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Identification of an intronic *cis*-acting element in the human dopamine transporter gene

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Abstract

The human dopamine transporter gene (*hDAT*) encodes the dopamine transporter in dopamine (DA) neurons to regulate DA transmission. *hDAT* expression varies significantly from neuron to neuron, and from individual to individual so that dysregulation of *hDAT* is related to many neuropsychiatric disorders. It is critical to identify *hDAT*-specific *cis*-acting elements that regulate the *hDAT* expression. Previous studies showed that *hDAT* Intron 1 displayed inhibitory activity for reporter gene expression. Here we report that the *hDAT* Intron 1 contains a 121-bp fragment that down-regulated both SV40 and *hDAT* promoter activities by 80% in vitro. Subfragments of 121-bp still down-regulated the SV40 promoter but not the *hDAT* promoter, as supported by nuclear

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protein-binding activities. Collectively, 121-bp is a silencer in vitro that might coordinate with transcriptional activities both inside and outside 121-bp in regulation of *hDAT*.

Keywords

DAT; *SLC6A3*; Medication target; Promoter activity; Cell type-dependent; Primate-specific

Introduction

Dopamine (DA) transmission plays essential roles in brain physiological states (voluntary movement, motivation, cognition, attention, working memory and behavioral organization) and pathological processes (attention deficit/hyperactivity disorder (ADHD), schizophrenia, bipolar disorder, Parkinson's disease and substance abuse). As a critical regulator of DA transmission, the dopamine transporter (DAT) regulates the spatio-temporal components of DA transmission, by sequestering DA into presynaptic neurons to terminate transmission. Therefore, DAT contributes to voluntary movement, reward and mnemonic functions of the brain and efficacy of therapeutic drugs targeted to DAT. Expression of the human DAT gene (*hDAT*) throughout the brain is highly circumscribed, varies among individuals and can be regulated by endogenous and exogenous factors [1–3]. Abnormal *hDAT* expression may confer risks for various neuropsychiatric diseases [4].

Only one in a million neurons is a DA neuron and *hDAT* is expressed in DA neurons only and at levels of great differences among different brain regions [5, 6]. Apparently, regional specificity of quantitative *hDAT* expression is finely controlled. Recent in vitro studies have shown that *hDAT* expression in DA cell lines is activated by common TFs including NurrI, ZFP161 and Sp [7–11]. However, *cis*-acting elements such as Intron 1 [12, 13] for controlling *hDAT* activity represent a rarely explored subject. We report an in vitro dissection of *cis*-acting activities that regulate *hDAT* promoter and represent an *hDAT*-specific medication target for correcting abnormal *hDAT* activity.

Materials and methods

Cell lines and RNA isolation

All human cell lines including four DA cell lines SK-N-AS, SH-SY5Y, BE(2)-M17, IMR-32 and one non-DA cell line HEK293T were purchased from ATCC and maintained at 37°C, 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/ml), and streptomycin (100 U/ml) (Invitrogen, Carlsbad, CA, USA).

Total RNA was isolated from cells with TRI Reagent solution (Ambion, Austin, TX, USA) according to the manufacturer's protocol and subsequently, subjected to DNase treatment.

Quantitation of mRNA levels by qRT-PCR

To prepare cDNA, reverse-transcription used Superscript II reverse transcriptase (Qiagen, Valencia, CA, USA) and oligo dT as the primer. Quantitative PCR reactions used the SYBR

Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and two pairs of primers listed in Table S1. *GAPDH* was used as an internal control. Relative expression levels were calculated by using an empirical $1.777^{-\Delta\Delta CT}$ method where the amplification efficiency value of 1.777 ± 0.183 (N = 10) was a qRT-PCR-based average for the *hDAT* primers [14].

