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Use of Flp-Mediated Cassette Exchange in the Development of a CHO Cell Line Stably Producing Erythropoietin

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The feasibility of the use of Flp-mediated cassette exchange in the development of a CHO cell line, which produces erythropoietin (EPO) stably and largely, was investigated. A stable, high enhanced green fluorescence protein (EGFP)-producing clone was screened by extensive flow cytometric analysis. An EPO expression unit was targeted into the premarked locus of the stable parental clone by Flp-mediated cassette exchange and a correctly targeted clone (FC28T7) was obtained. The EPO production of FC28T7 was proven to be stable in long-term culture. Furthermore, the Flp-mediated cassette exchange did not alter the stable parental clone's characteristics concerning transgene expression level and stability. Taken together, the data obtained here indicated that the establishment of CHO cell lines stably producing a desired protein is achievable using Flp-mediated cassette exchange.

Keywords: Flp-mediated cassette exchange, stable parental clone, long-term culture, EPO-producing CHO cell line

When considering the industrial use of mammalian cell lines producing therapeutic recombinant proteins, the stability of protein production is as important as protein productivity [2]. However, it is likely that there is a trade-off between the stability of the protein production and the production level. In the case of CHO cells, a substantial increase in production level can be achieved by several rounds of dihydrofolate reductase (DHFR)/methotrexate (MTX)-mediated gene amplification. The productivities of amplified pools or clones, however, usually decrease during long-term cultures owing to instability caused by the loss of genes [13, 18, 23] and repeat-induced transgene silencing [6, 11]. The unpredictable production stability of amplified cells makes the cell line development process more tedious and time-consuming since screening of stable clones during several rounds of transgene amplification is required to acquire a stable high-level producer. Therefore, it is important to consider both the expression level and the stability of transgene expression from the initial stages of cell line development. After screening of a chromosomal locus that conveys stable and relatively high expression of transgenes, precise targeting of the gene for a desired protein can be efficiently mediated by site-specific recombination.

Even though the efficient applications of the invertase/resolvase family to gene manipulation have been reported [3, 14, 24], Cre and Flp recombinases in the λ-integrase family are still the most widely used. Not many applications to cell line development using site-specific recombination have been performed thus far, even though the possibility was remarked some time ago [10]. Much work concerning site-specific recombination has focused on the methodology for improving the efficiency of recombination itself [1, 8] or on the functional study of individual genes and their *cis*-acting elements [4, 22, 25, 28]. One of the most practical attempts for cell line development was performed by Kito and his colleagues [19]. They reported that reasonably high expression levels of antibody with low copy numbers were obtained and, furthermore, the number of gene copies could become higher through gene amplification, resulting in higher expression levels. These were accomplished by the screening of a transcription-active and gene-amplifiable parental clone and the subsequent targeting of the antibody gene. However, the classical Cre/loxP system employed might have complications in recombination efficiency owing to excessive excision, and the investigation with regard to production stability has not been performed. Successful results on retroviral vector producer cell lines using Flp-mediated cassette exchange [5] and doxycycline-inducible transgene expression using Cre-mediated cassette exchange [29] have also been reported.

In this paper, random screening of a chromosomal locus, which guaranteed the stable and relatively high expression of a transgene, and subsequent targeting of an erythropoietin (EPO) gene into that locus, was conducted to obtain a

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stable high-level EPO producer. Here, we present the process of establishing a CHO cell line stably producing EPO, a glycoprotein that is one of the most important biopharmaceuticals [17], using Flp-mediated cassette exchange.

