Synthetic design of strong promoters

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We have taken a synthetic biology approach to the generation and screening of transcription factor binding sites for activity in human cells. All possible 10-mer DNA sequences were printed on microarrays as 100-mers containing 10 repeats of the same sequence in tandem, yielding an oligonucleotide library of 52,429 unique sequences. This library of potential enhancers was introduced into a retroviral vector and screened in multiple cell lines for the ability to activate GFP transcription from a minimal CMV promoter. With this method, we isolated 100 bp synthetic enhancer elements that were as potent at activating transcription as the WT CMV immediate early enhancer. The activity of the recovered elements was strongly dependent on the cell line in which they were recovered. None of the elements were capable of achieving the same levels of transcriptional enhancement across all tested cell lines as the CMV enhancer. A second screen, for enhancers capable of synergizing with the elements from the original screen, yielded compound enhancers that were capable of twofold greater enhancement activity than the CMV enhancer, with higher levels of activity than the original synthetic enhancer across multiple cell lines. These findings suggest that the 10-mer synthetic enhancer space is sufficiently rich to allow the creation of synthetic promoters of all strengths in most, if not all, cell types.

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direction and 10 in the reverse. As a result of this feature, all possible combinations of 10-mer repeats could be printed in only 50,000 unique sequences, although some had only nine full repeats (Fig. 1A). The approximately 5 Mb of oligonucleotides were cleaved from the microarray using a brief treatment with a basic solution and PCR-amplified before restriction cloning into the vector pSJ2. The pSJ2 vector is a self-inactivating murine stem cell virus based vector containing a multiple cloning site (MCS) upstream of a minimal CMV promoter driving EGFP and a puromycin resistance marker. The use of a retroviral vector allows individual constructs to be integrated into cells at single copy and, when used at a low multiplicity of infection (MOI), mitigates the occurrence of multiple integrations that would confound promoter analysis. EGFP provides a marker for overall levels of transcription from the minimal CMV promoter that can be easily assayed by FACS. The use of a self-inactivating vector prevents promoter interference from the viral LTRs, which contain a deletion in the 3′ LTR resulting in complete inactivation of the retroviral promoter upon integration into the genome. The MCS was designed such that it contained multiple restriction sites with compatible ends for the subsequent insertion of multiple enhancers simultaneously (Fig. 1B), which becomes important later in combinatorial experiments. For example, the EcoRI-XhoI enhancer fragments can be recloned into MfeI-Sall or EcoRI Sall to make double inserts. Enhancers could also be excised as BamHI-EcoRI fragments and inserted back into BglII-MfeI cleaved fragments.

To test the feasibility of using the 10-mer repeat sequence library to screen for enhancer elements, the plasmid library of repeats was packaged as retrovirus, infected onto HeLa cells at an MOI of 0.1, and puromycin-selected (Fig. 2A). If strong enhancers are being sought, single copy infection is particularly important, as double integration events will give greater GFP signal than single integrations. This contaminates the resulting library population with many enhancers that are far weaker than desired. Although the minimal CMV promoter gave transcription levels that were not significantly greater than those of uninfected cells, FACS on the enhancer-containing HeLa cells revealed that greater than 15% of the 10-mer repeat library inserts were capable of giving transcriptional enhancement greater than the background level of a minimal CMV promoter (Fig. 2B and C). Of the total population, fewer than 1% of cells were capable of driving GFP expression to levels that were within the range of WT CMV enhancer–driven constructs.

GFP-expressing cells were sorted based on their level of fluorescence into four populations. The cells were expanded, genomic DNA extracted, and their 10-mer repeat inserts were amplified by PCR and recloned into pSJ2 as four separate libraries for a second round of screening. The second-round libraries were then packaged and infected onto HeLa cells again, and their GFP fluorescence assessed by flow cytometry (Fig. 2D). In the second round, the percentage of cells from the highest GFP expressing sublibrary showed an increased numbers of cells capable of achieving levels of GFP fluorescence on par with the WT CMV enhancer. As a result of different FACS settings, the histograms in Fig. 2D are not on the same scale as those in Fig. 2B and C. The small fraction collected from the “high” population represents cells with fluorescence greater than the WT CMV enhancer median. To further enrich for strong enhancers, the highest expressing GFP population was sorted again and the inserts were recloned. The resulting library was run through a third round of screening, and again, as in the previous two rounds, clones that exceeded the mean GFP fluorescence of the WT CMV enhancer construct were selected.

