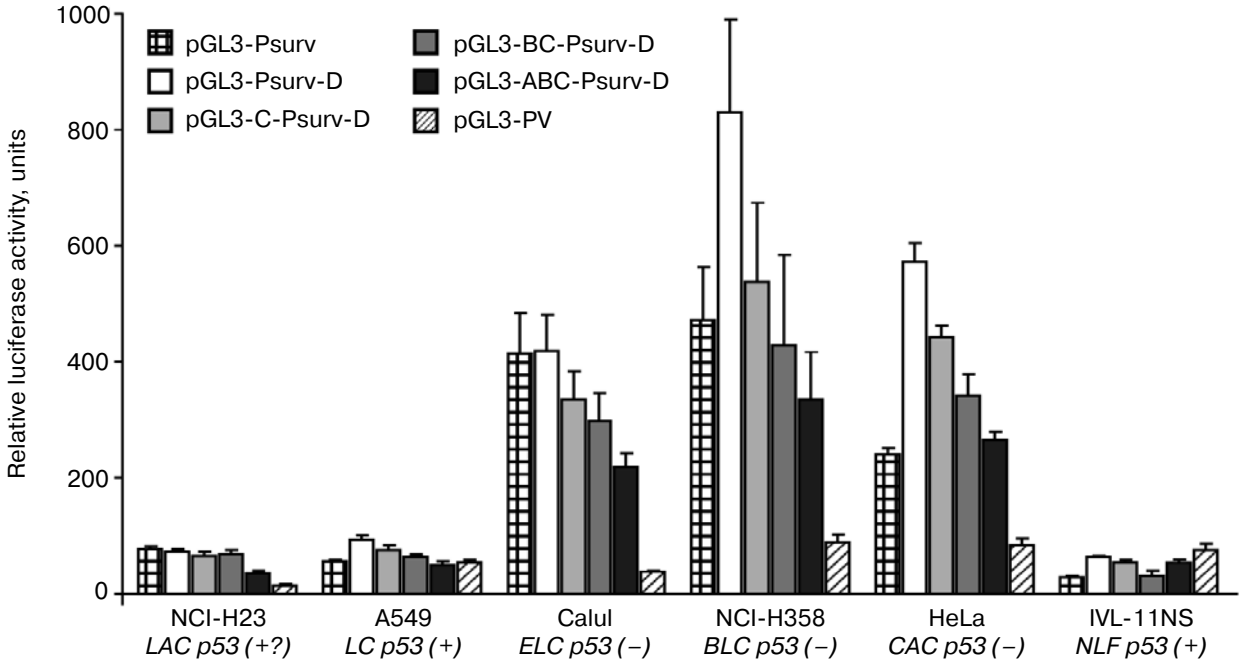


Fig. 1. Effect of sequences upstream adjacent to the 1498-bp *BIRC5* gene promoter on its activity in different cell lines. Y axis, luciferase activity relative to the basic activity in the cells transfected with a promoterless pGL3-BV vector. Histogram bars here and in Fig. 2 represent mean values of the luciferase activity measured in three independent experiments, and standard errors of mean (SEM) are given. The cell line types and the p53 status in each line are highlighted in italics.

promoter activity in cancerous and non-cancerous cells). Furthermore, the attachment of various fragments to the Psurv promoter did not affect its important feature—inhibition by the functionally competent p53 tumor suppressor. In cells with the wild type p53 (A549), the activities of all the promoters were considerably lower than in cells where the p53 activity was impaired (HeLa) or absent (Calu1, NCI-H358). This effect for Psurv is well known [21, 34] and is determined by functional p53 binding sites located within the survivin promoter. Thus, the factors interacting with the fragments under study do not interfere with p53 binding to its sites.

