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A Genome-Wide Activity Assessment of Terminator Regions in *Saccharomyces cerevisiae* Provides a "Terminatome" Toolbox

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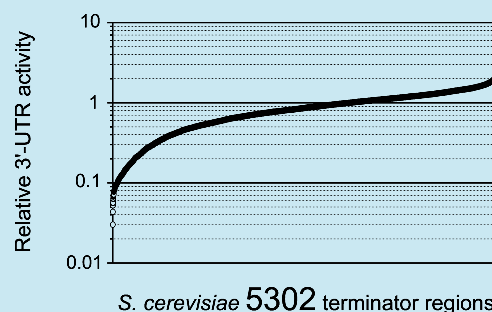
Supporting Information

ABSTRACT: The terminator regions of eukaryotes encode functional elements in the 3′ untranslated region (3′-UTR) that influence the 3′-end processing of mRNA, mRNA stability, and translational efficiency, which can modulate protein production. However, the contribution of these terminator regions to gene expression remains unclear, and therefore their utilization in metabolic engineering or synthetic genetic circuits has been limited. Here, we comprehensively evaluated the activity of 5302 terminator regions from a total of 5880 genes in the budding yeast *Saccharomyces cerevisiae* by inserting each terminator region downstream of the P_{TDH3}- green fluorescent protein (GFP) reporter gene and measuring the fluorescent intensity of GFP. Terminator region activities relative to that of the *PGK1* standard terminator ranged from 0.036 to 2.52, with a mean of 0.87. We thus could isolate the most and least active terminator regions. The activities of the terminator regions showed a positive correlation with mRNA abundance, indicating that the terminator region is a determinant of mRNA abundance. The least active terminator regions tended to encode longer 3′-UTRs, suggesting the existence of active degradation mechanisms for those mRNAs. The terminator regions of ribosomal protein genes tended to be the most active, suggesting the existence of a common regulator of those genes. The "terminatome" (the genome-wide set of terminator regions) thus not only provides valuable information to understand the modulatory roles of terminator regions on gene expression but also serves as a useful toolbox for the development of metabolically and genetically engineered yeast.



KEYWORDS: *terminator, 3′-UTR, post-transcriptional regulation, metabolic engineering, Saccharomyces cerevisiae, terminatome*

Terminator regions, which are located downstream of protein coding sequences, encode 3′ untranslated regions (3′-UTRs) of mRNA. Terminator regions are usually involved in two complex events: "transcriptional termination", which involves the cleavage of 3′-mRNA and poly(A) addition;[1,2] and "post-transcriptional regulation", in which the 3′-UTR determines the stability, translational efficiency, and localization of the mRNA.[3] Sequence and structural *cis*-elements located in the 3′-UTR of mRNA transcripts interact with RNA-binding proteins (RBPs) to post-transcriptionally regulate various aspects of gene expression including mRNA localization, translation, and decay.[3,4] The Puf family is one of the most investigated RBPs in eukaryotes, and Puf proteins function as specific or global regulators of mRNA degradation and translational repression.[5] In *Saccharomyces cerevisiae*, Puf family proteins are associated with many mRNA species containing Puf consensus motifs and are involved in the promotion of mRNA decay.[6−10] Another RBP in *S. cerevisiae*, Shed2p, binds to *ASH1* mRNA at four distinct sites, three in the coding region and one in the 3′-UTR.[11−13] The *ASH1* mRNA-She2p complex interacts with a motor protein, Myo4p, via an adaptor protein, She3p, to form a large ribonucleoprotein, which localizes *ASH1* mRNA to the budding daughter cell.[12] Another RBP, Khd1p, also binds to *ASH1* mRNA, in a C-rich region, to repress the translation of the transcript during its transportation from the mother cell to the tip of the budding daughter cell.[14] In addition, Khd1p meditates the enhancement of *MTL1* gene expression by binding to the gene's coding region.[15,16] However, for most of the 567 known or predicted RBPs encoded by the *S. cerevisiae* genome,[17] there is little information about function or binding sequences.

After the complete genome sequence and massive sets of mRNA expression data for *S. cerevisiae* became available, it became possible to use bioinformatics to explore the functional motifs in 3′-UTRs. Several 3′-end-processing signals have been identified, or predicted, on the basis that they are shared by many genes.[18−20] Searches for consensus regulatory elements have been conducted by comparing genome sequences from several related *Saccharomyces* species.[21,22] Many consensus sequences have been discovered upstream of a coding region (5′ *cis*-elements), and a small number has been found downstream of a coding region (3′ *cis*-elements).[22] Most of

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the 3′ *cis*-elements have not been verified experimentally. Expressed regions in yeast genomic DNA have been comprehensively investigated by various means including tiling arrays,[23] serial analysis of gene expression,[24] and next-generation sequencing.[25,26] The results of these studies indicate that each ORF is associated with a substantial 3′-UTR sequence; however, no functional motif has been directly predicted.

A comprehensive analysis of the effect of 3′-UTR activity on gene expression would facilitate the identification of functional motifs within terminator regions; however, only a small number of yeast terminator regions have been shown to influence the expression level of the upstream gene. In *S. cerevisiae*, the 3′-UTR activity of the mating pheromone a-factor (*MFA2*) gene has been investigated in detail. *MFA2* mRNA has a short half-life ($t_{1/2}$) of 3.5 min.[27−29] When the terminator region of *PGK1* ($t_{1/2}$ = 45 min) was replaced with that of *MFA2*, the stability of the chimeric *PGK1-MFA2* transcript was about half that of the authentic *PGK1* transcript.[28] Furthermore, the *MFA2* terminator region (*MFA2t*), coupled to a fluorescent reporter gene, green fluorescent protein (GFP) or red fluorescent protein (mKO2),[30] produced about half the amount of protein as that produced when the *PGK1* terminator region (*PGK1t*) was coupled to the same reporter gene; in contrast, use of the *TPS1* terminator region (*TPS1t*) produced 1.2 times the amount of product observed with the use of *PGK1t*.[31] These results suggest the existence of 3′-UTRs that variously influence the expression of upstream coding regions.

In this study, we evaluated and ranked the activity of terminator regions for 5302 of the 5880 genes in *S. cerevisiae* (∼90% covered). This snapshot data set could be used to find functional *cis*-elements in 3′-UTRs. In both metabolic and genetic engineering, the data set could also offer a means to regulate the level of transgene expression by exploiting the synergistic effects of 3′-UTRs and promoters.