Reporter construction

pGL3-hDAT7.9kb was generated by cloning a 7,777 bp *Bsa*BI/*Eco*RV fragment into the *Sma*I site of pGL3-enhancer. *Bsa*BI is located 5,930 bp upstream and *Eco*RV, 1,847 bp downstream of the transcription start site. PCR using primers 869f and 869r was carried out to clone a 869 bp fragment from a lambda clone (a gift of Dr. A. P. Kouzmenko of University of Melbourne, Australia), digested by *Bam*HI and replaced the 748 bp *Bam*HI/*Bgl*II fragment of pGL3-hDAT7.9kb, generating pGL3-hDAT7.9kb. Insertion orientation was confirmed by DNA sequencing with primer Luc170r. pGL3-hDAT7.9kb+121bp covered −5930 to +1968 and pGL3-hDAT7.9kb covered −5930 to +1847, assuming the transcription start site as +1 according to Genbank accession number NM_001044 (18-OCT-2009).

A 121 bp fragment was broken down into four subfragments and each subfragment was prepared by oligonucleotide (oligo) annealing to be boarded in *Hin*dIII and *Nco*I ends, followed by insertion into the *Hin*dIII/*Nco*I sites of Promega's pGL3-Promoter Vector, generating four SV40 subfragment plasmids pGL3-SV40-I, pGL3-SV40-II, pGL3-SV40-III and pGL3-SV40-IV. To replace the SV40 promoter in the subfragment plasmids with *hDAT* promoter, a 5,330 bp *Bsa*AI/*Eco*RV *hDAT* fragment was isolated from the pGL3-hDAT7.9kb construct by *Bsa*AI/*Hin*dIII digestion (*Hin*dIII located in the reporter vector), inserted into the *Sma*I/*Hin*dIII sites of the subfragment plasmids, generating four *hDAT* subfragment plasmids pGL3-hDAT5.3kb-I, pGL3-hDAT5.3kb-II, pGL3-hDAT5.3kb-III and pGL3-hDAT5.3kb-IV. Restriction enzymes were purchased from New England Biolabs (NEB, Ipswich, MA, USA).

Transfection and luciferase activity measurements

Transfection procedure has been described before except using a 24-well format in this study [15]. All transfection used plasmid DNAs mixed from at least two-three independent isolations (each with OD ratio of>1.7) in order to ensure that quality of DNA preparations did not complicate promoter activity signals. Transfection efficiency (mean ± SD) was 0.67 ± 0.05 for SK-N-AS, 0.46 ± 0.05 for IMR-32 and 0.78 ± 0.06 for HEK293T on average, based on using a plasmid of similar size and carrying GFP gene (4′-6-Diamidino-2-phenylindole or DAPI staining for total cells) and immunocytochemical staining with anti-luciferase antibodies [16]. Two days after transfection, cells were harvested and luciferase (Luc) activity measured by using a Luciferase Assay System Kit (Promega, Madison, WI, USA) in Bio-Tek Synergy HT/KC4 and a 96-well format, according to manufacturer's instructions. Cell number in each well was estimated by protein amount based on Bio-Rad's Protein Assay Reagent. Arbitrary unit of promoter activity is calculated as Luc activity readout/protein. Data are all presented as mean ± SEM.

Electrophoretic mobility shift assay (EMSA)

^{35}S-Labeling of double-stranded (ds) oligos. 20 μM of (equimolar concentrations of forward and reverse) oligoswere annealed in 200 μl of Annealing Buffer (10 mM Tris, pH 7.8, 50 mM NaCl and 1 mM EDTA) by incubation at 95°C for 2 min, followed by 45–60 min cooling down to room temperature and aliquots stored at −80°C. Labeling was carried out in a 30 μl solution containing 0.5 μl of 20 μM annealed ds oligos, 3 μl of 10× Reaction Buffer 2 (Invitrogen), 6 μl of ^{35}S-dATP (60 pmol, PerkinElmer Life and Analytical Sciences, Boston, MA, USA) and 1 μl of Large Fragment of DNA Polymerase I (NEB) and 19.5 μl of H_2O, incubated at room temperature for 28 min before cooling down on ice. To remove the unincorporated ^{35}S-dATP, the labeling mixtures were loaded into CHROMA SPIN-10 columns (Clontech, Mountain View, CA, USA), the columns centrifuged at 700×*g* for 5 min and the elute collected as ^{35}S-ds oligos.