MATERIALS AND METHODS

Plasmid Construction

An EGFP gene, a synthesized F_3 fragment, a synthesized *FRT* fragment, and a herpes simplex virus-thymidine kinase (HSV-TK) gene were inserted into the EcoRI site, HindIII and BamHI sites, NotI and SacII sites, and BamHI and EcoRI sites of pCMV-dhfr2 (Aprogen, Daejon, Korea), respectively, to yield an insertion vector, pEGFP-F_3 (Fig. 1A).

pIC-F_3-EGFP-*FRT*, one of the pIC family vectors, was used as a backbone vector to construct an EPO targeting vector, since the EGFP coding region flanked between F_3 and *FRT* sequences was proven not to be expressed at all by flow cytometric analysis in our previous work [16]. A promoterless hygromycin gene was replaced with the EGFP coding region to prevent unexpected expression of the hygromycin gene, resulting in the pIC-F_3-Hyg-*FRT*. An EPO gene was ligated into a unique XhoI and EcoRI site of pIC-F_3-Hyg-*FRT* to yield an EPO targeting vector, pIC-F_3-EPO (Fig. 1B).

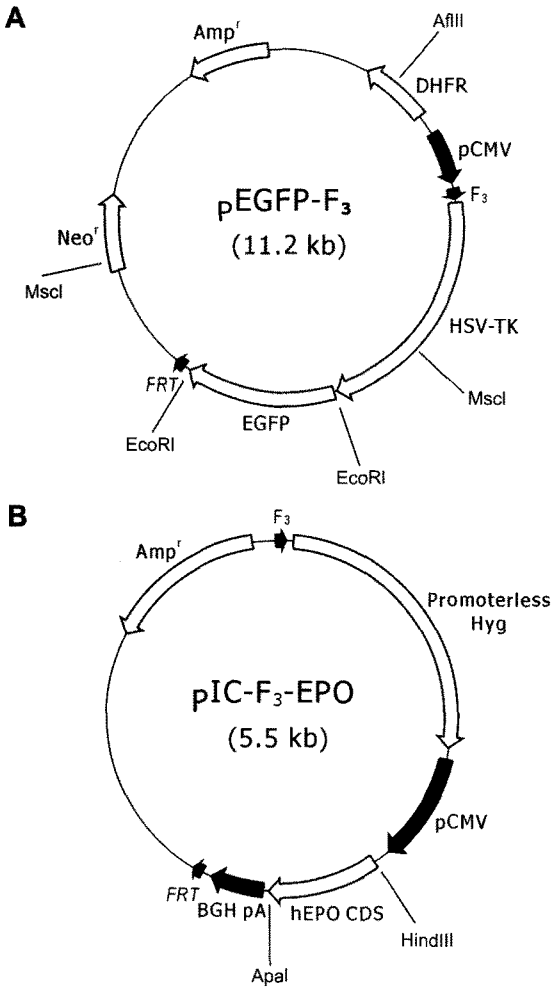

Fig. 1. Schematic map of pEGFP-F_3 (**A**) and pIC-F_3-EPO (**B**).
DHFR, dihydrofolate reductase gene; pCMV, cytomegalovirus promoter; HSV-TK, herpes simplex virus thymidine kinase gene; EGFP, enhanced green fluorescence protein gene; Neo^r, neomycin-resistant gene; Amp^r, ampicillin-resistant gene; Promoterless Hyg, promoterless hygromycin gene; hEPO CDS, coding region of erythropoietin gene; BGH pA, bovine growth hormone polyAdenylation signal. The AflII and MscI restriction sites, which were used for the investigation of rearranged DNA structures by Southern blot analysis, are displayed.

Selection of Parental Clones, Targeting of the EPO Expression Unit, and Drug Selection

The insertion vector, pEGFP-F_3, was transfected into DHFR-deficient CHO cells (DG44) using Lipofectamine 2000 (Invitrogen, Grand Island, NY, U.S.A.) following the manufacturer's recommendations. The transfectants were seeded into 96-well plates and the selection medium used was Isocove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 10% (v/v) dialyzed fetal bovine serum (dFBS; JRH, Lenexa, Kansas, U.S.A.) and 550 μg/ml of G418 (Invitrogen).