■ RESULTS AND DISCUSSION

Comprehensive Analysis of Terminator Region Activity. We explored the function of terminator regions as determinants of gene expression. The activity of each terminator region was quantitatively evaluated by assessing the level of protein production from a linked fluorescent reporter gene (GFP) under the control of a strong promoter (Figure 1). Because our primary aim was to isolate terminator regions that modulate gene expression in all genes, we chose this simple experimental setup.

The lengths of the 3′-UTRs of most *S. cerevisiae* genes have been experimentally estimated to be ≤300 bp;[23−26] therefore, for the first PCR step, we designed 5363 primer sets to amplify ∼500-bp DNA fragments that included the 3′-UTR of each gene. Terminator regions are AT-rich, and it can be difficult to design primers for such regions; however, the efficiency of PCR amplification was high in a preliminary experiment (98.9%, 375/379 amplified). In the second PCR step, vector sequences were added to the ends of the DNA fragments to make them suitable for gap-repair cloning. CEN plasmid transformants harboring each terminator sequence were then created with the use of gap-repair cloning, and GFP fluorescence intensity (FI) values were obtained for 5302 yeast transformants (Supplementary Table S1). After calibration, these FI values were normalized to that of the *PGK1t* strain (see Methods) to provide relative FI values, which were considered to be

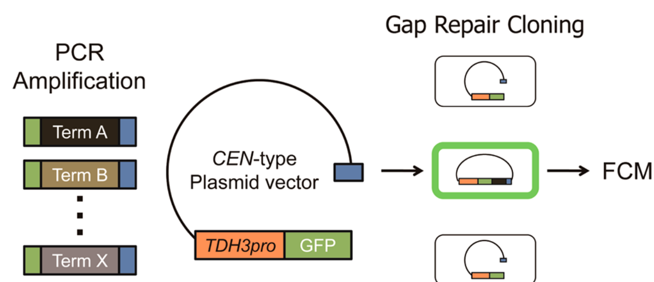

Figure 1. Scheme for comprehensive analysis of terminator region activity. Various DNA fragments containing a terminator region were amplified by PCR from the *S. cerevisiae* genome. A second PCR was conducted to introduce sequences homologous to a cloning vector at both ends of each fragment. The green region at the left end of each fragment is homologous to the green region at the right end of the GFP gene in the *CEN*-type plasmid vector, while the blue region at the right end of each fragment is homologous to the blue region in the same vector. A low copy *CEN*-type plasmid harboring the codon-optimized GFP (opGFP) gene[32] under the control of the *TDH3* promoter was digested with a restriction enzyme at a site just downstream of the stop codon and then mixed with an amplified DNA fragment and yeast competent cells. During incubation in selection medium, simultaneous cloning and transformation occurred (gap-repair cloning). Emission of GFP fluorescence from transformed yeasts was measured with the use of a flow cytometer.

measurements of the activity of each terminator region relative to that of *PGK1t*.

Each relative FI value is plotted in Figure 2A. The maximum relative FI value was 2.43 for the *NAT5t* strain, and the minimum value was 0.030 for the *ORC4t* strain; thus, terminator region activity varied over an 81-fold range. The distribution of the relative FIs is shown as a histogram in Figure 2B. The mean and median relative FI values were 0.87 and 0.86, respectively. The relative FI values for 4177 strains (79% of the 5302 strains) were between those of the *MFA2t* strain (relative FI, 0.49) and the *SAG1t* strain (relative FI, 1.9), which were used as standards. The relative FI values for 43 strains were extrapolated higher than the value for the *SAG1t* strain, and the relative FI values for 1084 strains were extrapolated lower than the value for the *MFA2t* strain. The distribution of relative FI values is skewed to the bottom.

Most and Least Active 30 Terminator Regions in Yeast. To verify the comprehensive FI data set investigated with a plasmid vector (Figure 1), especially for the strains where the relative FI values were extrapolated, we constructed and examined 95 genome-integrated transformants (see Methods). Of the 5302 plasmid strains included in the above global analysis, the 43 with the highest relative FI values (all higher than that of the *SAG1t* strain), 41 with the lowest relative FI values (all lower than that of the *MFA2t* strain), and 11 with mid-level values were chosen for verification (listed in Supplementary Table S2). The relative FI values of the genome-integrated strains were measured under the same growth conditions as those used for the plasmid strains. The relative FI values for equivalent terminator regions in the two types of transformants were highly correlated (R = 0.97; Figure 3), indicating that the results of the comprehensive analysis have high reproducibility.

Because the relative FIs of the genome-integrated transformants were measured with accuracy in three independent experiments, the data set from the integrated transgenic yeasts was thought to be more worthy of further study. We ranked the

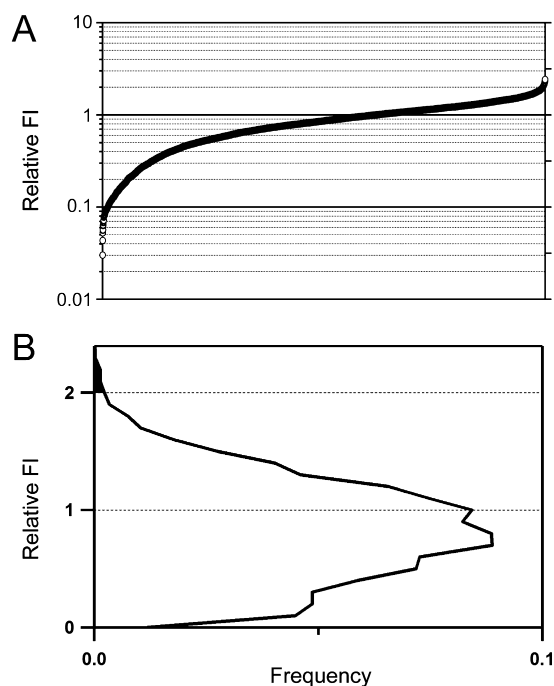

Figure 2. Comprehensive analysis of the yeast terminator region activities. Numerical values representing terminator region activity were obtained for 5302 transformed yeast strains produced by gap-repair cloning of terminator fragments into an assay plasmid (Figure 1). (A) Global ranking of yeast terminator region activity. The vertical axis indicates the GFP fluorescence intensity relative to that for *PGK1t* (relative FI value) on a log10 scale. Each circle represents a transformed yeast strain. (B) Histogram of yeast terminator region activity. The vertical axis indicates the relative FI value. The horizontal axis indicates the cumulative frequency of each FI value.

