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METHODOLOGY ARTICLE

Modulating ectopic gene expression levels by using retroviral vectors equipped with synthetic promoters

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Abstract The human cytomegalovirus and elongation factor 1α promoters are constitutive promoters commonly employed by mammalian expression vectors. These promoters generally produce high levels of expression in many types of cells and tissues. To generate a library of synthetic promoters capable of generating a range of low, intermediate, and high expression levels, the TATA and CAAT box elements of these promoters were mutated. Other promoter variants were also generated by random mutagenesis. Evaluation using plasmid vectors integrated at a single site in the genome revealed that these various synthetic promoters were capable of expression levels spanning a 40-fold range. Retroviral vectors were equipped with the synthetic promoters and evaluated for their ability to reproduce the graded expression demonstrated by plasmid integration. A vector with a self-inactivating long terminal repeat could neither reproduce the full range of expression levels nor produce stable expression. Using a second vector design, the different synthetic promoters enabled stable expression over a broad range of expression levels in different cell lines.

Keywords Synthetic promoters · Mammalian expression systems · Retroviral vectors

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Introduction

Most commonly-used mammalian expression vectors employ strong constitutive promoters. These promoters include the immediate-early promoter of the human cytomegalovirus (CMV), the promoter of human elongation factor 1α (EF1α), and other viral promoters. Vectors equipped with these promoters are useful for ectopically expressing genes at high levels. Yet it can be important to evaluate low and intermediate gene expression levels, since different expression levels may generate different phenotypes. For example, high over-expression of the Ras oncogene can lead to premature cell-cycle arrest and senescence (Serrano et al. 1997), while low over-expression can stimulate proliferation and induce tumor formation (Sarkisian et al. 2007). Additionally, in order to engineer and optimize genetic circuits or other synthetic devices in mammalian cells, it is paramount to be able to tune gene expression over a range of levels. To meet these needs, we set out to generate constitutive promoters that produce varying levels of expression.

The term "synthetic promoter" can refer to a constitutive promoter where the sequence has been modified so that it produces a different transcription level (Ruth and Glieder 2010; Jensen and Hammer 1998a). Jensen et al. were among the first to report a library of synthetic bacterial promoters (Jensen and Hammer 1998b). Since then libraries of yeast and mammalian promoters have been reported. In bacteria, synthetic promoter libraries have been employed to tune gene expression (Rud et al. 2006) and optimize lycopene production (Alper et al. 2005). In yeast, synthetic promoter libraries have been used to vary gene expression (Hartner et al. 2008) and produce different glycerol yields (Nevoigt et al. 2006). For mammalian cells, Tornoe et al. created a synthetic promoter library by generating chimeric promoters

assembled from SV40, beta-actin, and ubiquitin C sequences. The library was capable of a tenfold range in transcriptional activity (Tornoe et al. 2002).

To generate synthetic promoters of varying expression strengths, one can mutate promoter sequences immediately upstream of the transcription start site (TSS). This region contains cis-regulatory elements that affect the frequency of transcription initiation and consequently the level of gene expression. In bacteria, researchers have successfully generated promoter variants by changing the bases near, but not within, conserved regulatory sequences, e.g., changing the spacing between the bases −35 and −10 to the TSS (Jensen and Hammer 1998b). Yet in eukaryotic promoters, the regulatory sequences are not rigidly conserved and to generate mammalian promoters producing different levels of expression, one can vary consensus sequence elements. The TATA and CAAT boxes are two such elements found in promoters. TATA boxes have been identified in approximately 25% of human promoters (Xi et al. 2007). They are found approximately 30 bp upstream of the TSS and recruit the transcription pre-initiation complex and RNA polymerase. CAAT boxes are found in approximately 13% of human promoters (Xi et al. 2007) and bind the ubiquitous transcription factor NFY.

Here we report the generation of a library of synthetic promoters that can be used to evaluate and optimize ectopic gene expression over a range of levels. Synthetic promoters were generated by introducing mutations in and near TATA and CAAT sequences. To help ensure that the promoters would be compatible with vectors already used in the field, we generated variants of the commonly-used CMV and EF1α promoters. We demonstrated that retroviral vectors equipped with the synthetic promoters can be used to generate stable cell lines with desired levels of ectopic gene expression.

