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ARTICLE *in* BIOTECHNOLOGY PROGRESS · MAY 2005

Impact Factor: 2.15 · DOI: 10.1021/bp049658x · Source: PubMed

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Identification and Use of Zinc Finger Transcription Factors That Increase Production of Recombinant Proteins in Yeast and Mammalian Cells

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Randomized ZFP-TF libraries could induce a specific phenotype without detailed knowledge about the phenotype of interest because, theoretically, the libraries could modulate any gene in the target organism. We have developed a novel method for enhancing the efficiency of recombinant protein production in mammalian and microbial cells using combinatorial libraries of zinc finger protein transcription factors. To this end, we constructed tens of thousands of zinc finger proteins (ZFPs) with distinct DNA-binding specificities and fused these ZFPs to either a transcriptional activation or repression domain to make transcriptional activators or repressors, respectively. Expression vectors that encode these artificial transcription factors were delivered into *Saccharomyces cerevisiae* or HEK 293 cells along with reporter plasmids that code for human growth hormone (hGH) or SEAP (secreted alkaline phosphatase) (for yeast or HEK, respectively). Expression of the reporter genes was driven by either the cytomegalovirus (CMV) or SV40 virus promoters. After transfection, we screened the cells for increased synthesis of the reporter proteins. From these cells, we then isolated several ZFP-transcription factors (ZFP-TFs) that significantly increased hGH or SEAP synthesis and subjected these regulatory proteins to further characterization. Our results show that randomized ZFP-TF libraries are useful tools for improving the yield of heterologous recombinant protein both in yeast and mammalian cells.

Introduction

A prerequisite for the application of recombinant proteins in biotechnology and medicine is to develop efficient and faithful production systems. Guided by the characteristics and uses of the various recombinant proteins, researchers have chosen the following organisms for the production of recombinant products: *Escherichia coli* (*1*), yeasts, [such as *Saccharomyces cerevisiae* (*2*, *3*), *Pichia pastoris* (*4*), and *Hansenula polymorpha* (*5*)], insect cells (*6*), and mammalian cells [such as Chinese hamster ovary (CHO) cells (*7*)]. Most therapeutic proteins, such as human growth hormone (hGH) (*8*), monoclonal antibodies (*9*), and Factor VIII (*10*), are produced in one of these systems. The expiration of patents for expensive protein drugs and the development of monoclonal antibodies as therapeutics have intensified competitive efforts to improve the productivity of recombinant proteins. Various methods have been developed to increase the yield and reduce the production costs of recombinant proteins. In microorganisms, the standard approach to increase productivity is a chemical mutagenesis that creates high-producing strains (*2*). In *S. cerevisiae*, several "super-secreting" or "over-secreting" mutants have been isolated, and these yeast strains yield high concentrations of secreted heterologous proteins (*2*, *3*). An alternative approach has been to isolate several signal sequences from yeast secretory proteins, fuse them to the heterologous proteins, express the fusion proteins in yeast cells, and isolate the secreted recombinant proteins

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10.1021/bp049658x CCC: $30.25
Published on Web 03/30/2005

from the yeast cell media (*11*−*16*). In mammalian cells, amplification of the genes that encode recombinant proteins of interest is a key method used to enhance the productivity of these recombinant products (*17*, *18*). Other methods such as stabilizing the structure of a protein product (*5*), increasing its translation rate (*19*, *20*), or inhibiting apoptosis (*21*−*23*) have also been applied. Most of these approaches were carried out in CHO cells, which are a major producer of therapeutic recombinant proteins because of their genomic stability, their simple adaptation to serum-free medium, and the likeness of their post-translational processes to those of human cells (*24*). However, human cells are also used or are being developed as hosts for the industrial production of recombinant proteins. For example, a recombinant human activated protein C, which is used for severe sepsis treatment, is produced in a human embryonic kidney (HEK) 293 cell line (*25*). In addition, it has been reported that human PER.C6 cells can serve as an alternative host to CHO cells for the production of therapeutic monoclonal antibodies (*26*).