DNA binding—Each 17 μl of binding reaction was prepared by (EMSA Accessory Kit, EMD4Biosciences, Gibbstown, NJ, USA) mixing 5 μl of 4× binding buffer (80 mM HEPES, pH 8.0, 0.4 M KCl, 80% glycerol, 0.8 mM EDTA and 2 nM DTT), 1 μl of 500 ng/μl sonicated salmon sperm DNA (average size ~500 bp), 1 μl of poly(dI):poly(dC) (0.01 U dissolved in PBS), and 3 μl of yeast extract and 7 μl of sterile H_2O, followed by mixing with 1 μl of ^{35}S-labeled ds oligo (0.03 pmol) and incubation on ice for 2–3 h before loading for electrophoresis.

EMSA—6% non-denaturing DNA retardation gels (20 ml gel solution containing 4 ml of 29:1 acrylamide:bisacrylamide solution, 1 ml of 10× TBE, 15 ml of ddH_2O, 40 μl of 20% ammonium persulfate and 12 μl of TEMED) was prepared 1 day before pre-running in 0.5× TBE at 120 V for 0.5 h. After loading the binding reaction solutions, electrophoresis was carried out at 120 V for 1 h. Gels were dried on filter papers and exposed to X-ray film for 3–5 days for autoradiography.

Results and discussion

Endogenous hDAT expression in human cell lines

To study regulatory elements of the *hDAT* promoter, human cell lines that express endogenous *hDAT* were assessed among five human cell lines including four DA cell lines SK-N-AS, (BE2)-M17, IMR-32, SH-SY5Y and a non-neuronal cell line HEK293T by using quantitative real-time PCR (qRT-PCR). As a result, SK-N-AS expressed the highest levels of endogenous *hDAT* mRNA among the five cell lines and (BE2)-M17 the second highest (9.5% of SK-N-AS levels; Fig. 1). IMR-32 expressed 3.7% of the SK-N-AS levels. SH-SY5Y and HEK93 expressed <3% of the SK-N-AS levels. These data are consistent with the previous report that SK-N-AS expressed robust levels of hDAT [17]. We therefore used human DA cell line SK-N-AS as a main system and two to four other cell lines for *hDAT* regulation analysis that mimic different DA neurons to some extents.

Addition of an intronic 121-bp fragment decreased hDAT promoter activity

In a study on *hDAT* promoter regulation, we cloned a 7.9 kb promoter fragment (covered −5930 to +1847, assuming the transcription start site as +1 according to Genbank accession

number NM_001044 entered on PRI 18-OCT-2009) into Promega's pGL3-enhancer (a promoterless firefly luciferase reporter vector) by unique restriction sites *Bsa*BI and *Eco*RV. This reporter plasmid is now termed pGL3-hDAT7.9kb. We used the pGL3-enhancer, instead of the pGL3-Basic vector because the 7.9 kb in the later vector displayed very low activity and generated very unreliable results. The 7.9 kb was selected because that was the maximal size the vector could harbor. In order to cover more in the 3′ end (or Intron 1), we extended the 3′ end by adding 121 bp, generating pGL3-hDAT7.9kb+121bp (see "Materials and methods" section for construction). In order words, addition of a 121-bp into the 3′ end of the hDAT promoter fragment in pGL3-hDAT7.9kb resulted in pGL3-hDAT7.9kb+121bp (Fig. 2a). When assaying the promoter activity of these reporter plasmids, we found that pGL3-hDAT7.9kb expressed more promoter activity than pGL3-hDAT7.9kb+121bp consistently among three cell lines: 3.97-fold in SK-N-AS, 3.34-fold in SH-SY5Y and 4.42-fold in HEK293T (Fig. 2b–d). These data suggested that the 121 bp fragment inhibited the *hDAT* promoter.