Materials and methods

Promoter mutagenesis and expression vectors

The human cytomegalovirus immediate-early promoter (CMVwt, bases 156–744 of GenBank accession # K03104.1; our sequence has a G at base position 724, while the reference sequence has a C) and the human elongation factor 1α promoter (EF1αwt, bases 378–610 of GenBank accession # J04617.1) were mutated to generate promoters of varying expression strengths. Random mutagenesis of the CMV promoter region (from 62 bases upstream, to 7 bases downstream of the transcription start site) was performed by a degenerate primer PCR using the attB1-CM forward primer (all primers sequences are in Supplementary Table 1) and a degenerate reverse primer, attB2-CM-Deg, that was synthesized with nucleotide mixtures so that each base position had a 63.5% probability to be correct and 36.5% probability to be a mutation. Site-directed mutagenesis of the CMV TATA sequence was performed using the attB1-CM forward and attB2-CM-Txx reverse primers, where the x's in the primer sequence correspond to the mutations as listed in Supplementary Table 2. To introduce mutations in the CMV CAAT sequence, attB1-CM forward and CM-Cxx reverse primers were first used. Two additional rounds of PCR were necessary to generate the full length promoter mutant (1) using the attB1-CM forward and CM-Cextension reverse primers, followed by (2) using the attB1-CM forward and attB2-CM reverse primers. Site-directed mutagenesis on the EF1α TATA sequence was performed by first using the attB1-EF forward and EF-Txx reverse primers. Two additional rounds of PCR were again necessary (1) using the attB1-EF forward and EF-Textension reverse primers, followed by (2) using the attB1-EF forward and attB2-EF reverse primers. attB1 and attB2 recombination sites allowed plasmid construction using the Gateway system (Invitrogen, Carlsbad, CA, USA).

Using the Gateway protocol, mutant (i.e., synthetic) promoters were inserted into plasmid expression vectors at a Gateway destination site (DEST) upstream of an enhanced green fluorescent protein (GFP) reporter. The vectors also contained the gene for hygromycin resistance. Moloney murine leukemia virus (MLV) and self-inactivating murine stem cell virus (MSCV-SIN) vectors were constructed using standard molecular biology methods. For one set of MLV vectors, synthetic promoters were inserted upstream of GFP in-frame with an F2A sequence and a DEST site. The mCherry gene (a red fluorescent protein, RFP, variant) was inserted into this DEST site. F2A is a ribosome-slippage sequence (Donnelly et al. 2001) that allows bicistronic expression of GFP and the gene inserted into the DEST site. The retroviral long terminal repeat (LTR) contains a promoter that drives transcription of the puromycin resistance gene. The MSCV-SIN vectors were generated by modifying pMSCV-TMP (Dickins et al. 2005) (Open Biosystems, Huntsville, AL, USA). In these vectors, the retroviral transcriptional enhancer has been deleted. Synthetic promoters were inserted upstream of the GFP gene, F2A sequence, and DEST site. The mCherry gene was inserted into the DEST site, and an additional SV40 promoter was used to express the puromycin resistance gene. A 1.2 kb insulator sequence, chicken hypersensitivity site-4 (cHS, GenBank accession # U78775), was inserted upstream of some mutant promoters (Chung et al. 1997).

Cell culture and transduction

PD-31 cells were cultured in RPMI-1640 media with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, and 55 μM β-mercaptoethanol. NIH/3T3 fibroblasts were

cultured in DMEM with 10% newborn calf serum (NCS). HeLa cells expressing the murine ecotropic receptor (from pBABE zeo Ecotropic Receptor, Addgene #10687) were cultured in DMEM with 10% FBS. All media was supplemented with 1 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Expression constructs containing the synthetic promoters upstream of GFP were co-transfected with pOG44 (a Flp recombinase-expressing vector, Invitrogen) into HEK-293 Flp-In cells (Invitrogen) using calcium phosphate precipitation. Cells were selected with 100 μg/ml hygromycin, and clones were expanded.

Retrovirus was produced by co-transfecting plasmids encoding the MLV or MSCV-SIN vectors and pCL-Eco (Naviaux et al. 1996) into HEK-293T cells using calcium phosphate precipitation. Virus-containing supernatant was harvested and used to transduce cells. Virus was titered so that transduced cells received a single copy of the vectors. Polybrene (hexadimethrine bromide) was added to cultures at a concentration of 3 μg/ml (PD-31 cells) or 8 μg/ml (NIH/3T3 and HeLa cells). 24–48 h post-infection, cells were selected with 2 μg/ml (PD-31) or 1 μg/ml (NIH/3T3 and HeLa) puromycin.