After the third round of screening was complete, the remaining inserts were PCR-amplified and cloned as a plasmid library. Two 96-well plates of bacterial clones were then picked and plasmid DNA was sequenced. Sequencing revealed that many of the recovered clones contained highly similar sequences representing a few major classes of transcription factor binding sites. Individual representatives of the major classes of clones recovered were packaged as retroviruses and infected into cells. A variety of enhancer strengths were recovered in these individual clones. Strengths ranged from very low enhancement to enhancer activity on par with the WT CMV enhancer (Fig. 2E). Multiple related but distinct sequences were recovered in the final pool, supporting the reproducibility of the screen (Fig. S1).

The class of clones that contained consensus cAMP response element (CRE; bound by CREB protein) binding sites were recovered most frequently (Fig. 3A) and were found to exhibit the highest transcriptional enhancer activity (Fig. 2E and Fig. 3A and B). Enhancers predicted to contain AP–1–binding sites were also frequently recovered, but were found to exhibit less activity than CRE site–containing elements. The recovered transcription factor–binding sites occurred in both orientations with respect to the promoter. The most potent of the recovered CRE enhancers contained approximately seven to 10 predicted CREB half-binding.

**Fig. 1.** Design and synthesis of repeat library. (A) A single 10-mer DNA sequence printed in 10 head-to-tail repeats also contains 19 other potentially unique 10-mer repeats, for a total of 20 potentially unique sequence repeats in a 100-bp DNA fragment. DNA repeats were synthesized on microarrays, cleaved from the array surface, and PCR-amplified. The repeat oligonucleotides were cloned into a plasmid vector to generate a library of synthetic enhancers. (B) Map of plasmid vector pSJ2 for cloning/screening of 10-mer repeats in a 100-bp DNA fragment. DNA repeats were synthesized on a microarray, cleaved from the array surface, and PCR-amplified. The repeat oligonucleotides were cloned into a plasmid vector to generate a library of synthetic enhancers. Multiple restriction sites with compatible overhangs allow the cloning of multiple synthetic enhancers within the MCS. The MCS is upstream of a minimal CMV promoter driving GFP, and retroviral sequences are present for packaging as a self-inactivating retrovirus.
sites (14, 15), although some of the repeats were imperfect. To
close the F10 enhancer, against the activity of perfect
CRE repeats, constructs were generated that contained two, four,
six, eight, or 10 CRE consensus binding sites. These were tested
against the F10 enhancer in HeLa cells. The F10 enhancer
exhibited slightly less transcriptional activity than the construct
with eight CRE repeats (Fig. S2B). Constructs with 10 CRE repeats
were 60% stronger than CMV and were marginally stronger than
clones with eight CRE repeats, indicating that transcriptional
activation for CRE elements was approaching saturation in this
particular context or that binding sites more distant than 100 bp
were less effective. The recovered enhancers were tested for their
transcriptional activity in other cell lines, as one of the most
desirable characteristics of the CMV enhancer is its broad spec-
trum of activity in many cell lines. The CRE-based synthetic

![Fig. 2. Screening synthetic enhancers for transcriptional activation. (A) The synthetic enhancer plasmid library is packaged as retroviruses and infected into tissue culture cells at an MOI of 0.1, puromycin-selected, and screened by FACS for cells expressing the desired level of GFP. After sorting and genomic DNA preparation, the synthetic enhancer inserts are PCR-amplified and recloned for subsequent rounds of screening. (B) Histogram of GFP fluorescence of HeLa cells expressing GFP under the control of the WT CMV promoter and enhancer. (C) Histogram of GFP fluorescence of HeLa cells expressing GFP driven by a minimal CMV promoter with the synthetic enhancer library cloned upstream. Populations indicated on the FACS histogram were separately sorted, and synthetic enhancers present were recloned and reinfected into HeLa cells. (D) Histograms of GFP fluorescence for cells infected in the second round of screening, with arrows indicating their parental population of cells from the first round of screening. (E) After two rounds of screening, clones were tested individually for transcriptional activity. A clone representing an enhancer with relatively weak activity (clone E1) and one with very potent activity (clone F10) are compared with the WT CMV promoter/enhancer. The predicted transcription factor binding sites for each synthetic enhancer are indicated.]