Down-regulation of SV40 promoter by the 121-bp fragment independently of insertion location

Possibilities for the intronic 121-bp fragment to inhibit the *hDAT* promoter may include destabilization of the Luc RNA by the 121-bp fragment (because this fragment became a 5′ part of the Luc transcript) or this fragment served directly as a transcription factor (TF) binding site. To differentiate these two possibilities, we examined whether this fragment still inhibited Luc gene expression when inserted outside the Luc gene in Promega's reporter vector, pGL3-Promoter Vector. To do so, the 121-bp fragment was cloned into two different locations to generate two reporter plasmids pGL3-SV40+121bp (Fig. 3a): before and after SV40 promoter. We then transiently expressed these pGL3-SV40+121bp plasmids to examine 121-bp fragment regulation of SV40 promoter activity in two human cell lines, SK-N-AS and BE(2)-M17. The 121-bp fragment down-regulated the SV40 promoter activity by 30–40% when inserted either after or before the promoter in SK-N-AS (Fig. 3b); the down-regulation was 27–31% when inserted after or before the promoter in BE(2)-M17 (Fig. 3c). Importantly, the difference in down-regulation between "after" and "before" promoter was statistically significant in neither of these cell lines, suggesting that the 121-bp fragment likely served as a TF binding site and did not affect Luc mRNA activity. Overall, the down-regulation of the SV40 promoter was less significant than that of the *hDAT* promoter, suggesting that the 121-bp fragment be more effective on the native promoter.

We thus reported the identification of a unique 121-bp fragment, located from +1848 to +1968 near the 3′ end of the 2.2 kb Intron 1, as a *cis*-acting inhibitory element for the 7.9 kb *hDAT* promoter activity. Not found in the rest of the human genome, this sequence is conserved in chimpanzee (96% identity) but is not in rodents (only 61% in a localized and gapped 70 nucleotides around I and II), suggesting a possibility of species-related regulation of the DAT gene. *Cis*-acting elements that confer neuron specificity of *hDAT* expression are unlikely located in the DNA fragments examined in this study. The 7.9 kb *hDAT* promoter was expressed in all types of cells tested, including SY-SY5Y and HEK293T that expressed very little endogenous *hDAT*, suggesting that unlike the zebrafish gene [18], *cis*-acting elements that confer neuron specificity in humans are not located in this 7.9 kb. The 121-bp

fragment did not displayed the largest inhibitory activity in non-neuronal cells (Fig. 4), suggesting that this 121-bp fragment does not contribute to the neuron specificity either.

Regulation of the SV40 promoter by subfragments of the 121-bp

TFs usually recognize <20 bp DNA. To identify which regions of the 121-bp displayed the inhibitory activity, we broke down the 121-bp into four subfragments, I–IV (Fig. 4a) and analyzed their individual regulatory activity on the SV40 promoter after cloning by replacing the 33 bp *Hin*dIII/*Nco*I fragment of the Promega's pGL3-Promoter Vector (Fig. 4b). Subfragment I had 36 bp; II, 41 bp; III, 38 bp and IV, 22 bp with some overlapping between subfragments to reduce the possibility of losing TF binding activity (Fig. 4a).

As a result, in SK-N-AS, subfragments I, II, III and IV reduced the SV40 promoter activity by 53.6, 60.2, 63.1 and 17.4%, compared to the vector (Fig. 4c). In BE(2)-M17, subfragments II and III did not reduce the activity in a statistically significant manner; subfragment IV increased the activity by 38.3%, reaching statistical significance only by *t* tests, not by ANOVA posttest Tukey (Fig. 4d). In SH-SY5Y, subfragments I and II reduced the SV40 promoter activity by 48.1 and 29.4%; neither of III and IV regulated the SV40 activity (Fig. 4e). Similar to SH-SY5Y, in HEK293T, subfragments I and II reduced the SV40 promoter activity by 46.0 and 42.5%; neither of III and IV regulated the SV40 activity (Fig. 4f). In summary, I and II displayed the most consistent inhibitory activities and IV might display activating activity, depending on cell types.