Real-time quantitative PCR

Total mRNA from the HEK-293 Flp-In clones was purified (Trizol extraction, Invitrogen) and used to generate cDNA (QuantiTect Reverse Transcription Kit, Qiagen, Valencia, CA, USA). To determine GFP mRNA levels, cDNA was analyzed by real-time quantitative PCR (forward primer, 5′-CTGCTGCCCGACAACCA-3′; probe, 5′-FAM-TACC TGAGCACCCAGTCCGCCCT-Iowa Black FQ-3′; reverse primer, 5′-TGTGATCGCGCTTCTCGTT-3′, Integrated DNA Technologies, Coralville, IA, USA). GAPDH was analyzed as a reference (#4326317E, VIC-labeled, Applied Biosystems, Foster City, CA, USA). All reactions were performed in triplicate.

Flow cytometry

Cell fluorescence was analyzed on a LSRII flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA). Flow cytometry data was first analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Results

Mutagenesis and evaluation of promoters

To generate synthetic promoters of varying expression strengths, we made variants of the CMV and EF1α promoters. We introduced mutations by site-directed mutagenesis of the TATA or CAAT boxes or by random mutagenesis (Fig. 1a; Table 1 and Supplementary Table 2). We then inserted the mutated promoters into expression vectors containing a GFP reporter gene (Fig. 1b, Supplementary Material). Next, we stably integrated the vectors at a single site in the genome of HEK-293 cells. To measure the expression strength of the promoters, we quantified the fluorescence intensity of cells by flow cytometry (Supplementary Table 2). We observed that the mutant promoters demonstrated a 40-fold expression range. Based on the expression profiles (e.g., non-bimodal distribution with lower correlation of variance), we chose 23 promoters (Table 1 and Supplementary Fig. 1) for further characterization. With these promoters, the level of cellular fluorescence (Fig. 1c) correlated with the relative abundance of GFP mRNA (Fig. 1d), indicating that differences in expression were due to different transcription levels.

While our objective was not to study the relationship between transcription and promoter sequence, we generally found that more mutations led to decreased expression. Some of the weakest promoters were generated by the random mutagenesis method, which produced the highest frequency of mutations. Mutations to the TATA box were effective in producing a range of CMV and EF1α promoter strengths. Single mutations to the most conserved base (Bucher 1990) (underlined: TATATAA) decreased expression from the CMV promoter by 30–55%. In general, TATA box mutations affected expression more than CAAT box mutations in the CMV-derived promoters. However, the CAAT box motif in the CMV promoter has two atypical attributes: the actual sequence consists of the less common CAAAT sequence (Thomsen et al. 1984) (having three A's) and it is closer to the TSS (57 bp away) than it is in most promoters; thus results could be specific to the CMV promoter. Interestingly, three CMV promoters with CAAT box mutations produced expression levels that were >5% higher than wild-type expression.

Expression using retroviral vectors

Although site-specific plasmid integration was valuable in evaluating the strength of individual promoters, this method would be too time-consuming and laborious for more high-throughput experiments. Since retroviral vectors integrate into the host genome and stably transduce cells with high efficiency, we turned to retroviral vectors as an expression vehicle. Yet with this transduction method, vector genetic elements (e.g., retroviral enhancers) or host genetic elements near the site of vector integration (e.g., endogenous promoters) could interfere with ectopic gene expression. To minimize these issues, we needed to