Engineered zinc finger proteins (ZFPs), either alone or fused to transcriptional activation or repression domains, have been shown to function as transcriptional regulatory proteins for specific target genes in several organisms (*27*−*30*). Recent advances in this field now allow one to construct randomized ZFP-transcription factor (ZFP-TFs) libraries that consist of tens of thousands of distinct transcription factors (*31*−*33*). These libraries have been used successfully to screen for components that induce diverse phenotypes of interest, such as cellular differentiation, stress resistance (*31*), over-expression of surface antigens (*32*), or resistance to Taxol, an anti-cancer agent (33). The selected ZFP-TFs could bind to and modulate expression of their target genes, which would generate phenotypes of interest. The target genes of ZFP-TF could regulate genes directly or indirectly related to the phenotype. In the case of indirect regulation, cellular changes caused by ZFP-TFs would generate phenotypes of interest via regulation of one or two other genes in turn. These results clearly indicated that specific cellular phenotypes or regulations of specific genes could be induced by random screening of ZFP-TF libraries.

Seeking to exploit the capabilities of ZFP-TF libraries for random activation or repression of endogenous genes, we conducted experiments designed to determine whether a ZFP-TF library-based approach can be used to improve the yields of industrially important recombinant proteins. To this end, we chose two model experimental systems, hGH production in *S. cerevisiae* and secretory alkaline phosphatase (SEAP) reporter protein production in HEK 293 cells. Our results reveal that the ZFP-TF library approach represents an alternative tool for the engineering of host cells for the efficient production of recombinant proteins.

Material and Methods

Materials and Plasmids. Filter circles (82 mm) of 0.45 μm nitrocellulose, agarose type IV, and the hGH ELISA kit were purchased from Millipore (U.S.A.), Sigma Chemical (U.S.A.), and Roche (Germany), respectively. The hGH expression plasmid, pktXGH, was described previously (*16*). The expression cassette in pktXGH comprised the leader sequence of killer toxin of *Kluyveromyces lactis*, the KEX2 dibasic endopeptidase cleavage site, and the *hGH* gene. *S. cerevisiae* strain 2805 (*pep4:: HIS3, pro 1-δ, can1, Gal2, his3δ, ura3-52*) served as the expression host.

The pSEAP2-control reporter plasmid (Clontech, U.S.A.) contained the SEAP gene under the control of the SV40 promoter. To place the SEAP gene under the control of a promoter from cytomegalovirus (CMV), we isolated the CMV promoter sequences from pcDNA3.1 (Invitrogen, U.S.A.) by digestion with HindIII and MluI and used them to replace the SV 40 promoter in a pSEAP2-control vector fragment that had been digested with the same restriction enzymes. The resulting and the original pSEAP2-control plasmids were named CMV-SEAP and SV40-SEAP, respectively. A CMV-Luciferase plasmid, in which the luciferase reporter gene is under the control of the CMV promoter, was constructed by replacement of an SV40 promoter in pGL3-promoter (Promega) with the CMV promoter from pcDNA3.1 (Invitrogen) using HindIII and MluI sites.

Construction of a ZFP-TF Library. A ZFP library for use in yeast cells (*31*) was modified to carry the gene that renders yeast resistant to the drug blasticidin S. The blasticidin S resistance gene was amplified from pYES6/CT (Invitrogen, U.S.A.) and cloned into the NgoMIV site of pYTC-Lib, which was used for the expression plasmids in a previously described yeast-specific ZFP library (*31*). The ZFP expression cassette that carried the blasticidin S resistance gene was named pYTC-LB. DNA sequences that encoded four-finger ZFPs fused to either the *Gal4* transcriptional activation domain sequence or the *Ume6* transcriptional repression domain-encoding DNA (*34*), which were described previously (*31*), were subcloned into pYTC-LB. Yeast cells were transformed using the lithium acetate method. An hGH-producing yeast strain was selected from cells grown on SD *Ura*$^-$ agar plates [0.8 g/L Complete Supplement Medium-URA (BIO 101, U.S.A.) 6.7 g/L yeast nitrogen base without amino acids (DIFCO), and 2% glucose]. The hGH-producing strain was then transformed with a ZFP library, and transformants were cultured for 5 days at 30 °C on SD *Ura*$^-$ agar plates supplemented with 100 mg/L Blasticidin S (Invitrogen). Growing colonies were collected and stored at −80 °C as glycerol stocks and then analyzed further for a change in hGH production using an hGH ELISA kit (Roche, Germany).