![Fig. 3. Analysis of sequence motifs present in most commonly recovered synthetic enhancers. (A) Unsupervised clustering of sequence homology between enhancer elements recovered from screening in HeLa cells. The yellow bars indicate clones with CREB binding elements, whereas the blue bar represents all other classes of enhancer elements. For a larger version, see Fig. S3. (B) Relative expression levels of selected synthetic enhancer elements across a panel of cell lines. Percentages are expressed as percent of WT CMV enhancer/promoter. (C) Predicted transcription factor binding motifs within selected enhancer elements. Yellow boxes indicate half-CREB binding sites, green boxes indicate C/EBP binding sites, and blue boxes indicate TP53 binding sites. Clone F10 was recovered from HeLa cells and clones F4 and F5 were recovered from 293T cells.]

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enhancers were found to have the strongest transcriptional enhancement in the HeLa cells where they were originally screened, with relatively little activity in other cell lines (Fig. S2B).

To recover transcriptional enhancer elements with stronger activity in other cell lineages and test their activity in combination with HeLa enhancers, five additional cell lines (293T, U2OS, mouse embryonic fibroblast, FL5.12, and human mammary epithelial cells) were screened with the enhancer library to isolate additional strong enhancers. Two rounds of FACS screening were performed in each cell line, with the 2% most fluorescent cells isolated in each round. After the second round of screening, a subset of enhancer inserts were cloned and individually screened for GFP fluorescence in all cell lines in 96-well plates. Clones exhibiting strong enhancer activity were sequenced and recloned into the pSJ2 MCS, downstream of the previously characterized HeLa-F10 enhancer that contains CREB protein binding sites. Profiling the recovered enhancers revealed at least two classes of enhancers: (i) CRE-based enhancers that were strong in HeLa cells but not in other lines and (ii) enhancers with strong activity across several of the cell types tested. FL5.12 cells exhibited very low transcription from the CMV minimal promoter, regardless of with which enhancer it was paired. The class of sequences that exhibited transcriptional activation in 293T and MEFs but not HeLa cells were represented by multiple different sequence motifs. Enhancers exhibiting this transcriptional pattern were most frequently predicted to contain C/EBP binding sites, but many other sites were also recovered, such as P53 sites, and sequences with no known transcription factor binding motifs (Fig. 3B and C). The sequences of the recovered enhancers are shown in Table S1.

To determine whether the transcriptional level achieved by the strongest enhancer elements represented a fundamental limit of minimal CMV promoter, a second screen was performed to search for synthetic enhancers capable of acting synergistically or additively with already recovered clones. The original 50,000-mer repeat library was excised using EcoRI-XhoI and cloned into the MCS of EcoRI-SalI cut pSJ2 upstream of three different enhancers, representing each of the major sequence classes recovered in HeLa cells. The three resulting libraries were then screened for the strongest enhancers present with use of the same method as before. For already strong enhancers, such as clone F10, the addition of the library in the first round of screening gave little to no detectable transcriptional enhancement beyond their original levels. Enrichment was observed, however, for clones possessing greater transcriptional activity than the WT CMV enhancer after two additional rounds of screening and recloning. Multiple clones were found that possessed greater than twofold more transcriptional activity than the WT CMV enhancer upon picking individual clones to validate (Fig. 4A). Although the strongest double synthetic enhancer gave approximately twofold greater GFP transcription in HeLa cells than the WT CMV enhancer, it failed to achieve the same levels in other lines (Fig. 4B), although it did improve upon the levels of CRE-based enhancer alone. The sequences of the recovered double enhancers contained many of the same predicted binding sites as the individual HeLa enhancers, indicating that transcription was being improved by the same overall mechanisms, rather than specific synergy with the paired enhancer.