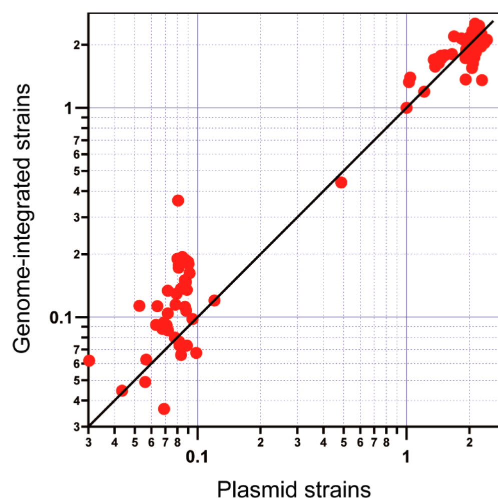

Figure 3. Verification of the comprehensive analysis of plasmid transformants. Both axes indicate GFP fluorescence intensity values relative to that for *PGK1t* (relative FI values) on a log10 scale. The vertical and horizontal axes represent the values for the genome-integrated and plasmid strains, respectively. Each of the 95 closed circles represents a terminator region. The linear regression line is shown as a solid line. The correlation efficient was 0.97.

transformants by relative FI value from the highest (top) to the lowest (bottom). The relative FI values of the top and bottom 30 terminator regions are displayed in Figure 4.

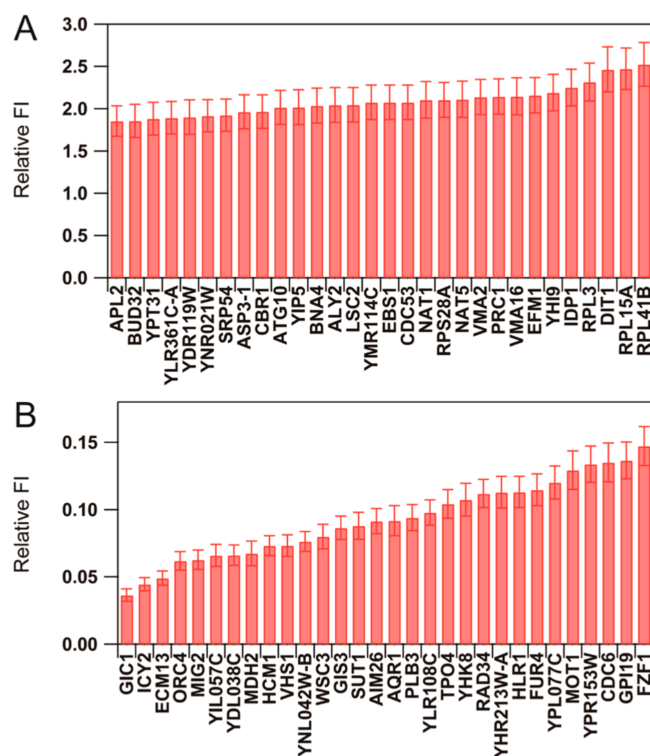

Figure 4. Most and least active 30 terminator regions in the budding yeast. The terminators in genome-integrated transformants were globally ranked from highest (top) to lowest (bottom) in terms of their activity relative to that of *PGK1t* (i.e., relative FI value). The relative FI values of the top 30 (A) and bottom 30 (B) terminator regions are displayed. Data are means ± standard deviation for 3 independent experiments.

The relative FI values and ranking of the values for genome-integrated transformants were slightly different from the results obtained in the global analysis with plasmid strains. For example, the relative FI for the *PRM7t* strain (bottom seventh in the global analysis) was not determined in the comprehensive evaluation. The maximum value in the comprehensive evaluation was 2.52 for the *RPL41Bt* strain, and the minimum was 0.036 for the *GIC1t* strain. The rank of the *NAT5t* and *ORC4t* strains (top and bottom, respectively, in the global analysis) was slightly changed to top 11th and bottom 4th, respectively, in the comprehensive evaluation. The 3′-UTR activity in the genome-integrated transformants was estimated to vary over a 70-fold range, which was comparable to that observed for the plasmid transformants in the global analysis.

To determine whether this comprehensive data set was significant in the context of other promoter and/or coding regions, we investigated the effects of promoter and reporter gene exchange on the terminator region activities. The GFP reporter gene was substituted for a reporter gene that encodes a red fluorescent protein, mKO2.[33] Nine mKO2 strains harboring the *TDH3* promoter and a corresponding terminator region were constructed (Figure 5A). The relative FIs of these mKO2 strains were significantly correlated with those of the respective GFP strains (R = 0.99).

With a strong promoter like the *TDH3* promoter, as used in this analysis, we might have overlooked or underestimated the subtle effects of the terminator regions because of over-production of transcripts and their functional *cis*-elements. To