For mammalian cells, a previously described ZFP-TF library was used (*31*). As reported, DNA sequences that encode either the p65 transcriptional activation domain (NCBI accession number NP_068810; amino acids 275−535) or the Kid transcriptional repression domain (NCBI accession number AAB07673; amino acids 12−74) were fused to ZFP-encoding sequences (*31*). For transfection of mammalian cells, the library plasmids were prepared individually using an Accuprep 96-well plate kit (Bioneer, Korea).

Screening for ZFP-TFs That Enhance the Productivity of hGH in Yeast. ZFP transformants were screened, using the filter immunoblot method, for cells that have enhanced productivity of hGH (*35*). The transformants (1×10^4) were grown on YPGal (1% yeast extract, 2% bacto-peptone, 2% galactose) plates for 3 days at 30 °C. Nitrocellulose filters were notched to aid in alignment and autoclaved for 20 min. Just prior to use, the filters were rinsed briefly in YPGal solution. A 0.4% agarose solution, sterilized by autoclaving, was heated to melting temperature and retained in a 45 °C water bath. The agarose solution (3 mL) was then applied as a thin layer over the colonies. It was necessary that the plates be on a level surface when adding agarose to ensure even layering. A nitrocellulose filter circle was then placed on the agarose layer, and the plates were incubated at 30 °C for 6 h to allow the secreted proteins

to diffuse through the agarose and be adsorbed to the filters. The filters were then removed, rinsed briefly in phosphate-buffered saline (PBS), and processed for immunoblotting analysis. The mouse anti-hGH monoclonal IgG1 (ABCAM, U.K.) and horseradish peroxidase-conjugated rabbit anti-mouse IgG (ABCAM, U.K.) were used as the primary and secondary antibodies, respectively, in the immunoblot assay.

Colonies identified as positive were isolated from plates and suspended in 2 mL of YPGal. The culture was replated on an YPGal plate at the density of 100 colonies per plate. The immunoblot assay was repeated to isolate single colonies that displayed enhanced production of hGH. The ZFP-TF expressing plasmids were rescued from the isolated colonies and retransformed into hGH-producing yeast cells to confirm the phenotypic alteration, and the plasmids were also sequenced to reveal the identities of the encoded ZFPs. To determine whether the ZFP-TF was responsible for the enhanced production of hGH, one of the identified ZFP-TFs, Z10, was mutated. To this end, we used polymerase chain reaction (PCR)-mediated mutagenesis to create Z10-M1, a defective form of the Z10 ZFP-TF that was generated by changing the amino acid isoleucine in the QSNI domain to alanine. Also, the activation domain of Z10 was removed by inserting a stop codon after the fourth finger to generate Z10-M2.

To measure the amount of hGH secreted into the yeast cell media, cells were inoculated into 3 mL of SD Ura^- Bla^{100} media and incubated at 30 °C overnight with shaking at 250 rpm. When an A_{600} of ~2 was reached, the culture broth was centrifuged, and hGH and ZFP-TF gene expression were induced by resuspending the cell pellet in YPGal media (50 mL). Induction was continued for 24 h at 30 °C, and the culture was then collected for further analysis.

To measure the activity of the isolated ZFP-TF in a bioreactor, a seed culture of the recombinant *S. cerevisiae* strain that had been grown in 50 mL of SD Ura^- Bla^{100} media was transferred to a 1-L jar containing 500 mL of YPGal media and grown in a BIOSTAT Q laboratory multi-fermenter (B. Braun Biotech International, Germany). The stirring speed was set at 200 to 1000 rpm. The bioreactor was operated at 30 °C, and dissolved oxygen was controlled above 50% air saturation to avoid oxygen limitation. Culture broth was removed every hour using an automatic biosampler, BIOMATE 2000A (Lokas, Korea), and the supernatant was stored at −20 °C before the expression level of the recombinant protein was analyzed. The amount of hGH in the collected samples was analyzed with an hGH ELISA kit (Roche, Germany).

