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 FACSArria 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.

![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.](https://www.pnas.org/content/10.1073/pnas.0914803107)
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)