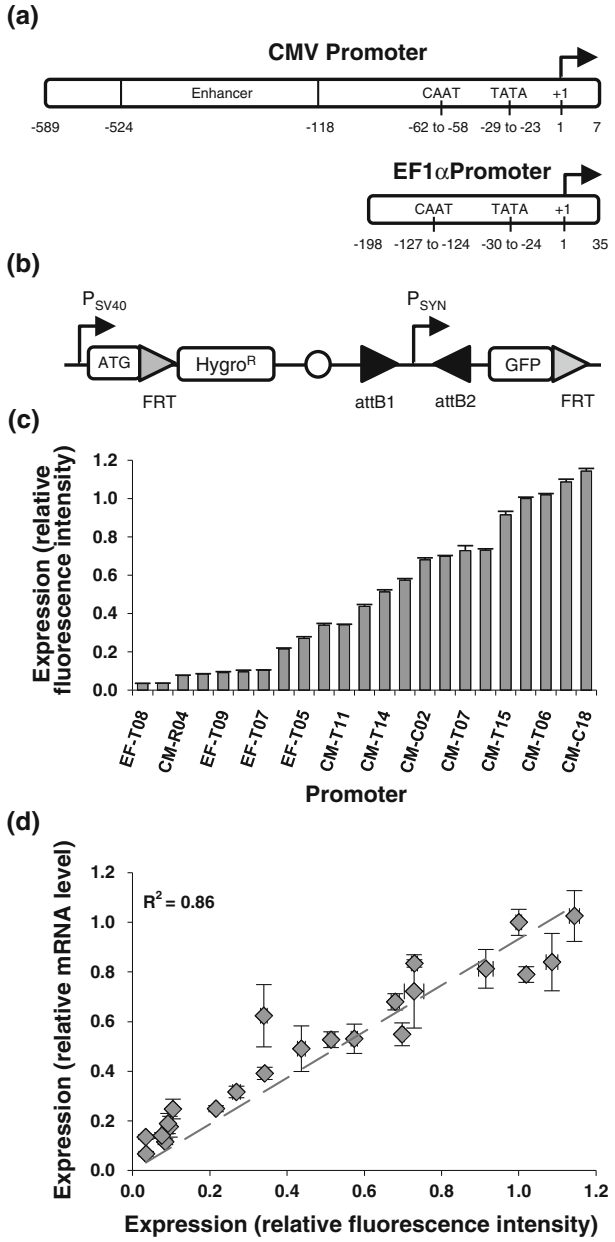

Fig. 1 Expression by synthetic promoters. **a** CMV and EF1α promoters mapped relative to their transcription start sites (+1). CAAT and TATA, transcription factor binding sites. **b** Plasmid vector integrated into recombination (FRT) site in HEK-293 Flp-In genome. P_{SV40}, SV40 promoter; P_{SYN}, synthetic promoter derived from either CMV or EF1α; GFP, green fluorescent protein gene; attB, recombination sites for cloning; $Hygro^R$, hygromycin resistance gene (translated at upstream ATG); circle, *E. coli* plasmid elements (**c**) GFP fluorescence intensity of HEK-293 cells. **d** mRNA level versus fluorescence intensity; *dashed line* represents linear regression ($y = 0.932x$). Expression was normalized to the wild-type CMV promoter (CMVwt). Values are arithmetic means ± s.d. ($n = 3$) calculated from geometric means of each sample population

identify a vector design where the relative expression strengths from the synthetic promoter would be preserved and not overshadowed by interfering genetic elements. At the same time, we needed to be able to express an antibiotic resistance gene to enable positive selection of stable transduction. Although many vectors express their genes of interest and antibiotic selection genes from the same promoter and transcript (e.g., by separating the genes by an internal ribosomal binding site), we avoided this vector structure because low expression of the antibiotic resistance gene could stifle positive selection.

We engineered two types of non-replicating retroviral vectors (Fig. 2a, representative provirus sequences with the parent promoters in Supplementary Material). The first vector was a Moloney murine leukemia virus (MLV) vector with the antibiotic resistance gene expressed by the long terminal repeat (LTR) promoter, upstream of the synthetic promoter. The second was a self-inactivating murine stem cell virus (MSCV-SIN) vector with a separate resistance cassette downstream of the synthetic promoter and gene of interest (Fig. 2a, Supplementary Material). The MSCV-SIN vector contained a self-inactivating (SIN) long terminal repeat (LTR), where the retroviral transcriptional enhancer was not integrated into the genome. To evaluate these vectors, the synthetic promoters were used to express GFP. With future applications in mind, we also engineered the vectors to contain an in-frame F2A ribosome slippage sequence and a cloning site that supports recombinase-mediated insertion of genes of interest. Because GFP and the gene of interest are expressed from the same transcript, GFP fluorescence intensity reports the expression level of the gene of interest.

We transduced several cell lines with a single copy of the expression vectors and measured GFP fluorescence. We found that the MLV vector was superior to MSCV-SIN on four counts: (1) the range of expression was greater (Fig. 2b); (2) selection for stable integration was more efficient (Fig. 2c); (3) expression correlated better with previously observed plasmid-based expression in HEK-293 cells (Fig. 3); and (4) the relative promoter strengths were better reproduced in the other cell lines: PD-31, NIH/3T3, HeLa (Fig. 3). These results were unexpected since we anticipated that eliminating the retroviral enhancer (using MSCV-SIN) would reduce the interference caused by transcriptional read-through from the LTR promoter. Instead, it is possible that with this particular MLV vector, the synthetic promoter is shielded from interfering host genome elements by the upstream LTR and antibiotic resistance gene. With the MLV vector, while the relative order of promoter strengths was generally preserved between different cell lines for promoters with the same parent (Fig. 4), we observed that expression from EF1α promoters was higher in the PD-31 pre-B cell line than in the other non-lymphocyte cell lines. Because the EF1α promoter sequence that we used did not contain its own transcriptional enhancer, transcription from the promoter