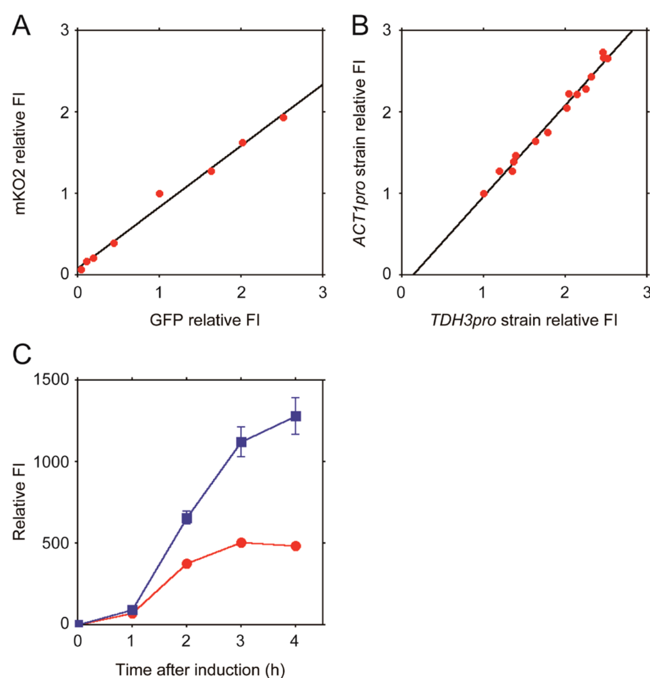

Figure 5. Effects of reporter gene and promoter exchange on terminator region activities. Relative activities were calculated as the ratio of the fluorescence intensity (FI) of the transformant containing the indicated terminator to that of the respective *PGK1t* transformant. (A) Effect of reporter gene exchange on terminator region activities. The horizontal axis shows GFP relative FI values, whereas the vertical axis shows mKO2 relative FI values. The linear regression is shown as a solid line. The correlation coefficient was 0.99. Values are the means of three independent experiments. (B) Effect of promoter exchange on terminator region activities. Both axes show GFP relative FI values. The vertical and horizontal axes represent the values for the *ACT1pro* and *TDH3pro* strains, respectively. Each of the 15 closed circles represents a terminator region. The linear regression is shown as a solid line. The correlation coefficient was 0.98. Values are the means of three independent experiments. (C) Terminator region activities under an inducible promoter. All constructs had a GFP reporter gene under the control of the *GAL1* promoter. The control *PGK1t* strain (circles) and the *RPL41Bt* strain (squares) were investigated. The FI of the *PGK1t* strain at 0 h served as the basal expression level. The error bar indicates standard deviation (±SD). The data shown are the average of three separate experiments.

avoid this limitation, we used the *ACT1* promoter (*ACT1pro*) instead of the *TDH3* promoter (Figure 5B) because the activity of the *ACT1* promoter is about 1/14th that of the *TDH3* promoter (data not shown). Fifteen terminator regions including the five most active terminator regions were tentatively selected (see Methods). The corresponding *ACT1pro* strains were constructed, and the relative GFP FIs were determined. We found no significant difference between the relative FIs of the *ACT1pro* and *TDH3pro* strains (R = 0.98).

To investigate the effects of terminator regions on transgene induction, we constructed two yeast strains that harbored either the *PGK1* or the *RPL41B* terminator region downstream of the GFP gene under the control of the *GAL1* promoter (Figure 5C). These yeast strains also harbored an improved galactose induction system, which we developed previously.[34] With this system, the transgene is fully induced within 4 h.[34,35] The kinetics of galactose induction during this first 4 h were quantitatively compared in the two recombinant yeast strains by using flow cytometry. The FI of the control strain at 0 h served as the basal expression level, and the fluorescence of both strains was comparable before induction. The ratio of the FI of the *RPL41Bt* strain to that of the *PGK1t* strain increased to 2.7 during induction (Figure 5C). The relative FI value at 4 h after induction was comparable to that for the corresponding genome-integrated strains harboring a constitutive promoter (i.e., *ACT1pro* or *TDH3pro*).

Least Active Terminators Reduce the mRNA Levels. To investigate whether the 3′-UTR-mediated regulation was at the post-transcriptional or translational level, quantitative RT-PCR analyses were performed for the bottom 30 transformants (Figure 6).

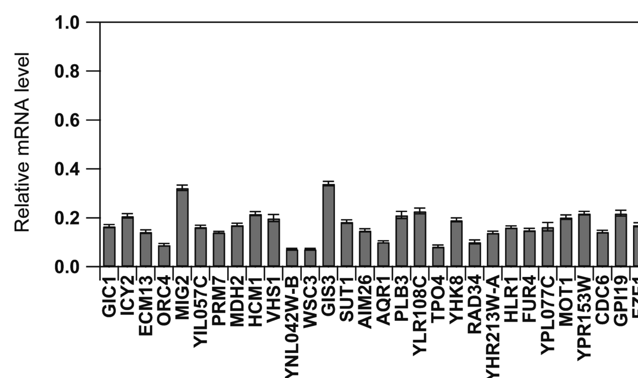

Figure 6. Low mRNA levels of the reporter GFP gene in the 30 strains with the least active terminator regions. GFP mRNA concentrations in the bottom 30 strains were analyzed by conducting quantitative RT-PCR. Relative GFP mRNA concentrations were then calculated as the ratio between the concentration in the transformant containing the indicated terminator and that in the *PGK1t* transformant. Data are means ± SD (n = 3).

No significant change in mRNA levels would suggest the existence of regulation at the translational level; however, the bottom 30 strains exhibited a clear reduction in transcript levels compared with the level in the *PGK1t* strain (Figure 6). The opGFP mRNA levels in the bottom 30 strains relative to that in the *PGK1t* strain ranged from 0.072 to 0.34 (*WSC3* and *GIS3*, respectively) (Supplementary Table S3). Taken together, the results suggest that the 3′-UTRs of the bottom 30 strains could contain *cis*-elements involved in the mRNA decay of the associated chimeric transcripts.

Biological Characterization of the Most and Least Active Terminator Regions. To relate the activities of the terminator regions to the natural context of gene/protein expression, we next compared our data sets with other global analysis data sets, such as mRNA abundance, protein abundance, mRNA half-life, and 3′-UTR length (Figure 7 and Table 1).

Overall, only mRNA abundance showed a weak correlation with terminator output (R = 0.241, Figure 6A and Table 1); no correlations were seen with the others. However, when the most and least active 100 terminator regions were compared, significant associations with these data sets were observed. Specifically, the genes with the most active terminators regions showed significantly higher mRNA expression levels ($P = 2.0 \times 10^{-5}$, Table 1) (but not protein expression levels) and had shorter half-lives ($P = 1.1 \times 10^{-2}$, Table 1) compared with all genes. Genes with the least active terminator regions significantly lowly expressed in both of their mRNA and