Screening for ZFP-TFs That Enhance Reporter Activity in Mammalian Cells. The procedure for screening the ZFP-TF library for increased production of the reporter protein SEAP is shown in Figure 3a. The screening was carried out in the human embryonic kidney (HEK) 293 cell line by co-transfection of a reporter plasmid and an individual ZFP-TF plasmid randomly isolated from either a ZFP-TF activation or a ZFP-TF repression library. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics, at 37 °C in a CO_2 incubator. Cells were seeded at 1×10^4 cells/well in 96-well plates, and after 1 day of incubation, we transiently transfected the cells with 50 ng of each ZFP-TF or an empty vector and 10 ng of a reporter plasmid using Lipofectamine Plus (Invitrogen, U.S.A.) as recommended by the manufacturer. After 3 days of incubation, the SEAP enzymatic assay was performed on the cells as previously described (*36*). 293F (Invitrogen, U.S.A.) cells were then used to confirm the activities of each positive ZFP-TF. To this end, 293F cells were seeded at 5×10^4 cells/well in 48-well plates. Using Lipofectamine 2000 (Invitrogen, U.S.A.), the cells were then transfected with 160 ng of a ZFP-TF expression plasmid (referred to as SS8-p65, CS5-Kid, or CS6-Kid) and 20 ng of the reporter SEAP expression plasmid. After 2 days of incubation, we performed the SEAP enzymatic assay, and the results were calculated as the fold activity increase relative to the activity of cells transfected with the empty control vector.

To determine whether the ZFP-TF was responsible for the enhancement of the SEAP activity, mutagenesis of isolated ZFP-TFs were carried out. Polymerase chain reaction (PCR)-mediated mutagenesis was used to create a defective form of the CS6-Kid that was generated by changing the amino acid arginine in the RDNQ domain to alanine. Also, functional domains of ZFP-TFs were replaced with one that had opposite functionality. To this end, the Kid repression domain of CS6-Kid was replaced by the p65 activation domain.

Results and Discussion

Screening for ZFP-TFs That Enhance hGH Production To screen a large number of ZFP-TFs that could increase production of hGH in *S. cerevisiae*, we modified the filter immunoassay that had been applied previously for the isolation of mammalian cell lines that secrete the recombinant protein (*35*). A schematic representation for this assay is shown in Figure 1a. hGH secreted from colonies on the plate diffused through a thin layer of agarose and adhered to the nitrocellulose membrane. The membrane was then incubated with antibodies to mouse hGH and horseradish peroxidase-conjugated rabbit anti-mouse IgGs. Then, on the basis of the detected spot's size, clones that exhibited a higher production of hGH than control cells were isolated. From a screen of about 100,000 transformants, we identified several clones that exhibited increased hGH production in the immunoblot assay. Of these, we chose for further characterization a strain with a particularly strong signal, named GH-10. As shown in Figure 1b, the spot detected on the immunoblotted filter that corresponded to GH-10 was well discriminated from the spots that represented other colonies. A ZFP-TF expressing plasmid purified from GH-10, called Z10, was used to transformed the same hGH-producing yeast strain, which confirmed the stimulation of hGH production by Z10.

The level of hGH production in the GH-10 yeast strain was quantitatively analyzed in a 50-mL flask culture. Because hGH was supposed to be secreted, we analyzed the amount of hGH in the culture media by ELISA. GH-10 showed an approximately 2.5-fold higher production of hGH compared to the control strain, which had been transformed with a control vector, pYTC-LB (Figure 2a).

We next examined whether the DNA binding moiety and the transcriptional activation domain of the ZFP-TF are essential for the enhancement of hGH production form yeast cells. The Z10 ZFP-TF was composed of four zinc finger domains (Table 1). Site-directed mutagenesis of an isoleucine residue in the QSNI domain of Z10 yielded the Z10-M1 mutant protein, and this mutation abolished the ability of the ZFP-TF to increase hGH production (Figure 2a, Table 1). Function of the Z10 ZFP was also dependent on the transcriptional activation activity of the Gal4 domain, as the Z10-M2 variant, which is the ZFP DNA binding domain of Z10 without the Gal4 activation domain, failed to increase hGH production