Table 1 Summary of mutations and expression for selected synthetic promoters

Name	Sequence	Mutagenesis Method	GFP Expression (relative fluorescence intensity)
CMVwt	(-62)**CAAAT**GGGCGGTAGGCGTGTACGGTGGGAGGTC**TATATAA**GCAGAGCTGGTTTAGTGAACCGTCAGATC(+ 7)	n/a	1.00 ± 0.007
CM-C18	---G----------	site-directed	1.14 ± 0.013
CM-C07	T---C----------	site-directed	1.09 ± 0.015
CM-T06	----------G----------	site-directed	1.02 ± 0.007
CM-T15	----------GGGG----------	site-directed	0.91 ± 0.019
CM-C23	GG----------	site-directed	0.73 ± 0.008
CM-T07	----------GG----------	site-directed	0.73 ± 0.025
CM-T18	----------A----------	site-directed	0.70 ± 0.005
CM-C02	-CCCC----------	site-directed	0.68 ± 0.011
CM-R02	--C-----T-T---------C---G--C-CT---------A--C---A-C-G----CG---A-ACCC--	random mutagenesis	0.57 ± 0.008
CM-T14	----------GGG----------	site-directed	0.51 ± 0.010
CM-T08	----------GGG----------	site-directed	0.44 ± 0.010
CM-T11	----------GGGGGGG----------	site-directed	0.34 ± 0.002
CM-R03	----------C----G----C--AC-T-CCA--G---C-TG--C-A--AAG----T----A-C---G	random mutagenesis	0.22 ± 0.004
CM-R05	---C-T-----C---G--AG--AA-CA--------G-GC---C------A-ACTT---C-T-A--T---	random mutagenesis	0.08 ± 0.001
CM-R04	--C----C---A----T--A--T---A----------C----TGA-----T-A-----C---T---	random mutagenesis	0.08 ± 0.003
EF1αwt	(-30)**TATATAA**GTGCAGTAGTCGCCGTGAACGTTC(+ 1)	-	0.34 ± 0.009
EF-T05	CC----------	site-directed	0.27 ± 0.010
EF-T07	----CCC----------	site-directed	0.10 ± 0.001
EF-T01	CCCCCCC----------	site-directed	0.10 ± 0.008
EF-T09	--CCCCC----------	site-directed	0.09 ± 0.005
EF-T08	---CCCC----------	site-directed	0.03 ± 0.002
No promoter	Vector without promoter	-	0.04 ± 0.002
Cells only	No vector	-	0.00 ± -

Promoter naming convention: CM, CMV promoter mutant; EF, EF1α promoter mutant; C, T, or R—CAAT, TATA, or random mutagenesis, respectively. The TATA and CAAT box sequences of the wild-type promoters (CMVwt or EF1αwt) are in bold, and the transcription start site (TSS) is underlined. Base position is given in parentheses at the ends of the sequence and corresponds to the distance from the TSS. Mutations listed under the wild-type sequences: –, no mutation; Δ, base deletion. GFP expression values from HEK-293 cells are arithmetic means ± s.d. ($n = 3$) calculated from geometric means of each sample population