The relatively conserved regions A, B, and C sequentially added to the Psurv-D promoter led to a progressive decrease of its activity in lung tumor cells and in HeLa cells. When added jointly, the three conserved regions approximately twofold decreased the activity of the initial promoter. This result is in line with an earlier work [17] having shown that a 1430-bp promoter region in HeLa cervical adenocarcinoma cells was twofold more active than a 6500-bp region that included all the cloned here conserved DNA fragments. These findings might suggest that the conserved regions contain binding sites of non-tissue-specific negative transcription regulators. However, these data alone do not allow resolving a general challenge: to ascertain whether they belong to this gene and which gene or genes are regulated by these conserved and putatively regulatory elements [33]. The distance limit from a regulatory element to its cognate gene is not well understood, and looping of chromatin over a 40 Mb length to sites of transcriptional activity has been demonstrated [35]. Therefore, the question about the involvement of these fragments in *BIRC5* promoter activity modulation remains to be investigated.

The situation looks more promising with the D fragment, which is located just next to the promoter and most probably involved in interactions with the transcriptional machinery recruited by the promoter regulatory modules.

Regulatory properties of the D fragment and its constituent subfragments. Using HeLa (impaired p53 activity) and A549 (wild type p53) cell lines, we examined the regulatory properties of the D fragment in some more details, including localization of active structural elements and the fragment enhancer properties. The D fragment was divided into three segments: a 30-bp 5′-nontranslated region, a 110-bp translated region of the *BIRC5* gene first exon, and a 191-bp part of the first intron (designated in Scheme 1c as D1, D2, and D3, respectively). Each of them was cloned into the pGL3-Psurv reporter vector (Scheme 2). Plasmid constructs pGL3-Psurv-D1, pGL3-Psurv-D2, and pGL3-Psurv-D3 were used to transfect the A549 and HeLa cells. Despite a greater activity of all the Psurv promoter derivatives in $p53^-$ HeLa cells as compared to $p53^+$ A549 cells, the expression profiles directed by various promoters in these two cell lines were quite similar (Fig. 2). The Psurv-D1 and Psurv-D2 pro-