To target the EPO expression unit into the premarked locus of a selected parental clone, a total of 10 μg of the EPO targeting vector pIC-F_3-EPO and the Flp expression vector pΔEGFP-Flp were electroporated into 5×10^6 cells at 250 V 960 μF using the GENE PULSER II (BIO-RAD, Hercules, CA, U.S.A.). The molar ratio of pIC-F_3-EPO and pΔEGFP-Flp was adjusted to 1:3. Three days after electroporation, targeted cells were seeded onto 100-mm dishes (Nalge Nunc Intl., Rochester, NY, U.S.A.) at a concentration of 2×10^5 cells/dish. The selection medium consisted of IMDM, 10% dFBS, 200 μg/ml of hygromycin (BD Biosciences Clontech, Palo Alto, CA, U.S.A.), and 3 μM ganciclovir (GCV; Sigma, St. Louis, MO, U.S.A.). Colonies were picked between 2 to 3 weeks after drug selection. Genomic DNA of each clone was isolated for Southern blot and PCR analyses.

All cells were maintained as monolayer cultures in a 5% CO_2/air mixture, humidified at 37°C.

Selection of EPO-Producing Subclones by the Traditional Method

In order to compare the EPO production level and stability between clones established by Flp-mediated cassette exchange and those obtained by the traditional method, development of a CHO cell line producing EPO was performed according to the traditional procedure [15]. The pCMV-dhfr2-EPO vector, which has exactly the same EPO expression unit as pIC-F_3-EPO, was transfected into CHO DG44 cells. Thereafter, transfected cells were cultivated under the treatment of G418. Surviving cells were pooled and gene amplification was achieved by stepwise increase of the MTX concentration (5 nM and 20 nM). Since the cell pool amplified at 5 nM MTX had a similar EPO production level compared with the correctly targeted clone (FC28T7) obtained by Flp-mediated cassette exchange, these cells were cloned by the limiting dilution method. One subclone, which had similar characteristics of cell growth and EPO production (specific growth rate (μ)=0.54 day^{-1}; specific EPO productivity (q_{EPO})=3.0 μg/10^6 cells/day) with those of FC28T7 (μ=0.61 day^{-1}; q_{EPO}=2.1 μg/10^6 cells/day), was selected among the various subclones to compare the stability of EPO production during long-term culture with the targeted clone. The subclone was designated as EPO 05-8.

Flow Cytometric Analysis

The FACSCalibur system (Becton Dickinson, San Jose, CA, U.S.A.) was used for estimating the green fluorescence levels of various clones. Green fluorescence at 525 nm was detected through FL1 set at a PMT voltage of 400, with a logarithmic gain. Ten thousand cells were analyzed for each sample. Data analysis was performed using WinMDI 2.8 software.

Southern Blot Analysis

To investigate the copy number of the insertion vector (pEGFP-F_3) present in the parental clones, genomic DNAs (5 μg) of each clone were digested with EcoRI overnight at 37°C and loaded on a 0.6% agarose gel for electrophoresis. After electrophoresis, the separated DNA was blotted onto a positively charged nylon membrane (Roche, Mannheim, Germany). A neomycin-resistant gene (795 bp) obtained by PCR was used as a template for a probe. The probe was labeled by randomly primed incorporation of digoxigenin (DIG)-labeled deoxyuridine triphosphate (Roche). Prehybridization, hybridization, and subsequent luminescent detection using CSPD substrate (Roche) were conducted as described in the DIG user's guide (Roche).

Southern blot analysis was also employed for the investigation of rearranged genomic DNA structure after targeting of the EPO targeting vector (pIC-F_3-EPO). Genomic DNAs of targeted clones were digested with AflII and MscI. The partial fragment of DHFR CDS (355 bp) obtained by PCR was used as a template for a probe. The strategy of detecting Southern blot products is shown in detail in Fig. 4.

In an effort to investigate the change in EPO gene copy numbers during long-term culture, genomic DNAs from 10^6 cells of the correctly targeted clone (FC28T7) and EPO 05-8 at the beginning and end of long-term culture were digested by ApaI and HindIII. The EPO CDS (582 bp), obtained by ApaI and HindIII digestion of pIC-F_3-EPO, was used as a template for a probe. Known amounts of template DNA were loaded on the same gel as standards. The band intensities of samples were normalized to the standards, using TINA v2.09g software.