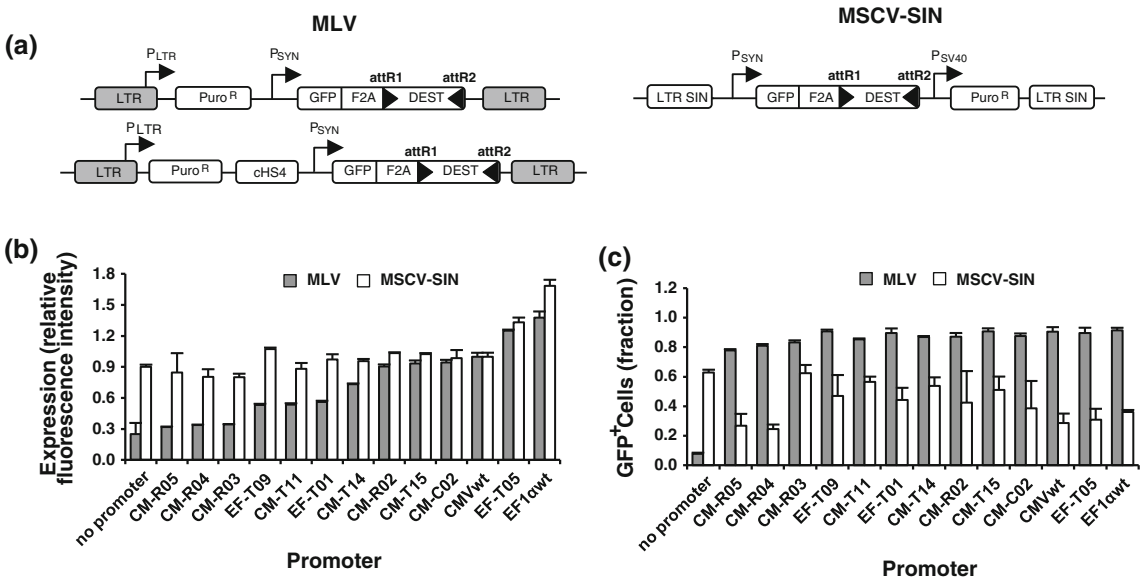

Fig. 2 Expression from synthetic promoters using retroviral vectors. **a** MLV and MSCV-SIN (self-inactivating) vectors depicted as integrated into genome. LTR, retroviral long terminal repeat; P_{LTR}, LTR promoter; $Puro^R$, puromycin resistance gene; P_{SYN}, synthetic promoter derived from CMV or EF1α promoters; P_{SV40}, SV40 promoter; LTR SIN, retroviral long terminal repeat with enhancer sequences deleted; F2A, ribosomal slippagesequence; DEST, recombinase-mediated cloning site; cHS, chicken hypersensitivity site-4. F2A allows bicistronic expression of GFP and a gene of interest inserted in the DEST site. In this study, the mCherry gene was inserted into the DEST site. **b** Expression by synthetic promoters (GFP fluorescence intensity). **c** Positive selection of transduced cells: GFP^+ fraction after addition of puromycin. MLV (*gray bars*) and MSCV-SIN (*white bars*) vectors were evaluated in PD-31 cells. Values are arithmetic means ± s.d. ($n = 3$) calculated from geometric means of each sample population

was likely bolstered by the enhancer in the MLV LTR, which is thought to be particularly potent in lymphocytes like the PD-31 line. Because the CMV promoter sequence contains its own strong transcriptional enhancer (Fig. 1a), transcription from the CMV promoter is likely less affected by the LTR enhancer, and consequently the promoter strengths were less varied between lymphocyte and non-lymphocyte cells. In support of this notion, we found that when the CMV enhancer was deleted, expression in the PD-31 cells increased comparably (data not shown). Lastly, we also found that adding a 5′ transcriptional insulator (chicken hypersensitive site-4, cHS4, Fig. 2a and