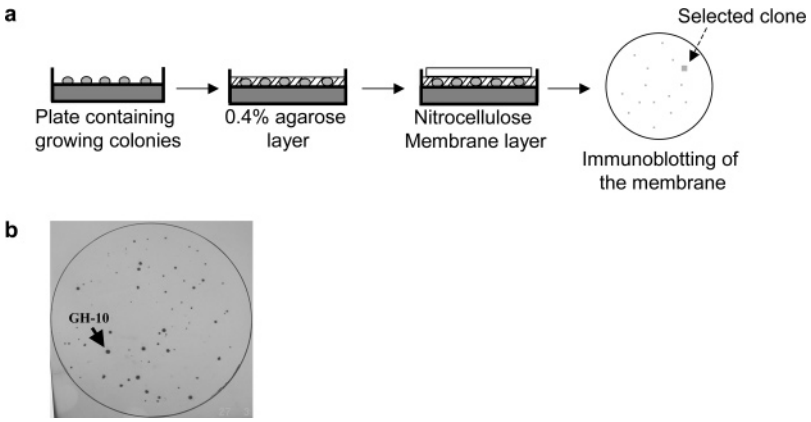

Figure 1. (a) Diagram of the filter immunoblot assay method. Yeast cells that secrete hGH are grown on plates and overlayed with agarose. The secreted protein diffuses through a thin layer of agarose and is adsorbed by a nitrocellulose filter prepared as indicated in Materials and Methods. hGH is detected on the filter through a series of immunological binding steps. (b) Selection of an hGH-overproducing colony, GH-10, on the plate after the second round screening of filter immunoblot assay.

Table 1. ZFP-TFs Characterized in This Study

ZFP-TFs	zinc finger domains (N to C termini)				functional domain	putative binding sequences
Z10	RDNQ	QSNI	QTHQ	RDER	Gal4 (activation)	5′ GHGHGAMAAAAG3′
SS8*	QSNR1	DSNR	DSNR		P65 (activation)	5′ GACGACGAA 3′
CS5	QSHV	QSNI	QTHR1	CSNR1	Kid (repression)	5′ GAVRGAMAAHGA 3′
CS6	QSHV	VSTR	RDNQ	QTHR1	Kid (repression)	5′ RGAAAGGCWHGA3′

The sequences of the various zinc finger domains and their DNA binding residues were described previously (*37*). *SS8 is a three-finger ZFP-TF. W=A or T; V= A, C or G; M=A or C; H=A, C or T; R=A or G.

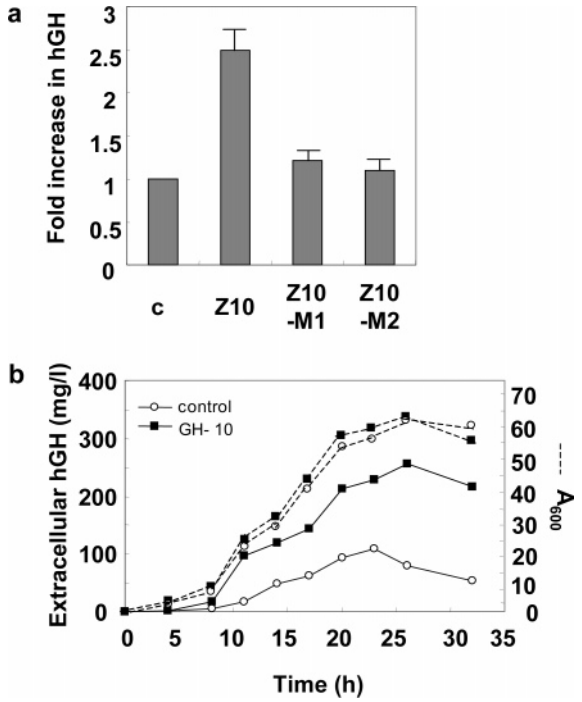

Figure 2. (a) Analysis of Z10 ZFP-TF activity during flask culture (50 mL). ZFP activity is presented as fold increase in the amounts of secreted hGH compared to amounts achieved with the control vector, pYTC-LB. Yeast cells that express hGH were transformed with the indicated expression vectors, and the amount of hGH secreted from each group of cells was divided by that of control cells. The experiments were done twice (three times?) separately. (b) Activity of the Z10 ZFP-TF in a typical fed-batch culture. Samples were harvested at the indicated time after switching from feeding medium to induction medium. The amount of hGH in the samples was analyzed by ELISA. GH-10 indicates hGH-expressing yeast cells transformed with the Z10 ZFP-TF and control hGH-expressing yeast cells transformed with pYTC-LB. The solid line indicates extracellular amount of hGH and the dotted line optical density of cells. The identities of the ZFP-TFs characterized in the experiments are summarized in Table 1.