PCR-Based Genomic DNA Analysis

PCR analysis was performed to investigate whether the survived parental clones have intact recognition sequences (F_3 and *FRT*), which are essential for proper cassette exchange in cooperation with the site-specific recombinase, Flp. Primers were designed to yield DNA fragments approaching 0.7 kb, which included the *FRT* sequence, and DNA fragments approaching 0.5 kb, including the F_3 sequence. Amplification was performed at 95°C for 10 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final elongation step at 72°C for 10 min.

Rearranged DNA structures after targeting of the pIC-F_3-EPO vector into the selected parental clone were also examined using PCR analysis. Fig. 4 shows the possible rearranged DNA structures after targeting of pIC-F_3-EPO and the primers used. Two combinations of primers were employed to investigate the rearranged DNA structures. The first combination was designed to amplify the genomic DNA region approaching 1.1 kb nearby the F_3 mutant sequence. The forward primer has an exact homology only with the front of the F_3 mutant sequence of pEGFP-F_3, which has already been integrated into a chromosome. The reverse primer matches exactly the partial sequence of the hygromycin gene of the incoming pIC-F_3-EPO vector. The other combination of primers was intended to yield DNA fragments approaching 0.6 kb including the *FRT* sequence. The forward primer matches exactly the partial sequence of the EPO gene of the incoming pIC-F_3-EPO vector, and the reverse primer has an exact homology only with the rear of the *FRT* sequence of pEGFP-F_3. No bands result in the case of promoter trap of the pIC-F_3-EPO (Fig. 4A). When only one reciprocal crossover occurs *via* the *FRT* sequence (Fig. 4B) or F_3 mutant sequence (Fig. 4C), only the DNA fragment approaching 0.6 kb or 1.1 kb should be amplified, respectively. Both of the DNA fragments approaching 1.1 kb and 0.6 kb may be yielded only when complete cassette exchange occurs by two reciprocal crossovers (Fig. 4D). In both combinations of PCR primers for the investigation of rearranged DNA, amplification was performed at 95°C for 10 min, followed by 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by a final elongation step at 72°C for 10 min.

Long-Term Culture

For long-term culture, the correctly targeted clone (FC28T7) was cultivated as a monolayer culture in the absence as well as in the presence of the selection pressure (hygromycin and GCV) in 25-cm^2 T-flasks (Nalge Nunc Intl.). Basal medium was IMDM supplemented with 10% dFBS. At the same time, EPO 05-8 was also cultivated as a monolayer culture in 25-cm^2 T-flasks containing 5 ml of IMDM supplemented with 10% dFBS in the absence as well as in the presence of 5 nM MTX. The initial cell concentration in each batch culture was adjusted to 10^5 cells/ml. Cells were passed and harvested every 4 days for determination of viable cell concentration. Culture supernatants for the EPO assay were taken at every passage and frozen at −80°C for further analyses.

RESULTS

Experimental Scheme

The success of recombinase-mediated cassette exchange (RMCE) depends mainly on the use of two heterospecific recognition sites, which do not recombine with each other. Based on our previous work [16], a F_3 spacer mutant [27] was used in cooperation with Flp recombinase to develop a stable high-level producer, owing to its incompatibility with the wild-type sequence (*FRT*) and its ability to mediate efficient site-specific recombination in a transient state.

Flp recognition targets (F_3 and *FRT*), the negative selection marker (HSV-TK), and the reporter gene (EGFP) were introduced into CHO DG44 cells by random integration of pEGFP-F_3 (Fig. 1A). Transcription-active clones were selected by the limiting dilution method and flow cytometric analysis taking into account their fluorescence levels [19]. Subsequently, Southern blot analysis was employed to screen clones harboring a single copy of the insertion vector. The stability of EGFP production of these clones during prolonged culture (up to 60 days) was investigated by flow cytometric analysis, which allowed the selection of a "stable" parental clone. Thereafter, pIC-F_3-EPO (Fig. 1B), which is necessary for site-specific recombination and the production of EPO, was introduced into the parental clone and targeted into the

the premarked chromosomal locus flanked by recognition sequences using Flp-mediated cassette exchange. Targeted cells were selected by the combined use of positive and negative selection methods. Productivity of a correctly targeted clone and targeting efficiency were investigated. Cell growth and EPO production during long-term culture were also examined. These parameters facilitated feasibility studies regarding the use of the Flp-mediated cassette exchange system in the development of a CHO cell line stably producing EPO.