We also examined regulation of the 5.3 kb *hDAT* promoter by the subfragments but did not observe any significant inhibitions in SK-N-AS or HEK293T (*data not shown*). An interesting question is how the inhibitory activity on the *hDAT* promoter disappeared after the 121-bp broke down into four smaller elements. One explanation is that the inhibitory activity of the 121-bp was achieved by coordination among the TFs that bind to the individual subfragments. It has been reported that a TF can function differently depending on the environments [19–22]. TF scan has predicted that 121-bp could be recognized by more than 30 TFs, suggesting that multiple TFs might regulate this fragment. To better understand the intronic and significant regulation of *hDAT*, future studies will need to identify these TFs so that we may further examine whether these TFs recognize each other and/or undergo different posttranslational modifications in response to external or internal regulations. Cell type- as well as promoter-dependences were observed in subfragment regulation, suggesting that same subfragments may regulate different promoters in different ways and in different cells [23, 24]. These data thus cautioned that in vitro assays on transcriptional regulations need to be careful in choosing target promoters and that the assays should not depend upon single easy-to-assess (high activity) promoters such as the SV40 promoter.

Nuclear protein binding to the subfragments

To examine whether any potential TFs could bind to the subfragments, we have carried out EMSAs and investigated whether nuclear proteins isolated from the four cell lines bind to double-strand oligos of the four subfragments. We prepared double-strand oligos with five extra "T" at 5′ end of each forward strand, followed by filling-in in the presence of ^{35}S-

dATP to label the oligos. Labeled oligos were each mixed with nuclear proteins isolated from three DA cell lines (SK-N-AS, BE(2)-M17, SH-SY5Y) and one non-DA cell line (HEK293T). It turned out that subfragments II and IV both displayed binding activities for all four cell lines (see arrows in lanes 3, 11, 19, 27 and 7, 15, 23 and 31 of Fig. 5). Subfragment IV displayed a binding activity that could not be inhibited completely by including 30-fold excess of the unlabeled oligo in the binding mixture (see two lanes under "IV"). Such information suggests that this particular protein is expressed abundantly but has low affinity for IV. Nevertheless, those binding activities are consistent with the inhibitory effects of both subfragments on the SV40 promoter in most of the cell lines. Subfragment I showed two bands for SK-N-AS but lacked evident binding activity for other cell lines (see lanes 9 vs. 10, 17 vs. 18 and 25 vs. 26). Subfragment III did not show any binding activity.

This *hDAT* 121-bp may evolve into an HTS platform to develop *hDAT*-based genetic medicine. In humans, lower *hDAT* expression levels seem to correlate with higher risks for diseases, as reviewed before [4]. Consistently, animal studies have also shown that reduced DAT expression is a risk factor. For example, mice carrying a reduction in DAT expression display liability for drug addiction [25–28]. Both clinical and pre-clinical findings suggest that up-regulation of *hDAT*, perhaps via modulation of the 121-bp, may alleviate related brain disorders such as substance abuse in humans.

In conclusion, this 121-bp is a silencer of *hDAT* promoter. 121-bp-mediated inhibition appears to involve a transcriptional complex, providing a novel platform to screen for *hDAT* modulators.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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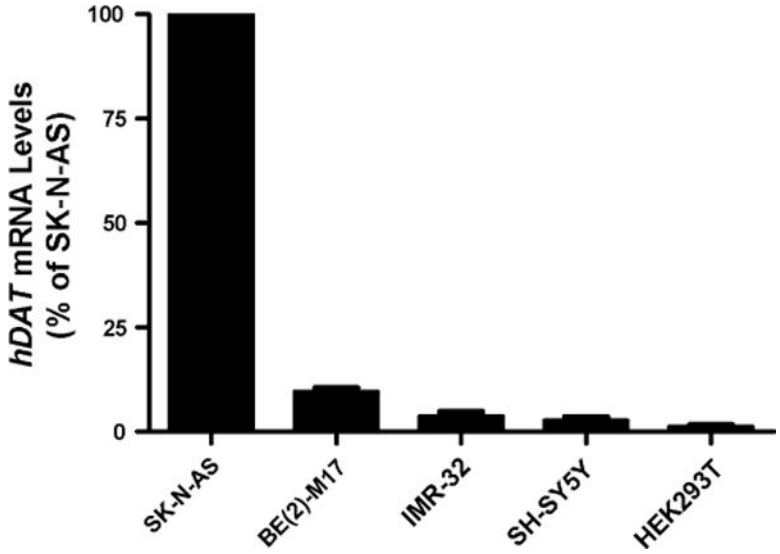

Fig. 1.