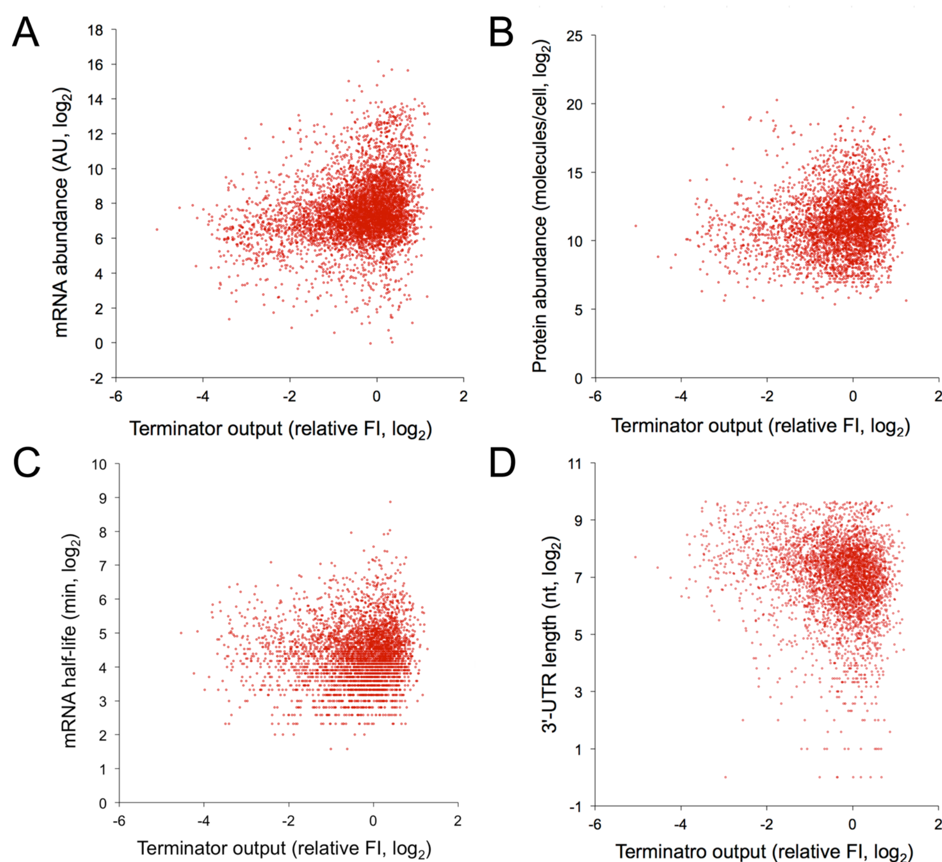

Figure 7. Comparison of terminator activities with large-scale data sets. The scatter plots show the following comparisons: (A) terminator output vs mRNA abundance,[26] (B) terminator output vs protein abundance,[36] (C) terminator output vs mRNA half-life,[37] and (D) terminator output vs 3′-UTR length.[26]

Table 1. Relationships between the Terminator Data Sets and Other Global Data Sets

	mRNA abundance (AU, log_2)	protein abundance (molecules/cell, log_2)	mRNA half-life (min)	3′-UTR length (nt)
total (5302)				
hit count[a]	5248	3633	3938	3962
average (SD)	7.56 (1.87)	11.27 (2.23)	25.17 (20.60)	177.37 (145.45)
R^b	0.241	0.003	0.045	−0.183
most active (100)				
hit count[a]	98	66	77	78
average (SD)	8.71 (2.28)	11.63 (2.72)	27.62 (19.10)	175.46 (141.99)
P-value[c]	**2.0×10^{-5}**	4.1×10^{-1}	**1.1×10^{-2}**	9.5×10^{-1}
least active (100)				
hit count[a]	98	55	54	56
average (SD)	6.19 (1.53)	10.57 (1.68)	29.15 (14.40)	261.50 (186.72)
P-value[c]	**1.4×10^{-14}**	**2.1×10^{-2}**	6.9×10^{-2}	**6.1×10^{-5}**

[a]The number of genes found in each data set is shown. [b]Pearson's correlation coefficient. [c]Mann−Whiteny U-test; $P < 0.05$ are shown in bold letters.

protein levels ($P = 1.4 \times 10^{-14}$ and $P = 2.1 \times 10^{-2}$, Table 1). These results indicate that the activity of the terminator region is a determinant of mRNA and protein expression levels at least for some genes in the natural context.

The above comparison also indicated that the genes with the least active terminator regions contained longer 3′-UTRs ($P = 6.1 \times 10^{-5}$, Table 1), suggesting that the 3′-UTRs of these genes might contain RNA elements that actively degrade these mRNAs. In fact, we found some Puf protein-binding sites, which have previously been reported to play a role in the activity of 3′-UTRs,[7] in these least active 3′-UTRs. However, the number of Puf sites observed in the least active 3′-UTRs was not statistically different from the mean number of Puf sites for the total number of 3′-UTRs examined (data not shown). It is therefore unclear whether these Puf sites are actual determinants of the activity of these 3′-UTRs.

We next performed gene ontology (GO) analysis to connect the activities of the terminator regions with cellular functions. We examined whether the genes with the most and least active 100 terminator regions were concentrated in any function, process, or component GO term by using the GO term finder (ver. 8.3) on the *Saccharomyces* Genome Database (http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl). Only the 100 genes with the most active terminator regions gave

Table 2. GO Terms Significantly Concentrated in the 100 Genes with the Most Active Terminator Regions

GO term (component)[a]	observation (out of 100 genes)	background (out of 5302 genes)	P-value[b]	genes annotated to the term
cytosolic part	15 (15.0%)	210 (4.0%)	9.7×10^{-4}	*VPS15, NAT1, RPL41B, RPP2B, RPL29, RPT6, RPS20, RPL15A, REH1, RPL3, RPS28A, NAT5, RPS1, VPS30, RPL5*
cytosolic ribosome	12 (12.0%)	151 (2.9%)	3.0×10^{-3}	*NAT1, RPL41B, RPP2B, RPL29, RPS20, RPL15A, REH1, RPL3, RPS28A, NAT5, RPS12, RPL5*

[a]GO term finder (ver. 0.83) was used for this analysis. [b]GO terms with a *P*-value <0.01 are listed.

significantly concentrated GO terms (components), namely, "cytosolic part" and "cytosolic ribosome" (Table 2). Of the 15 genes that annotated to the GO term "cytosolic part", 12 also annotated to the GO term "cytosolic ribosome". Ribosomal proteins are among the most transcriptionally active in *S. cerevisiae*,[38,39] and the terminator regions of the ribosomal protein genes appear to contain a common element to increase gene expression.