We sought to determine whether an enhancer with activity in HeLa cells could be combined with enhancers that were strong in other lines to yield enhancers that are functional in multiple lines. When the F10 enhancer was combined with enhancers recovered from 293T, U2OS, or HME cells, the strong transcriptional activity of the F10 enhancer in HeLa cells was masked by the addition of a downstream enhancer. The activity across the cell line panel of the resulting double enhancer invariably resembled the downstream enhancer most closely (Fig. S2).

![Fig. 4. Screening for secondary enhancers of initial screen hits. (A) Double enhancer clone Syn1 recovered from the screen for secondary enhancers. The WT CMV enhancer is represented in red, the Syn1 double enhancer is represented in blue. (B) Median fluorescence values for the WT CMV, F10, and Syn1 enhancers. F10 and Syn1 are represented below as percentage of WT CMV values. Values colored in red represent low GFP fluorescence and values colored in green represent the highest values of fluorescence.](http://www.pnas.org/content/early/2010/02/04/0908215107.full)
Discussion
In this study we describe a method for the synthetic optimization of promoters. Using an unbiased screen of a synthetic 10-bp repeat sequence library we identified many synthetic enhancer elements capable of increasing transcription from a minimal CMV promoter. In several cases, the recovered synthetic enhancers had transcriptional activation activity on par with the WT CMV enhancer with only two rounds of enrichment. Thus a 100-bp synthetic enhancer can be engineered to match or exceed the promoter strength of the strongest known mammalian promoters.

We found that transcription levels from synthetic enhancers can be further modulated by altering the number of repeats present after an element with activity is isolated. The maximum of 10 repeats of transcription factor binding sites present in the synthetic enhancers were chosen for manageable microarray printing size. As evidenced by the improvement of recovered CREB enhancers by adding additional CREB elements, this was not always sufficient to achieve saturating levels of transcriptional activation. These experiments also show that very specific levels of transcription can easily be achieved by finding an active enhancer element through unbiased screening and increasing or decreasing the number of repeats. An “allelic series” of promoters of differing strengths could easily be generated for any cell line in this manner, allowing more careful fine-tuning of gene expression in genetic studies.

The elements recovered had a strong bias for the cell line in which they were initially screened, frequently exhibiting lower activity in other lines. Each cell line showed a distinct pattern of sequences that showed maximal activation reflecting the underlining complexity of the transcriptional milieu from cell type to cell type. From these data, it is clear that strong promoters will need to be designed for individual cell types depending on the particular application. This could be achieved more rapidly by the combination of FACS enrichment with massively parallel sequencing to determine how sequence space is distributed over bins of different expression levels, at the same time generating a fingerprint of the transcription factor activity pattern for that cell type. Characterization of the proteins binding specific enhancers, through MS or other means, could be instructive in determining the mechanism underlying the differential activity of enhancers across cell lines.

Although enhancers with significant levels of activity were recovered from most cell lines, transcriptional levels from all enhancers in the mouse pre-B-cell line FL5.12 were very low. Further, the WT CMV enhancer caused little to no increase in transcription from the minimal CMV promoter. Based on these data, it is possible that the minimal CMV promoter used for the screening is largely inactive in FL5.12 cells. However, we cannot rule out the possibility that other differences exist, such as effects on GFP folding or microRNA expression patterns that artificially mask the apparent transcription of this construct in FL5.12 cells. The WT CMV promoter is known to be poorly expressed in some stem cell lineages, although the factors responsible are unknown (16). This phenomenon may be specific for the CMV minimal promoter, as the CMV enhancer is functional in hematopoietic stem cells in concert with other promoters (17). These data suggest that a specific repressive factor may act upon the minimal CMV promoter. It may therefore be necessary to screen cell types with poor CMV activity with different minimal promoters to find their most active enhancer elements.