(Figure 2a). These mutagenesis analyses suggest that Z10 functions as a transcription regulator in vivo to increase the synthesis of hGH in *S. cerevisiae*.

The activity of the Z10 ZFP-TF was further characterized under bioreactor condition. Ten hours after adding the induction media containing galactose to the yeast cells growing in the bioreactor, the amount of hGH produced by GH-10 was ~3-fold higher than that produced by control cells (Figure 2b). We speculate that a certain amount of time is necessary for the Z10 ZFP-TF to be expressed and then go on to activate the transcription of genes that presumably are involved in recombinant protein production or protein secretion. Extracellular accumulation of hGH increased with increasing cell mass, reaching a value of ~250 mg/L when A_{600} was ~65 (Figure 2b).

Despite the increased production of hGH protein in the GH-10 strain, we did not detect a significant difference in the amount of hGH mRNA in GH-10 relative to that of control cells (data not shown). It is possible that the Z10-ZFP has an effect on the secretory machinery of *S. cerevisiae* and in this way serves to increase the amount of hGH detected in the media. Certain recombinant proteins are difficult to be secreted from yeast cells and the ZFP library approach might be an efficient way to engineer the secretory machinery of *S. cerevisiae*.

Screening for ZFP-TFs That Enhance SEAP Activity in Mammalian Cells To test whether ZFPs can increase production of recombinant proteins in mammalian cells, we used as a model system HEK 293 cells that produce SEAP. SEAP was chosen as a reporter protein because most therapeutic proteins produced in mammalian cells are secreted into medium. Cells were transiently transfected with randomly chosen plasmids from a ZFP-TF library. The procedure for screening of the resulting cells is shown in Figure 3a.

We screened two different sets of ZFP-TF libraries; transcriptional activators and repressors. First, we co-transfected, into HEK 293 cells, a plasmid encoding SEAP whose expression is under the control of the SV40

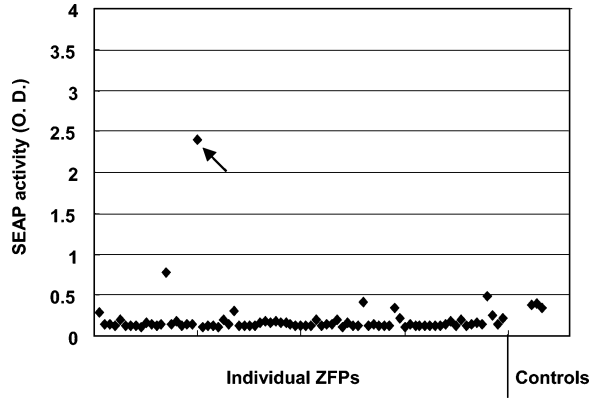

Figure 3. An example of the SEAP assays. HEK 293 cells were transfected with SV40-SEAP and an expression vector that produced one of 81 ZFP-TFs and the transfected cells were incubated for 3 days. Optical densities (O.D.) of the supernatants of the transfected samples are shown along with those of empty vector-transfected samples (control, triplicates). The arrow indicates the (positive) SS8-p65-transfected sample.

promoter and each of 1,000 individual plasmids that encode ZFP activators each of which consists of three zinc fingers fused to the p65 transcriptional activation domain (*31*). One ZFP-TF, SS8-p65, was selected because it yielded cells that produced the highest SEAP activity observed in our experiments (Figure 3b). SS8-p65 showed a more than 5-fold activation of the plasmid-borne SEAP gene, whose expression is under the control of the SV40 promoter, compared to the empty vector control. In contrast, SS8-p65 showed no effect on expression of the SEAP whose expression was under the control of the CMV promoter (Figure 4a). One possible explanation for these results is that SS8-p65 binds directly to the SV40 promoter and activates the reporter gene. Alternatively, SS8-p65 may bind to an endogenous gene whose product in turn affects transcription of the SV40 promoter, but not the CMV promoter. The latter appears more plausible, because there are no sequences in the SV40 promoter and enhancer regions that match the expected binding site of SS8-p65. In present, the endogenous target gene of SS8-p65 and its exact mechanism of SEAP activation are not known.