To determine whether any DNA/RNA motifs are common to the most or least active terminator regions, we next performed a motif survey with the MEME suite (http://meme.nbcr.net/meme/) and the *Saccharomyces* Genome Database by using the following criteria: (1) find motifs that are significantly concentrated in the most or least active terminator regions (30 or 100) but are not significantly concentrated in the background terminator regions, (2) ensure that the found motifs are not located within the downstream ORF, and (3) determine whether the found motifs are conserved among closely related yeast species. We also performed this survey by restricting the target to ribosomal protein genes with the most active terminator regions. To date, neither survey has yielded any motifs that satisfy the above criteria.

■ DISCUSSION

Synthetic genetic circuits are considered to be a key technology in the design and creation of artificial organisms. Compared with the explosive development of synthetic circuits in *Escherichia coli*,[40−44] few studies have been conducted in *S. cerevisiae*.[45,46] One of the current major limitations in the implementation of synthetic circuits is a dearth of appropriate modular biological parts. In *E. coli*, distinct ribosome binding sites (RBSs) have been exploited to optimize the function of a genetic system by variously modulating the protein synthesis rate of the downstream gene.[47−49] In eukaryotes, no simple modules like RBSs have been used to control the rate of protein synthesis. Here, we propose that terminator regions could be used as an alternative to RBSs.

Our global analysis of terminator region activity presents a new perspective on the 3′-UTR role in eukaryotes. The activity of the yeast terminator regions varied within a 70-fold range, whereas the levels of mRNA and protein expression in yeast have been reported to vary by more than a 1000-fold and 10,000-fold, respectively.[36,50] This global data set of the terminator region activities was associated with the abundance of mRNA and protein (Table 1), indicating that the activities of the terminator regions analyzed in this study somehow reflects the natural context of gene expression. We therefore think that terminator region activity could be a determinant of the level of gene expression, at least for some genes, albeit not a major determinant.

It is well-known that some elements in the 3′-UTR determine mRNA stability.[3,5] We were therefore surprised to find that the mRNA half-lives of the genes with the least active terminator regions did not have significantly shorter half-lives ($P = 6.9 \times 10^{-2}$, Table 1). However, the results of previous studies on *MFA2t*[28] and our current qRT-PCR analysis suggest that 3′-UTR activity *per se* might not be a major determinant of mRNA half-life.

Some 3′-UTRs might be more active when grown under conditions of stress or severe nutrient deficiency. For maximum yields of target protein, a 3′-UTR might need an authentic 5′-UTR or coding region or both. Most non-repetitive genomic DNA sequences are transcribed, and many neighboring genes share a 3′-UTR in reverse directions,[23−25] suggesting the existence of a regulatory function for the 3′-UTR. Otherwise, these results might imply that the regulation of gene expression via the 3′-UTR has a relatively minor role; however, the lack of correlation might result from the difference in the host strains or growth conditions used in the experiments. The dynamic range of relative FI values observed in our study seemed rather small, perhaps because the cells were grown under favorable conditions.

How would a series of terminator regions with various 3′-UTR activity levels be of practical use? Metabolically engineered microorganisms that produce biofuels and bioplastics are required to help produce a sustainable society.[51−55] In these transgenic organisms, exogenous genes required for the biosynthesis of target chemicals must be strongly expressed. Therefore, inclusion of both a strong promoter and a strong terminator region is important. In contrast to promoters, little attention has been paid to the selection of terminator regions for transgenes even though the promoters and 3′-UTRs in the terminator regions synergistically regulate the expression of genes at the transcriptional and post-transcriptional levels, respectively. In the next generation of genetically modified yeast producing complicated target products, such as phenolics, isoprenoids, and polyketides, terminators could be used as potent genetic tools to regulate the expression of heterologous genes.[56] In *S. cerevisiae*, only a few studies have examined which terminator region is appropriate for transgene regulation.[31,57,58] To construct metabolically engineered yeast or a synthetic genetic circuit, terminator regions containing a functional 3′-UTR could be used in various artificial promoter and coding region contexts. In this study, we investigated the effects of reporter gene exchange (GFP/mKO2) on gene expression (Figure 5A). We also examined the relative activities of several terminator regions with the highest or lowest activities in the context of the *ACT1* and *GAL1* promoters (Figure 5B and C). Together these results suggest that these terminator regions work well as a genetic component for modulating transgene expression.

GO analysis indicated that ribosomal protein genes have significantly active terminator regions. The genes of ribosomal proteins are highly expressed,[38,39] and this expression is primarily regulated by the transcriptional rate to coordinate equimolar ribosomal protein synthesis.[59−63] Post-transcriptional and translational regulation of ribosomal protein genes has been reported in eukaryotes,[64,65] suggesting that the 3′-

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UTRs of these genes might be involved in the control of ribosomal protein synthesis. Ribosome-related genes with highly active terminator regions, such as *EFM1* and *YMR114C*,[66,67] might also be involved in this coordination.

Identification of the *cis*-elements of specific regulators is difficult because the target single-stranded RNA molecules are too fragile and scarce to examine biochemically. Systematic investigation of RBP-binding elements has been performed only for a few general global regulators,[8,68,69] whereas chIP-on-chip (combined chromatin immunoprecipitation and microarray) analysis of genomic DNA has been used to comprehensively examine which *cis*-elements bind to which transcriptional factors.[70−72] The low reporter mRNA levels observed in the strains that harbored the 30 least active terminator regions (Figure 5) indicate that the 3′-UTRs included in these regions caused mRNA destabilization, leading to the transient expression of the upstream reporter gene. However, so far, we have been unable to find any motif that is significantly associated with the most or least active 3′-UTRs. Further experimental studies, such as mutagenesis of the 3′-UTRs and the identification of *trans*-elements that regulate the activity of these 3′-UTRs, are required.

Our experiments could not distinguish between transcriptional termination and post-transcriptional regulation events. A more detailed experimental approach, such as the measurement of mRNA half-life, translational efficiency, and observation of mRNA localization, might decouple these events. To assess transcriptional terminator activity in isolation, we would need to establish an experimental scheme such as a dual-reporter assay. Since no translational coupling (translational restart) has been observed in *S. cerevisiae*, we would need to develop a new downstream reporter that would be suitable for such an assay.