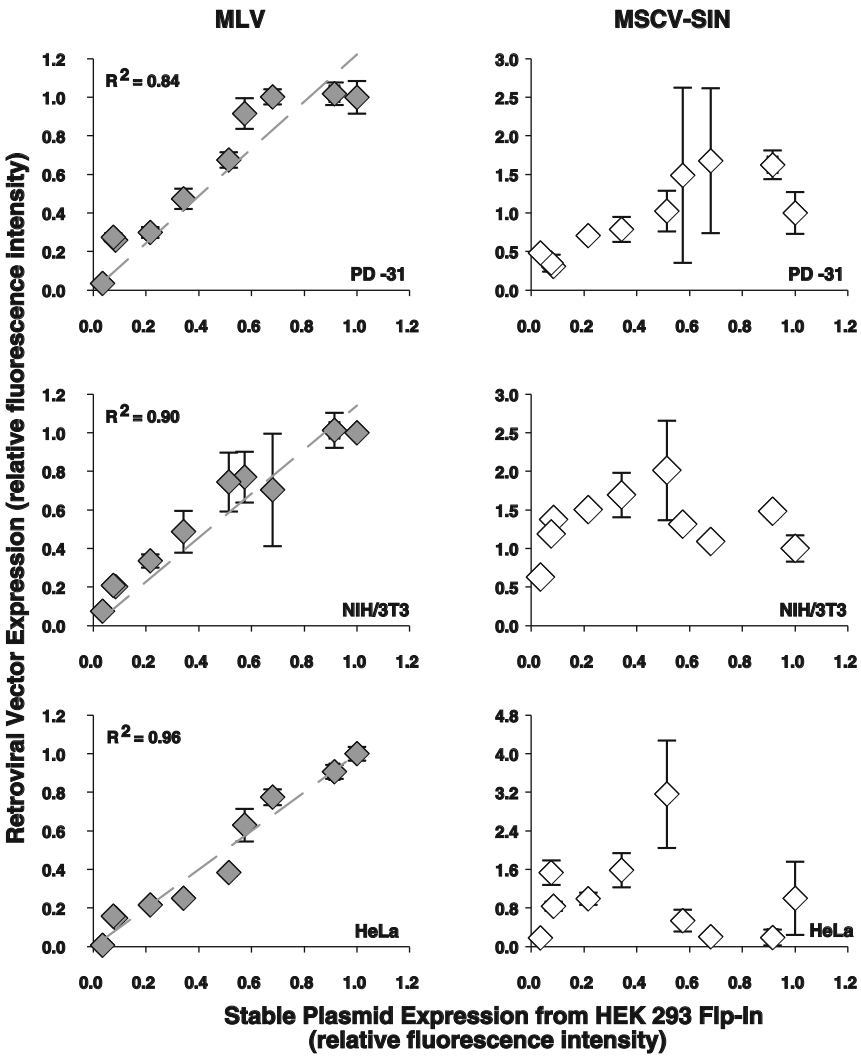

Fig. 3 Comparison of expression from synthetic variants of the CMV promoter in different cell lines and vectors. Expression levels (GFP fluorescence intensity) from MLV (*left*) and MSCV-SIN (*right*) vectors in PD-31, NIH/3T3, and HeLa cells compared against those from plasmid vector expression in HEK-293 cells. Fit to linear regression (*dashed line*; $y = 1.223x$ for PD-31, $y = 1.141x$ for NIH/3T3, and $y = 1.000x$ for HeLa) indicates whether relative promoter strengths were maintained between different cell types. Values are arithmetic means ± s.d. ($n = 3$ for PD-31, HEK-293 Flp-In and $n = 2$ for NIH/3T3, HeLa) calculated from geometric means of each sample population

Supplementary Material) can decrease expression levels by more than 50% (Fig. 4). Although this effect was not as large for weaker promoters, in general the cHS sequence decreased expression when inserted upstream of the synthetic promoters.

Discussion

We generated a library of synthetic promoters by mutating constitutive promoters commonly used in mammalian expression vectors. While the parent promoters normally produce high, possibly supra-physiological, expression levels, mutants demonstrated a range of low and intermediate levels. We also identified a retroviral vector construct that could produce desired expression levels in a generally predictable manner. The retroviral vector is equipped with antibiotic resistance for positive selection, a GFP reporter gene, and a cloning site for convenient recombinase-mediated insertion of genes of interest. Because the cloning site is compatible with other publically available cDNA libraries, e.g., that from the Human ORFeome project (Matsuyama and Yoshida 2009), we envision that the vector will be immediately useful to many biologists and engineers.

When using the promoters, one should bear in mind that expression levels can vary between cell types. While promoters derived from the same parent likely will preserve expression levels relative to themselves, users should still evaluate promoters in each cell type before proceeding with experiments. Users of our synthetic promoters should initially choose several promoters covering a wide expression range to test whether expression in their cells resembles that of the cell lines tested here (Fig. 4). Whenever possible, we suggest using a reporter gene so expression levels can be continuously monitored. Vector components can affect the relative strength of the promoters and if users choose to deviate from our MLV vector design, we recommend including and evaluating a buffer region (e.g., another expressed gene as with our puromycin