We also screened 5000 individual plasmids that encode ZFP transcriptional repressors each of which consists of four zinc fingers fused to the Kid transcriptional repression domain (*31*) to identify clones that enhance the production of SEAP whose expression is under the control of the CMV promoter. Two ZFP-TFs, CS5-Kid and CS6-Kid, selected from this screening increased SEAP activity. Interestingly, these two ZFPs activated SEAP activity in cells transfected with the SEAP reporter plasmid whose expression is under the control of either the SV40 or CMV promoter at a level of 2- to 3.5-fold (Figure 4b). When the SEAP gene was replaced with a luciferase gene, both CS5-Kid and CS6-Kid also increased luciferase activity at a level of 2- and 6-fold, respectively, in 293T cells (data not shown). Because CS5-Kid and CS6-Kid contained a repression domain, Kid, and activated SEAP activity, this result clearly indicated that these ZFP-TFs bound to and regulated endogenous genes rather than direct regulation of the SEAP reporter gene by their binding to the SV40 or CMV promoter. Unknown target genes directly repressed by CS5-Kid and CS6-Kid would result in activation of SEAP activity via unidentified mechanism yet.

We performed methylthiazoletetrazolium (MTT) assays to examine whether the three ZFP-TFs merely increase cell numbers and thus increase the SEAP levels. No significant difference was observed between cells transfected with ZFP-TF plasmids and those transfected with the empty control vector (data not shown). We also tested whether the transfection efficiencies of the various cell samples were similar, by co-transfecting cells with a plasmid encoding LacZ and then staining the cells to detect LacZ activity. The transfection efficiency of each