Endogenous *hDAT* expression in five human cell lines including four DA cell lines SK-N-AS, BE(2)-M17, IMR-32, SH-SY5Y and one non-neuronal cell line HEK293T, based on qRT-PCR that used two different pairs of *hDAT* primers ($N = 3$)

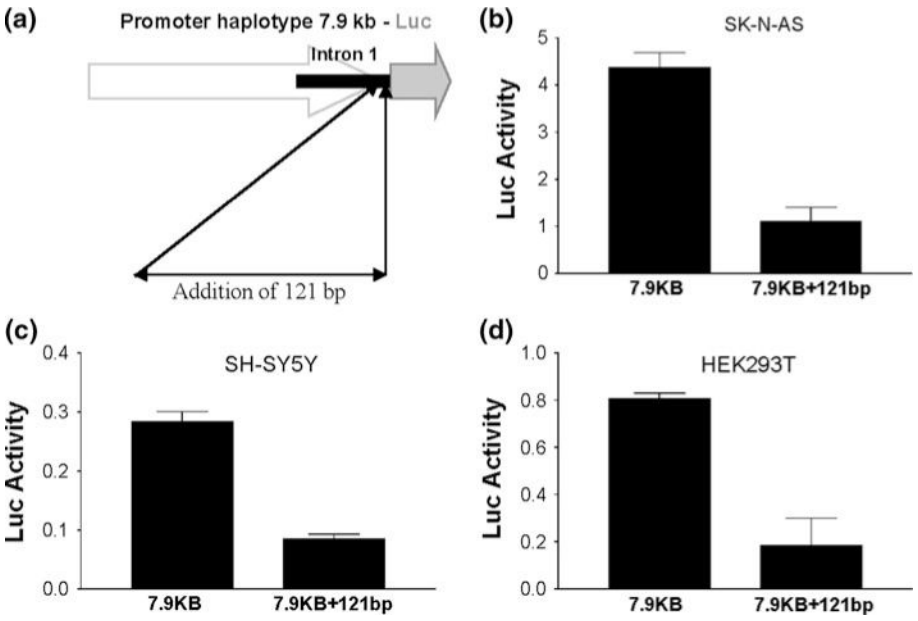

Fig. 2.

Promoter activity decreased by addition of (extending) a 121-bp fragment into Intron 1. **a** Schematic of 7.9 kb-Luc expression constructs and addition of a 121-bp fragment. **b–d** Addition of 121-bp fragment decreased the promoter activity in SK-N-AS, SH-SY5Y and HEK293T. Luc activity (arbitrary units) represents promoter activity of 7.9 kb *hDAT* fragment before (7.9 kb for pGL3-hDAT7.9kb) and after (7.9kb+121bp for pGL3-hDAT7.9kb+121bp) adding 121-bp. $P < 0.0001$ for SK-N-AS ($N = 6$) and SH-SY5Y ($N = 14$) or $P = 0.0342$ for HEK293T ($N = 2$ each in duplicate) all by *t* tests