It is currently unclear what the rate-limiting factors are for gene expression, but they are likely to include RNA polymerase recruitment, chromatin modification, nucleosome remodeling, promoter clearance and pausing, abortive transcription, and rates of RNA polymerase transcription after initiation. Each of these could potentially be optimized to promote strong transcription, and a subset of these might be affected by the synthetic enhancers. In principle, if a particular pair of factors operate to promote transcription through different mechanisms, one would expect that they would behave synergistically. Our attempts to identify such synergy among different elements failed to discover strong synergy, although in all cases we could continue to marginally improve upon the promoter strength. For example, we observed that most of the recovered enhancers failed to show additive effects with the likely CREB-based F10 enhancer. This may be because we are nearing a theoretical limit to promoter strength or that we simply have not varied enough other promoter elements. It is also possible that the sequence space larger than 10 bp would contain additional elements capable of synergy. Adding the F10 enhancer, which was strong in HeLa cells, to an enhancer with activity in 293T cells yielded an enhancer that was strong in 293T but not HeLa. This indicates that the CREB-based F10 enhancer may be strongly dependent on close proximity to the minimal CMV promoter to activate transcription, or that the enhancer elements used for combinations interfered with each other. Although strict proximity requirements would make the design of promoters with combination enhancers more difficult, combinations could be designed that alternate the transcription factor sites with different spacing or in different orders to allow for more optimal promoter geometry. Screening for more precise spatial requirements or interference between enhancer elements could be performed by generating libraries of many possible combinations and spatial arrangements of elements already known to possess activity from the first round of screening. It is possible that strong enhancer elements could be compressed substantially in size, or combined with minimal promoters that are smaller than CMV to generate very short complete promoter elements for specialized applications in which size may be an issue. With very small elements, it may be possible to knock-in promoters or enhancer elements at a specific locus using homologous recombination (18) with only synthetic oligonucleotides, without the difficulty of generating longer targeting constructs.

Although screening 10-mer sequence space is feasible, many transcription factors have binding sites that are larger than 10 bp. Although these sequences are too complex to screen in completely unbiased fashion, it is possible to use gapped or inverted repeats to take advantage of the symmetrical nature of transcription factor binding sites (19). With more complete, unbiased site libraries, sites, strong or called “allelic series” of sites, strong or called “element-in-element” interactions could be performed by generating libraries of many possible combinations and spatial arrangements of elements already known to possess activity from the first round of screening. Sequence space could also be rationally constrained by improving predictions of binding site specificity for known DNA binding factors in the human genome. Although predictions have improved markedly for zinc finger transcription factors (20), many other classes of DNA binding domains have unknown specificities. Predicting exact specificities, however, would be unnecessary for the purpose of designing new libraries. General predictions of which spatial arrangements of nucleotides are being “read” by proteins could be sufficient to reduce the complexity of libraries to screenable sizes.

The synthetic biology approaches described here could be applied to any biologic process mediated by short DNA/RNA binding sites. For example, the current library could be used to search for transcription factors regulated in response to a particular stimulus by FACS sorting with and without stimulus adding the F10 enhancer, which was strong in HeLa cells, to an enhancer with activity in 293T cells yielded an enhancer that was strong in 293T but not HeLa. This indicates that the CREB-based F10 enhancer may be strongly dependent on close proximity to the minimal CMV promoter to activate transcription, or that the enhancer elements used for combinations interfered with each other. Although strict proximity requirements would make the design of promoters with combination enhancers more difficult, combinations could be designed that alternate the transcription factor sites with different spacing or in different orders to allow for more optimal promoter geometry. Screening for more precise spatial requirements or interference between enhancer elements could be performed by generating libraries of many possible combinations and spatial arrangements of elements already known to possess activity from the first round of screening. It is possible that strong enhancer elements could be compressed substantially in size, or combined with minimal promoters that are smaller than CMV to generate very short complete promoter elements for specialized applications in which size may be an issue. With very small elements, it may be possible to knock-in promoters or enhancer elements at a specific locus using homologous recombination (18) with only synthetic oligonucleotides, without the difficulty of generating longer targeting constructs.