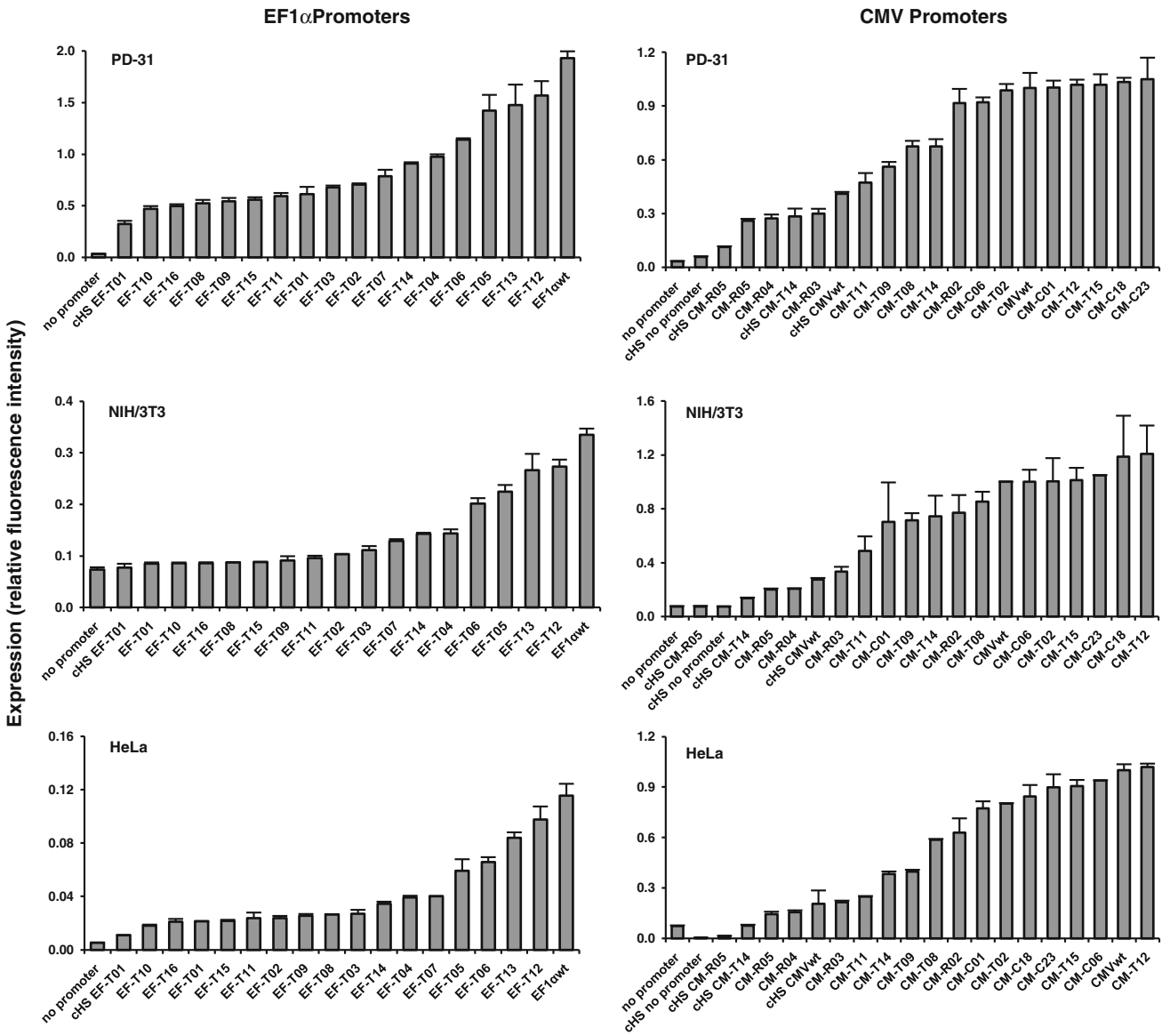

Fig. 4 Expression levels from MLV vectors employing synthetic promoters in various cell lines. GFP expression from PD-31, NIH/3T3, and HeLa cells. Promoters were derived from parent EF1α (EF1αwt) or CMV (CMVwt) promoters. cHS indicates promoters preceded by chicken hypersensitive site-4, a putative transcriptional insulator. Values are arithmetic means calculated from geometric means of each sample population. *Error bars* for PD-31 represent s.d. ($n = 3$); for NIH/3T3 and HeLa the high value of duplicate samples is represented by *error bars* above the mean ($n = 2$)

resistance cassette or a transcriptional insulator) upstream of the synthetic promoters.

We believe that using our synthetic promoters to specify different expression levels can have advantages over an inducible expression system, e.g., expression using the tetracycline-responsive promoter. First, inducible promoters can be "leaky" in expression and even when switched "off" to the maximum extent, this uninduced level of expression may already be too high for one's purposes. Second, using inducible systems to express multiple genes requires an orthogonal system for each additional gene; i.e., one is limited by the number of available inducible systems. In contrast, when using different synthetic promoters to drive multiple genes at different expression levels, there is no such limitation. Third, when using inducible systems, each different expression level requires a different culture with a different inducer chemical concentration. In contrast, since the expression level from the synthetic promoters are independent of any supplemented chemical, multiple expression levels can be evaluated in a single culture. We envision transducing cells with a mixture of retroviral vectors equipped with the library of synthetic promoters. One can generate a single heterogeneous culture where a broad range of expression levels are

represented. One could then use single-cell analysis, sorting (e.g., fluorescence-activated cell sorting), or enrichment methods (e.g., selection for proliferation) to determine a relationship between expression level and phenotype (Ferreira et al. 2011). Finally, synthetic promoters can be useful in "hard-wiring" a genetic circuit or device so that it can function without chronic supplementation with inducer chemicals.

We anticipate that the synthetic promoters and vectors reported here will be helpful to researchers seeking to evaluate genes at levels comparable to those in nature. They also will be useful in synthetic biology applications that require tuning of gene expression and system optimization.

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