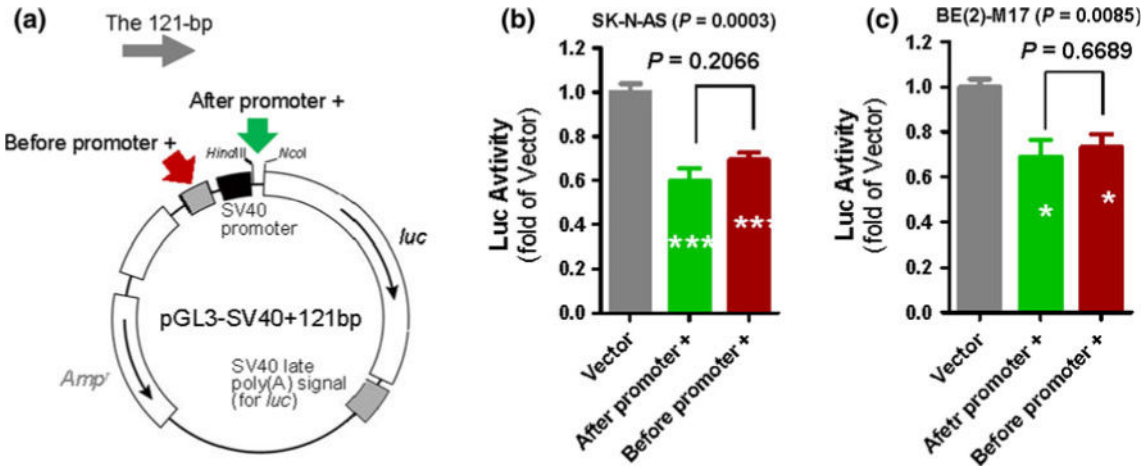

Fig. 3.

Down-regulation of SV40 promoter activity by *hDAT* intronic 121-bp fragment. a Cloning of the 120-bp fragment into two different locations (indicated by *two arrows*) in pGL3-Promoter Vector (Vector), resulting in pGL3-SV40+121bp. 121-bp inhibition of SV40 promoter activity, based on Luc activity from Vector versus pGL3-SV40+121bp in two different human cell lines SK-N-AS and BE(2)-M17 (**b, c**). *Arrows* indicate two different insertion locations. ANOVA and Tukey tests: $*P < 0.05$; $***P < 0.001$, compared to Vector ($N = 4$, each in duplicate)