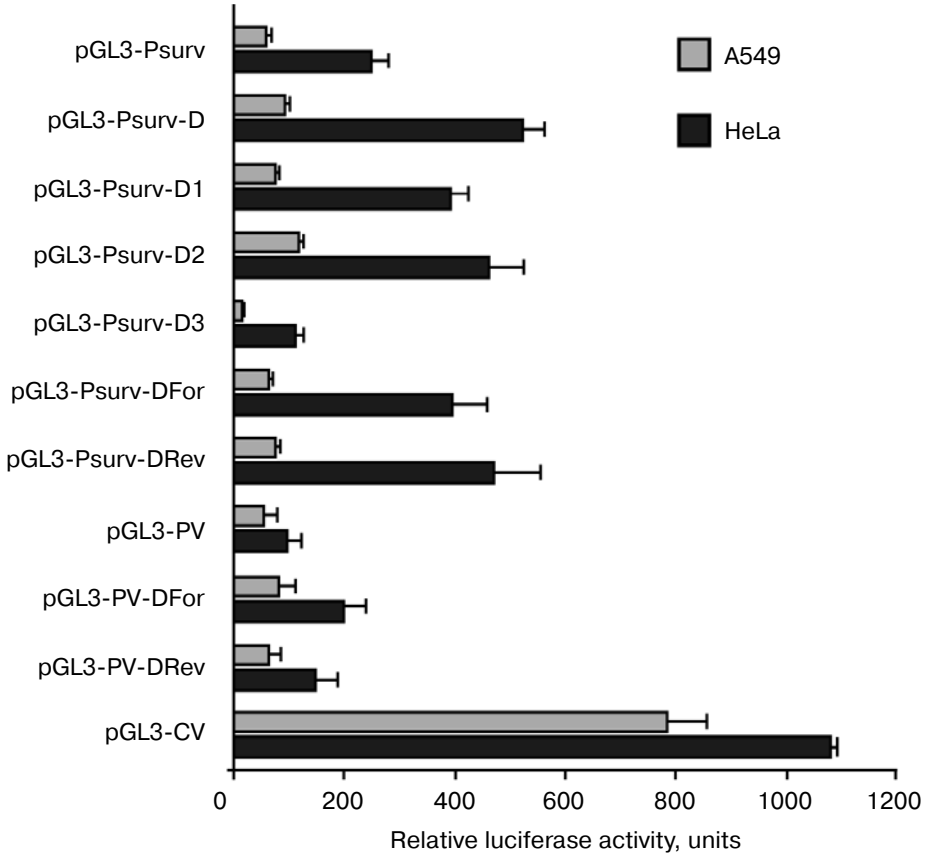


Fig. 2. Effect of DNA sequences from the transcribed region of the *BIRC5* gene on the activity of the survivin promoter (Psurv) and the SV40 early promoter. The cells were transfected with reporter plasmids that harbored the luciferase gene under the control of either the 1498-bp survivin gene promoter (Psurv) or the SV40 early promoter. Y axis, names of the reporter constructs used for transfection. X axis, promoter activity in the A549 and HeLa cells measured as relative luciferase activity. One unit of the luciferase activity was defined as the activity in extracts of cells transfected with plasmid pGL3-BV.

moters were approximately twice more active ($p < 0.05$), whereas the Psurv-D3 promoter was twofold less active ($p < 0.001$) than the initial Psurv promoter (Fig. 2). The activities of the D1 and D2 subfragments were quite comparable with the whole D fragment. This comparison suggests that the total activity of the D fragment is a non-additive effect of the three subfragments and that the enhancing capacity is linked to the D1 and D2 subfragments. The D3 subfragment decreased the Psurv promoter activity, which suggested a possible presence of transcription factor binding sites negatively regulating the *BIRC5* gene transcription within the D3 sequence; however, this supposition requires additional verification.

To characterize possible enhancer properties of the D fragment, the latter was cloned in two orientations into pGL3-Psurv and pGL3-PV plasmids at the distance of about 2000 bp from either the Psurv or SV40 promoters, respectively (Scheme 2). The pGL3-Psurv-D, pGL3-Psurv-DFor, and pGL3-Psurv-DRev plasmids introduced in the A549 or HeLa cells (Fig. 2) caused an equal reporter gene expression, which was in all cases twofold higher than that caused by the Psurv promoter ($p < 0.05$). Thus, the D fragment orientation and/or position did not affect its activating capacity. Such a behavior is characteristic of transcriptional enhancers. Moreover, we investigated the question about the D fragment ability to enhance heterologous promoter activity using SV40 virus promoter. To this end, we transfected HeLa and A549 cells with the pGL3-PV-DFor and pGL3-PV-DRev constructs. When attached to the SV40 promoter, the D fragment did not significantly enhance its promoter activity, irrespective of the orientation. Therefore, the uncovered enhancer activity of the D fragment can be promoter restricted, at least in the *in vitro* test. A drawback of such tests is that reporter constructs for regulatory element activity analysis often contain a minimal or modified promoter, which may lack the full set of transcription factor binding sites needed to form a fully functional protein complex. Moreover, enhancers were found to display different regulatory activity with different promoters [33], for example, with TATA-less and DPE (downstream promoter element)-containing promoters and with TATA-containing promoters [36]. The promoter-specific effects of enhancers can be explained by differential ability of

enhancer-binding proteins to interact with different protein complexes formed on different core promoters [37]. Psurv is a TATA-less promoter [17], whereas the SV40 promoter is a classical TATA-box-containing promoter [38], so the difference observed is not unexpected. It follows that the results of the *in vitro* test should be interpreted with caution: the enhancing effect of the D fragment on Psurv in this case might be in favor of its *in vivo* enhancer function in the survivin gene promoter regulation. Nevertheless, further studies are necessary to make reliable conclusions about the enhancer specificity of this element. Moreover, possible structural elements including two putative CDE elements (Scheme 1d) that confer the enhancer ability on the D fragment also remain to be identified and investigated.

Thus, in present work we have detected and characterized in detail a 3′-fragment of the CpG island localized in the transcribed region of the *BIRC5* gene that in enhancer-like way stimulated gene promoter activity in normal cells and in cancer, in particular lung cancer, cell lines. In addition to earlier reports [19, 26, 39], the results provide new information on the *BIRC5* gene expression regulation and demonstrate that the survivin gene protein coding sequences of its first exon might play an essential role in this gene expression regulation, as shown also for some other genes [40]. The data obtained open one more possibility to enhance survivin promoter activity while maintaining its cancer specificity. The promoter improved with the identified enhancer element might be used in cancer and, in particular, lung cancer gene therapy for directed therapeutic gene expression in tumor cells.

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