Our global screening method could be applicable to other model eukaryotes such as *Schizosaccharomyces pombe*, in which both a low-copy plasmid vector and recombination cloning are available.[73] *S. cerevisiae* has been used as a versatile platform for metabolic engineering.[74−76] In this study, several *S. cerevisiae* terminator regions showed 2.5-times the activity of the traditional terminator region, *PGK1t*, and twice that of *TPS1t*, which is classed as a strong terminator region.[31] Further investigations are currently underway to elucidate the characteristics of these novel strong 3′-UTRs. In a model plant, *Arabidopsis thaliana*, combining a strong terminator region from *HSP* with an exogenous 5′-UTR produced 10 times the yield of target product as that achieved with a standard promoter-terminator combination.[77] Alternatively, after identification of functional *cis*-elements, combinatorial screening would enable us to develop artificial terminator regions.

■ METHODS

Strains and Media. *S. cerevisiae* strain W303-1a (MATa *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) was used as the wild type. Yeast transformants were grown in synthetic complete glucose medium (SD) or SD-derived selection medium (SD-URA or SD-TRP); the medium contained a 0.67% Yeast Nitrogen Base (YNB) without amino acids (Difco, NJ, USA), 0.082% Complete Supplement Mixture (CSM), CSM-URA, or CSM-TRP (FORMEDIUM, Norfolk, U.K.), 2% glucose, and adenine (40 mg/L).

PCR-Primer Design. Gene-specific primers for terminator sequences were designed with the use of the Primer3 program (http://primer3.sourceforge.net/) and genomic DNA sequences retrieved from the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org/). Perl version 5.8 (http://www.cpan.org) was used to process data, reformat results, and automate monotonous tasks. We developed all scripts. Positions of open reading frames in the genome were extracted from the protein translation tables that were provided with the genomic DNA sequences. Primer3 was used to design each pair of gene-specific primers (i.e., forward and reverse primers). STEP 1: An approximately 1000-bp sequence, starting a few nucleotides before the termination codon of an ORF, was extracted. STEP 2: Primer3 calculated a pair of gene-specific primers for the extracted sequence, with restrictions as listed below. If no suitable pair of primers was generated, the Primer3 calculation was repeated with a different amplification size (450−600 bp) and primer length (16−45 bp). STEP 3: Each primer was searched against the entire genome sequence to determine whether the sequence was unique. If the primer hybridized at multiple points, STEP 2 was repeated with a different amplification size, longer primer length, and a different starting position. Gene-specific primers are listed in Supplementary Table S1. The first sets of PCR primers (1st primers) were designed as 5′-GAATTGTATAACTGAGGTACC-3′ plus a gene-specific forward primer and 5′-ATCAGTTATTACCCGGGTACC-3′ plus a gene-specific reverse primer. The resultant PCR product was then amplified with a second set of PCR primers (2nd primers): 5′-CCGCTGCTGGCATTACCCATGGTATGGAT<u>GAATTGTATAACTGAGGTAC</u>-3′ for the forward primer and 5′-CTCACAAATTAGAGCTTCAATTTAATTAT<u>ATCAGTTATTACCCGGGTAC</u>-3′ for the reverse primer. The underlined sequences in these primers annealed to the sequences added to the ends of the fragments in the first PCR amplification. The second primers contained additional vector sequences for use in gap-repair cloning, as described below.

First and Second PCRs To Amplify Terminator Regions. PCR amplification was performed using PrimeSTAR HS DNA Polymerase (Takara Bio, Shiga, Japan) in 96-well plates (Optical 96-Well Reaction Plate, Life Technologies, CA, USA). Terminator DNA fragments of length ∼500 bp were amplified from the *S. cerevisiae* genome by using the first primers (see previous section) under the following thermal cycling conditions: 10 s at 98 °C, followed by 35 cycles of 10 s at 98 °C, 15 s at 55 °C, and 15 s at 72 °C. Amplification of samples in the four corner wells of each plate was tested by separating the products by agarose gel electrophoresis. To produce DNA fragments suitable for gap-repair cloning, a second PCR reaction was performed in a 10-μL mixture containing the second primers (see previous section) and 1 μL of 1:200 diluted product from the corresponding first PCR reaction. The thermal cycling conditions were as follows: 15 s at 98 °C, followed by 30 cycles of 10 s at 98 °C, 15 s at 61 °C, and 15 s at 72 °C. The quality of the amplification was tested as described above. The reaction mixtures were vacuum-dried and preserved at 4 °C until further use.

Gap-Repair Cloning. Gap-repair cloning, which was originally reported by Botstein's group,[78] has been widely used in global analyses of *S. cerevisiae*.[79−81] Briefly, the sequence to be cloned is amplified with primers containing plasmid vector sequence, so that the ends of the resultant DNA fragments are homologous to the ends of the linearized plasmid vector. Both the insert DNA fragments and the linearized plasmid vector are simultaneously introduced into yeast competent cells, where they are subject to homologous recombination. Transient transformants are selected by

identifying the plasmid vector marker and phenotype. In this study, pAUR112 (Takara Bio) with the *URA3* selection marker was used as a parental plasmid vector, and the codon-optimized GFP gene (opGFP)[32] under the *TDH3* promoter (−796 to −1 relative to the start codon) was inserted between the *Sac*I and *Kpn*I sites. Transformation was performed by using a Frozen-EZ Yeast Transformation II Kit (Zymo Research, CA). Forty micrograms of *Kpn*I-linearized vector, 10 μL of competent cells, and 100 μL of solution III from the kit were added to each dried DNA fragment from the second PCR reaction in separate wells of a 96-well deep well plate (3960, Corning Inc., NY) and mixed. After incubation at 30 °C for 1 h, the reaction mixtures were mixed with 1 mL of SD-URA selection medium. The plate was sealed with an air-permeable cover and shaken at 160 rpm in a shaking incubator (MBR-022UP, TAITEC, Saitama, Japan) at 30 °C for 48 h. With the exception of the first 24 clones, we did not check the sequences of the DNA fragments that were cloned into the plasmids. For the clones that were checked, the DNA sequences correctly matched the corresponding terminator regions (data not shown).