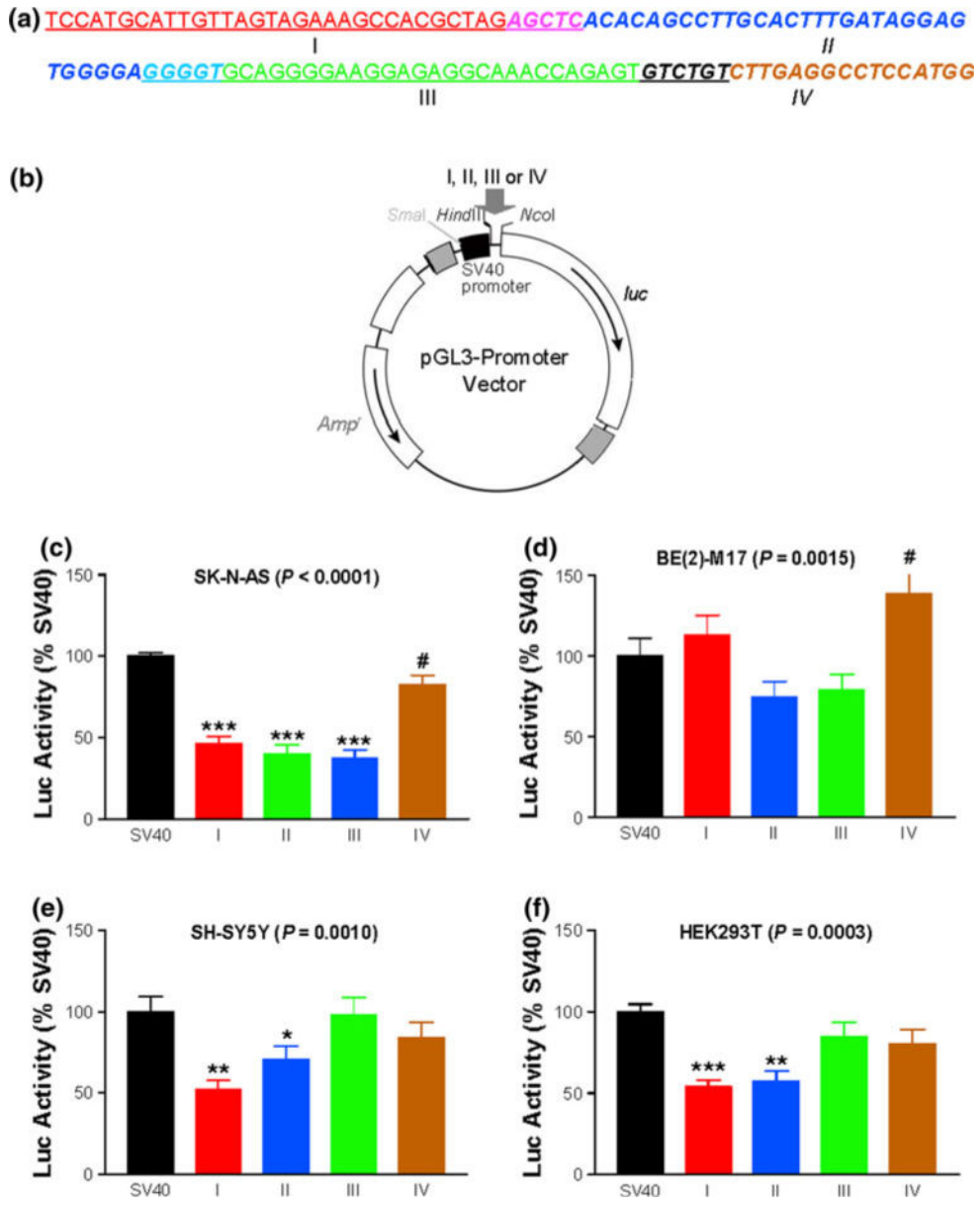

Fig. 4.

Inhibition of SV40 promoter activity by subfragments of 121-bp. a DNA sequences of four subfragments (I–IV) throughout 121-bp. **b** Reporter pGL3-Promoter Vector. *Gray arrow* indicates subfragment insertion site. **c–f** Influences of subfragments on SV40 promoter activity in four different cell lines. *SV40* pGL3-Promoter Vector; *I* pGL3-SV40-I; *II* pGL3-SV40-II; *III* pGL3-SV40-III; *IV* pGL3-SV40-IV. Indicated *P* values are based on ANOVA: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; Student's *t* test: $^{\#}P < 0.05$, comparing to SV40 ($N = 4$–5, each in duplicate)

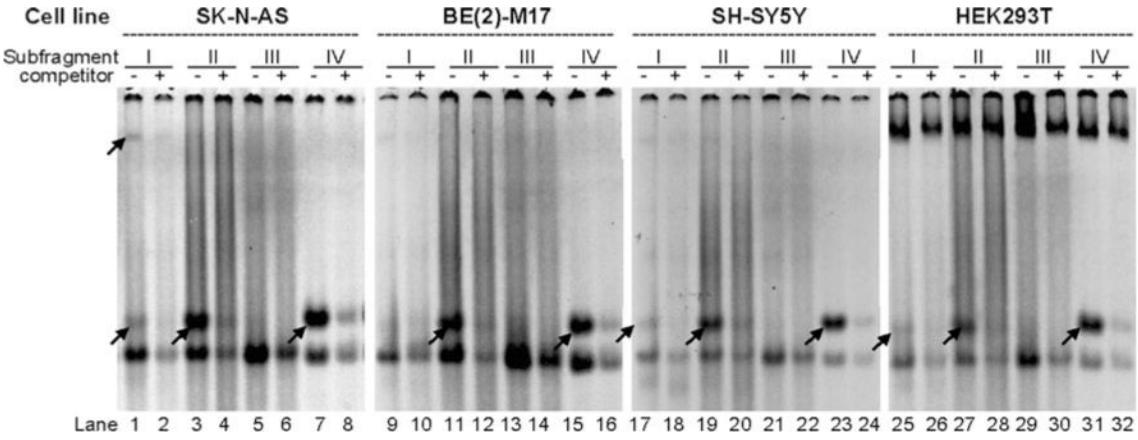

Fig. 5.

EMSA of nuclear protein binding to four subfragments (I–IV) of 121-bp. Nuclear proteins were isolated from SK-N-AS, BE(2)-M17, SH-SY5Y and HEK293T, as indicated on top. DNA sequences of I–IV are shown in Fig. 4a and ds oligos were labeled by ^{35}S-dATP. Competitor is 30-fold excess of unlabeled double-strand oligo. *Arrow* specific binding activity by comparing to next lane with competitor; *lowest band* poly(A/T, generated by ^{35}S-A filling-in)-binding activity (control not shown)