Plasmid Construction and Selection Strategies

Two kinds of vectors are necessary for efficient cassette exchange and EPO gene expression. One is an insertion vector, which is used to insert the recognition sequences (F_3 and *FRT*) into a CHO chromosome. The other is an EPO targeting vector for targeting of the EPO expression unit into a premarked locus.

Fig. 1A shows the structure of pEGFP-F_3. The expression of the EGFP gene was confirmed by the observation of green fluorescence using an IX71 fluorescence microscope (Olympus, Tokyo, Japan). The expression of the HSV-TK gene, which serves as a negative selection marker, was also investigated by RT-PCR (data not shown). Cells transfected with pEGFP-F_3 died with the treatment of 3 μM GCV and the cytotoxic effect of the same concentration of GCV, on DG44 cells, which have no HSV-TV gene, was negligible (data not shown). These observations indicate the feasibility of the negative selection strategy.

The structure of pIC-F_3-EPO is given in Fig. 1B. It has an exchange cassette approaching 3.2 kb, and the exchange cassette has a promoterless hygromycin gene, which serves an essential role in the positive-selection strategy, and an EPO expression unit. DG44 cells transfected with only the pIC-F_3-EPO vector experienced cell death when treated with hygromycin, which supports the practicability of a positive selection strategy using the promoterless hygromycin gene.

Establishment of a Stable Parental Clone

After transfection with pEGFP-F_3, 54 parental clones, which had high green fluorescence levels, were selected using flow cytometric analysis. Thereafter, PCR analysis was performed to investigate whether these clones had intact recognition sequences (F_3 and *FRT*), which are essential for precise cassette exchange through site-specific recombination. In 4 clones of the 54 parental clones, either the 0.7 kb or the 0.5 kb DNA fragment was not detected. Therefore, these clones were rejected. The PCR result for investigating recognition targets of 5 representative clones is shown in Fig. 2A.

Parental clones, which were selected based on their fluorescence levels and the presence of recognition targets, were analyzed by Southern blot to estimate the copy number

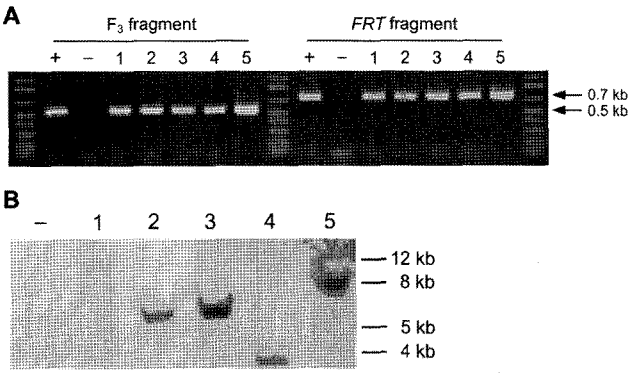

Fig. 2. Investigation of recognition targets (F_3 and *FRT*) and copy number of pEGFP-F_3 vector constructs shown for (+), the pEGFP-F_3 vector as a positive control; (−), DG44 as a negative control; Lanes 1–5, the 5 selected parental clones harboring a single copy of the vector construct (FC7, FC15, FC28, FC50, and FC53, respectively).

A. PCR analysis. The DNA band approaching 0.5 kb, which includes the F_3 sequence, and the DNA fragment approaching 0.7 kb, which includes the *FRT* sequence, were detected in 5 selected parental clones. **B.** Southern blot analysis. It was designed that the number of fragments digested by EcoRI exactly matched with the number of inserted vector constructs.

of the pEGFP-F_3 vector construct on the chromosomal loci. Based on the result of the Southern blot, 45 clones out of the 50 remaining parental clones were discarded since they had more than 2 copies of the vector construct on their chromosomes. The Southern blot results of 5 clones, which harbored single-copy vectors, are shown in Fig. 2B.