Flow Cytometry and Data Processing. The flow cytometry procedure was basically the same as that described previously.[34] Data processing was performed by using IgorPro software (version 6.1, Wavemetrics, Inc., OR, USA). The GFP fluorescence intensity (FI) of approximately 3,000 cells in each nondiluted sample culture was measured with the use of a Cell Lab Quanta SC MPL flow-cytometer (Beckman-Coulter, CA, USA) equipped with a filter set (510/10) for GFP and a 488 nm laser (Supplementary Figure S1).

Because only 96 clones could be simultaneously measured on each plate, interplate normalization was required to quantitatively compare the average FI values from multiple plates. We selected three transformants harboring *PGK1t*, *MFA2t*, and *SAG1t*, respectively, as internal standards because our previous study[27] and preliminary experiments with stable transgenic yeasts indicated that the average FI value in the *MFA2t* strain is about half that in the *PGK1t* strain,[31] while that in the *SAG1t* strain is twice that in the PGK1t strain (Supplementary Figure S2). The FI value of each strain relative to that of the *PGK1t* strain was termed the relative FI value. The reproducibility of sequential experiments was confirmed by conducting repeated experiments for a set of 96 terminators (Supplementary Figure S3). The results were highly correlated (R = 0.92) indicating that the experimental procedures worked well.

Construction and Evaluation of Stable Transformants. To verify the comprehensive FI data set, especially extrapolated values (i.e., values higher than that of the *SAG1t* strain, or lower than that of the *MFA2t* strain), 94 genome-integrated transformants at *PDC6* locus were constructed as described in our previous study[31] except that a codon-optimized GFP, opGFP,[32] was used instead of GFP (Supplementary Figure S4). All cloning procedures were performed with an In-Fusion Advantage PCR Cloning Kit (Clontech, Mountain View, CA, USA). Each PCR primer set was designed as 5′-AATTGTATAACTGAGGTACC-3′ plus a gene-specific forward primer, and 5′-TAATGTCGTTGGATCC-3′ plus a gene-specific reverse primer. Each cloned terminator region was inserted into the vector and identified by sequencing analysis. Subtle differences were found between these sequences and the reference sequences registered in the SGD (data not shown). Transformation was performed by using a Frozen-EZ Yeast Transformation II Kit with SD-TRP medium. Cells were cultured in SD medium at 30 °C in test tubes shaken at 70 rpm. Overnight cultures were diluted with 6 mL of fresh SD medium to a final OD_{660} of ~0.1, and then the diluted cultures were incubated until they reached an OD_{660} of 0.6−0.7. After the cell cultures were diluted 10-fold with physiological saline, the FI value of approximately 10,000 cells in each sample was measured as described above. The relative FI values for 97 genome-integrated yeast transformants (including the *MFA2t*, *PGK1t* and *SAG1t* strains) are listed in Supplementary Table S2.

Effects of Promoter and Reporter Gene Exchange on 3′-UTR Activities. All transformants were constructed as described above except for those involving the mKO2 gene (Amalgaam, Tokyo, Japan), the *ACT1* promoter, or the *GAL1* promoter. Nine terminator regions (*FUR4t*, *GIC1t*, *IDP1t*, *MFA2t*, *PGK1t*, *RPL41Bt*, *STD1t*, *TYR1t*, and *YIP5t*) were tentatively selected and cloned downstream of the mKO2 gene under the control of the *TDH3* promoter. The FI values of the mKO2 strains were measured as described above except for a filter set (570/15) as a red fluorescence detection. Fifteen terminator regions (*BNA4t*, *DIT1t*, *FMP52t*, *IDP1t*, *PGK1t*, *RPL3t*, *RPL15At*, *RPL41Bt*, *SCW4t*, *TPS1t*, *TUB1t*, *TYR1t*, *UBC1t*, *VMA2t*, and *YIP5t*) were tentatively selected and cloned into the downstream region of the *opGFP* gene under the control of the *ACT1* promoter. The two terminator regions *PGK1t* and *RPL41Bt* were also tentatively selected and cloned downstream of the *opGFP* gene only this time under the control of the *GAL1* promoter.

The kinetics of galactose induction during the first 4 h was quantitatively compared between the *GAL1pro* strains by using flow cytometry. Overnight cultures were diluted with 5 mL of fresh SD medium to a final OD_{660} of ~0.1, and then the diluted cultures were incubated until they reached an OD_{660} of 0.6−0.7. The cells were collected by centrifugation and the supernatant was discarded. The cell cultures were induced with 5 mL of 0.5% galactose medium, sampled at the indicated times, and diluted 10-fold with physiological saline; the GFP FI of at least 10,000 cells in each sample was then measured. Three independent measurements were averaged, and the standard deviation was calculated. The FI of the control *PGK1t* strain at 0 h served as the basal expression level.

Quantitative PCR Analysis. Total RNA was isolated from the same cultures as those used for FI measurements by using a High Pure RNA Isolation Kit (Roche, Basel, Switzerland). cDNA templates were synthesized from 1 μg of each total RNA by using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). To quantify target cDNAs, real-time PCR analysis with SYBR Green I was performed with the use of a SYBR Green PCR Master Mix in an ABI PRISM 7000 Sequence Detection System (both from Life Technologies). *TUB1* expression was used as the internal standard. The PCR primer sequences were as follows: 5′-CCAACTGGTTTCAAGATCGGTA-3′ and 5′-TCCACAGTGGCCAATTGTGA-3′ for the *TUB1* gene, and 5′-CCAATTGGTGATGGTCCAGTCT-3′ and 5′-CGGTGACGAACTCCAACAAAA-3′ for the opGFP gene. Thermocycling conditions consisted of 40 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. A threshold value to determine the threshold cycle number (Ct) value was set manually. The level of opGFP mRNA expression in each transformant was calculated based on the level of *TUB1* mRNA expression. The values were normalized to that of the *PGK1t* strain. Three replicates of each sample were processed in three independent experiments.

ASSOCIATED CONTENT

Supporting Information

Representative GFP fluorescence data from flow cytometry of a sample culture expressing the CEN/ARS plasmid; intra- and interplate normalization of FI values to produce relative FI values; reproducibility of the experimental procedure; schematic diagram of the genome-integrated gene constructs; list of genes, relative FIs, pimers and amplified terminator regions; list of genes, relative FIs from the transient and stable transformants; mRNA levels in the least active 30 terminator region strains relative to that in the *PGK1t* strain. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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