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The synthetic biology approaches described here could be applied to any biologic process mediated by short DNA/RNA binding sites. For example, the current library could be used to search for transcription factors regulated in response to a particular stimulus by FACS sorting with and without stimulus looking for a shift in the expression levels, much like we have done for protein stability (21). One could also take different enhancers and combine them with a synthetic minimal promoters either designed de novo or taken from the 20,000 known genes. This entire complement could then be used to look for optimization or for pairing between particular enhancers and minimal promoters. Synthetic repeat libraries could be placed in 5’ UTRs to look for sequences capable of enhancing splicing, translation, or even acting as internal ribosome entry site elements. Such libraries placed in 3’ UTRs could also be designed...
Materials and Methods

Synthesis and Cloning of Synthetic Enhancer Library. Fifty thousand 10-mer repeat sequences were synthesized on a NimbleGen microarray (Roche) flanked by EcoRI-XhoI sites and primer sequences. The sequences were cleaved from the array by a brief basic wash and PCR-amplified. The PCR amplification used the following primers: SJ2 forward, TCTAGGCGCCGGAATTAGAT; and SJ2 reverse, CGCCTACCTCGACATACGTT. PCR was performed at 95 °C for 5 min, at 55 °C for 30 s, and at 72 °C for 30 s with HS Taq according to the manufacturer’s recommendations (Takara). Amplified product was digested overnight with EcoRI and XhoI and gel purified with a Qiagene spin kit (Qiagen). The cut and gel-purified PCR material was then cloned into XhoI-EcoRI cut λSM2C phage DNA and packaged using a λ-phage packaging extract (Epilcare). The resulting library of more than $10^8$ recombinants was then amplified as phage and converted to plasmid form using a Cre recombinase expressing bacteria. The resulting plasmid library was amplified and prepared using a Maxiprep kit (Sigma). The XhoI-EcoRI fragment was then cut from this library, gel-purified, and cloned into XhoI-EcoRI cut pSJ2. The resulting library was in excess of 2 x $10^6$ recombinants.

PCR Recovery and Recloning of Synthetic Enhancers. Cells were trypsinized and resuspended at 10 million cells/mL in 10 mM Tris/10 mM EDTA, pH 8.0, and Proteinase K and SDS were added at final concentrations of 200 ng/mL and 0.5%, respectively. Lysis mixture was incubated overnight at 55 °C. Lysates were extracted twice with phenol/chloroform/isoamyl alcohol and once with chloroform in PhaseLock tubes (5 Prime). Residual chloroform was evaporated for 1 h at 55 °C and RNase A was added at 37°C for 1 h to remove any RNA remaining. Lysate was then extracted twice more with phenol/chloroform and precipitated with 1.5 volumes of ethanol. After centrifugation and washing with 70% ethanol, the resulting pellet was resuspended in 10 mM Tris, pH 8.0, 1 mM EDTA.

Purified genomic DNA then underwent PCR in 400-μL reactions containing 20 μg of DNA. One hundred nmol of each primer was added, and TaKaRa HS Taq was used at the manufacturer’s recommended concentrations of buffer and dNTPs. Primers were SJ2FWD and SJ2REV, and the PCR program was 95 °C for 5 min, 55 °C for 30 s, and 72 °C for 30 s for 35 cycles. The resulting PCR product was precipitated and gel-purified for the correct size using a TAE agarose gel and Qiagene gel extraction kit (Qiagen). The gel-purified PCR product was digested with EcoRI and XhoI overnight, and gel-purified again before ligation into EcoRI-XhoI cut pSJ2. The ligation mixture was electro-transformed into MegaX DH10B ultracompetent Escherichia coli (Invitrogen) and plated on four LB-carbenicillin 150-mm plates. Colonies were scraped from the plates and grown in liquid culture for 8 h before analysis with a Maxiprep kit (Sigma). For cloning of second-site library into clones F10, A11, and E1, the complete synthetic enhancer library was digested with SalI and EcoRI and cloned into XhoI-EcoRI digested pSJ2 already containing the listed inserts.