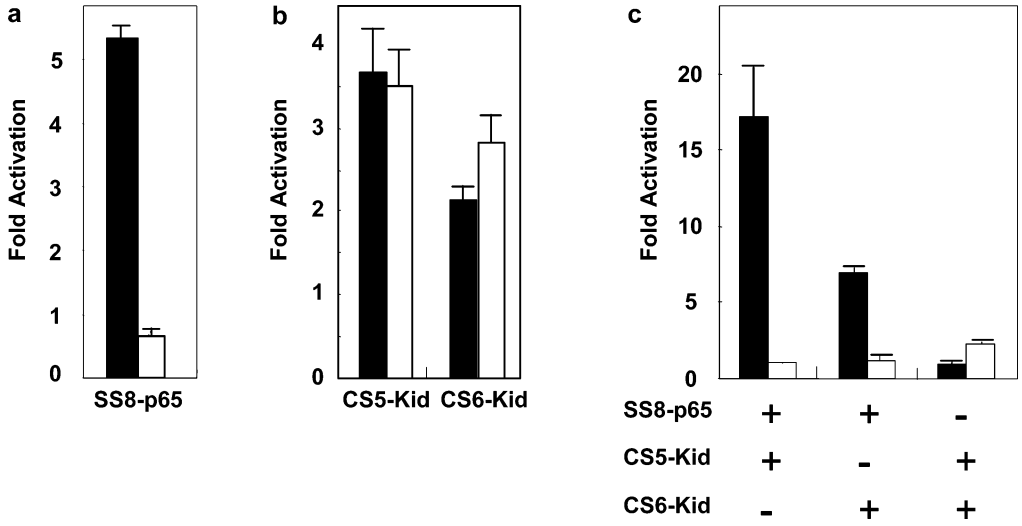

Figure 4. Activation of the SEAP gene, which was under the control of either the SV40 (black bars) or CMV promoter (white bars), by specific ZFP-TFs in 293F cells. (a) Fold increase in the activity of SS8-p65, which was selected from screening with the SV40-SEAP reporter, compared with activity achieved with a control vector lacking a ZFP-TF. (b) Fold increase in the activity of CS5-Kid and CS6-Kid, which were selected from screening with the CMV-SEAP reporter, compared with activity achieved with a control vector lacking a ZFP-TF. (c) Synergistic effect of positively acting ZFPs. 293F cells were seeded at 5×10^4 cells/well in a 48-well plate and transiently transfected with Lipofectamine 2000 (Invitrogen) after 1 day of incubation. The medium was assayed for SEAP activity after 2 additional days of incubation. For transfections, a plasmid encoding a ZFP-TF and an empty parental plasmid (80 ng for each in the case of a and b) or two plasmids each encoding a distinct ZFP (80 ng for each in the case of c) were used along with 40 ng of one of the reporter plasmids. For a control, 160 ng of the empty vector was used with the same amount of reporter, and its SEAP activity was set at 1-fold. For each series of transfections, 3 wells were tested, and the average value is shown. The experiments were carried out at least twice and showed similar changes in fold activities. The identities of the ZFP-TFs characterized in these experiments are summarized in Table 1.

sample was between 60% and 70% (data not shown). These results suggest that the enhancement of SEAP activity by ZFP-TFs did not result simply from an increase in the number of cell divisions or a difference in the samples' transfection efficiencies. In addition, the positive ZFP-TFs were tested in two different but related cell lines, namely 293T and 293F cells (Invitrogen, U.S.A.), as well as in HeLa and CHO cells. As expected, all ZFP-TFs exhibited comparable positive effects in human cells (that is, 293T, 293F and HeLa) but not in CHO cells (data not shown). These results imply that the target binding sites of these ZFP-TFs are maintained in the human genome sequences, but not in the Chinese hamster genome sequence.

We further tested whether the three positive ZFP-TFs exhibit a synergistic effect when two of them are co-transfected into cells. When SS8-p65 was co-transfected into HEK 293 cells together with CS5-Kid or CS6-Kid, we observed a synergistic effect on the SEAP activity (Figure 4c). However, co-transfection of CS5-Kid and CS6-Kid did not show any synergistic effect (Figure 4c). These observations suggest that not all the ZFP-TFs regulate the same target genes in the pathways related to the enhancement of protein production/secretion. A combination of ZFP-TFs that target different sets of endogenous genes could further enhance the efficiency of protein production.

We next examined whether the DNA binding moiety and the transcriptional activation domain of the ZFP-TF are essential for the enhancement of SEAP production form 293 cells. To study this, we generated ZFP mutants with one of the following alterations: (1) a key amino acid residue (an arginine residue of the CS6-Kid RDNQ domain) that was expected to function in DNA base recognition was replaced with an alanine, or (2) the transcriptional repression domain was replaced with an activation domain. As expected, these mutants, when expressed in 293 cells, failed to increase the SEAP activity (data not shown).

Taken together, our data suggest that the ZFP-TF library approach is an effective tool for improving recombinant protein production in mammalian cell lines or in microbial cells. A similar library was also used successfully to increase production of a humanized monoclonal antibody encoded by a gene that was amplified by treatment of CHO cells with methotrexate (manuscript in preparation).

Conclusions

Metabolic engineering of cells is one of the most notable recent advances in the realm of biotechnology. The method described here, which uses libraries of artificial transcriptional factors, yields a significant improvement in the production of a protein of interest. This increase in protein production is achieved through the regulation of endogenous gene expression, altering processes ranging from the transcription of the delivered genes to secretion of the final protein product. These findings suggest that the ZFP-TF library approach is an effective tool for improving recombinant protein production in mammalian cell lines or in microbial cells.

Acknowledgment

We thank our colleagues at ToolGen Inc., especially Mr. Jin Woo Park, for their input related to experiments described herein. This work was partially supported by the Korea Health 21 R&D Project (02-PJ10-PG4-PT02-0027) of the Korean Ministry of Health & Welfare to W.S.; the National Research Laboratory program (M1-0140-00-0048) of the Korean Ministry of Science and Technology to J.-S.K.; and the Microbial Genomics research grant to B.L.S. from the Ministry of Science and Technology (MOST).

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Accepted for publication February 15, 2005.

BP049658X