Informatics. Transcription factor motif searches were performed using the TFSearch (http://www.cbr.jpl.nasa.gov/pfsearch) and PMatch (http://www.gene-regulation.com/cgi-bin/pubprograms/pmatch/bin/p-match.cgi) programs. The dendrogram of sequence similarities and multiple alignment of CRE sequences were generated using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html).

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Supporting Information

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SI Materials and Methods

Tissue Culture and Retroviral Methods. HeLa, 293T, and U2OS cells were purchased from the American Type Culture Collection (ATCC). Human mammary epithelial cells were from a normal reduction mammoplasty (Lonza) and were immortalized with hTERT by T. Westbrook. FL5.12 cells were a gift from C. Thompson (Philadelphia, PA). MEFs were large-T antigen-immortalized mouse embryonic fibroblasts, and were a gift from Ji Luo (Boston, MA). HeLa, 293T, and MEF cells were cultured in high-glucose DMEM with 10% FBS (Invitrogen). Human mammary epithelial cells were cultured in MEGM media (Lonza). FL5.12 cells were cultured in DMEM with 10% FBS and mouse recombinant IL-3 supplement (Sigma). Cells were passaged using trypsin whenever they reached 90% confluence, except for FL5.12 cells, which were passaged when the cell density exceeded 1 million per milliliter.

Retrovirus was produced by transfecting 5 μg of retroviral backbone plasmid, 1 μg of pCMV-VSVG, and 1 μg of pCG-GagPol onto 70% confluent 293T cells using TransIT-293T reagent according to manufacturer’s instructions (Mirus). Media were changed 24 h after transfection, and viral supernatant was collected 48 h after transfection, filtered at 0.45 μm, and stored at −80 °C until use. Before use in library screening, retroviral supernatant was titered on U2OS cells. For infection, 60% confluent cells were incubated in the presence of viral supernatant overnight at 37 °C in 4 to 8 μg/mL Polybrene (Sigma). Retroviral supernatant was infected onto cell lines at an MOI of 0.1 for library screening, and selected with puromycin for 1 week.

Flow Cytometry. The cell line of interest was infected with pSJ2-enhancer library retroviral supernatant with a minimum of 5 million infection events at an MOI of 0.1, and puromycin-selected for 4 to 7 d at a concentration of 0.3 to 2.0 μg/mL, depending on cell line. The selected population was then harvested by trypsinization, pelleted by centrifugation, and resuspended in growth media at a concentration of 10 million cells per milliliter. This cell suspension was sorted for the GFP fluorescence level of interest on a BD FACS Aria cell sorter, such that at least 2 million cells were recovered for the population of interest. This population was then cultured for 1 week to increase cell numbers, before harvesting for genomic DNA.

For testing of individual enhancers by FACS analysis, virus was generated in 24-well plates and infected onto the cell line of interest in 96-well plates. Media were changed 24 h after infection, and cells were trypsinized and resuspended in 200 μL PBS solution for FACS analysis 48 h after infection. The 96-well plates were analyzed using an HTS module on a BD LSRII, and the resulting files were analyzed for fluorescence intensity in the FlowJo software package.

![Fig. S1.](www.pnas.org/cgi/content/short/0914803107) Relative expression levels of selected synthetic enhancers. (A) Median flow cytometry fluorescence values for WT CMV-GFP in indicated cell lines. (B) Levels of GFP fluorescence for indicated cell lines and enhancers. Values are expressed as percentage of WT CMV enhancer-promoter. Values colored in green indicate stronger expression and values in red indicate weaker expression.
Fig. S2. Multiple alignment of HeLa enhancers predicted to contain CRE sites. Similarity between multiple clones is shaded blue, with darker blue representing more frequent similarity. The histogram at bottom represents percentage identity among all clones, with the higher black bars signifying greater identity. The CRE half site is TTGACG.
Fig. S3. Cladogram of HeLa synthetic enhancer elements. Unsupervised clustering arranged HeLa synthetic enhancer elements into the cladogram based on sequence similarity.
Other Supporting Information Files

Table S1 (XLS)