The EGFP production stabilities of these 5 candidates were examined by flow cytometric analysis during prolonged culture over 60 days. The changes of EGFP expression levels are shown in Table 1. When considering the production stability and the final EGFP expression level, FC28 was thought of as the most promising candidate for the targeting of pIC-F_3-EPO to obtain a stable high-level EPO producer. Fig. 3 shows fluorescence levels of FC28 at day 7 and at day 62. Even after prolonged cultivation for 62 days, there was no significant broadening of the peak, which means homogeneity of the population was retained.

Table 1. Mean fluorescence intensities of 5 parental clones at day 7 and day 62 of cultivation.[a]

	7 days	62 days
FC7	342.2	249.8 (0.73)[b]
FC15	286.0	229.0 (0.80)
FC28	354.3	338.3 (0.95)
FC50	173.9	175.6 (1.01)
FC53	429.7	206.2 (0.48)

[a]Green fluorescence at 525 nm was detected through FL1 set at a PMT voltage of 400, with a logarithmic gain.

[b]The numbers in parentheses represent the ratio of mean fluorescence intensity at the end of culture (day 62) compared with that at the beginning of culture (day 7).

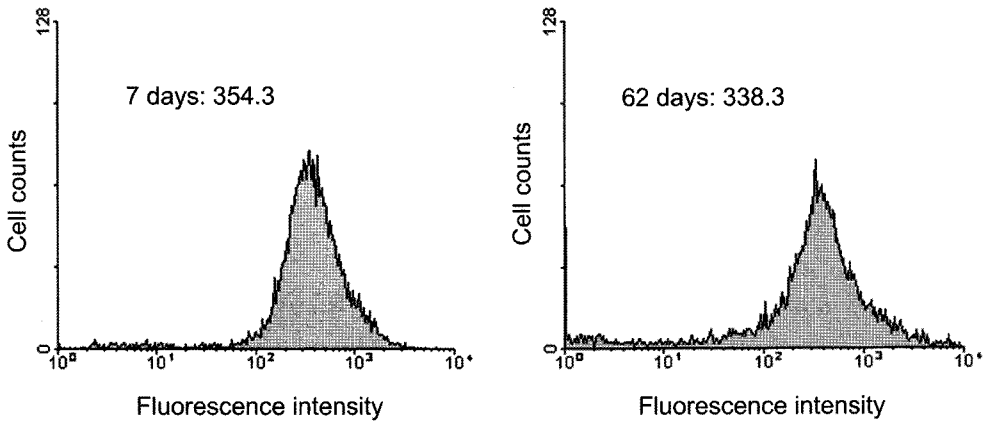

Fig. 3. Histogram for fluorescence levels of the FC28 parental clone at day 7 and at day 62 (FL1=400).

Targeting of pIC-F_3-EPO into the Stable Parental Clone

pIC-F_3-EPO and pDEGFP-Flp were electroporated into the FC28 parental clone, which indicated the most stable EGFP expression during prolonged culture. Three days after electroporation, targeted cells were seeded onto 100-mm dishes (Nalge Nunc Intl., Rochester, NY, U.S.A.) at a concentration of 2×10^5 cells/dish. Transfectants were seeded at such a low density to avoid death of properly targeted cells by the bystander effect when they were exposed to GCV [9, 12].

Thirty-one colonies, which were of average size and well isolated from the other colonies, were chosen, expanded, and analyzed. These 31 clones, separated from the selected colonies, were designated FC28T1-FC28T31 (T stands for "targeting").

Investigation of Rearranged DNA Structures and Targeting Efficiency

Fig. 4 shows the experimental design for the Southern blot analysis. pEGFP-F_3 has only one AflII site and two MscI sites inside and outside its exchange cassette. On the other hand, pIC-F_3-EPO has neither the AflII nor the MscI sites. Four kinds of genomic DNA structures are possible after introducing pIC-F_3-EPO: no exchange by promoter-trap

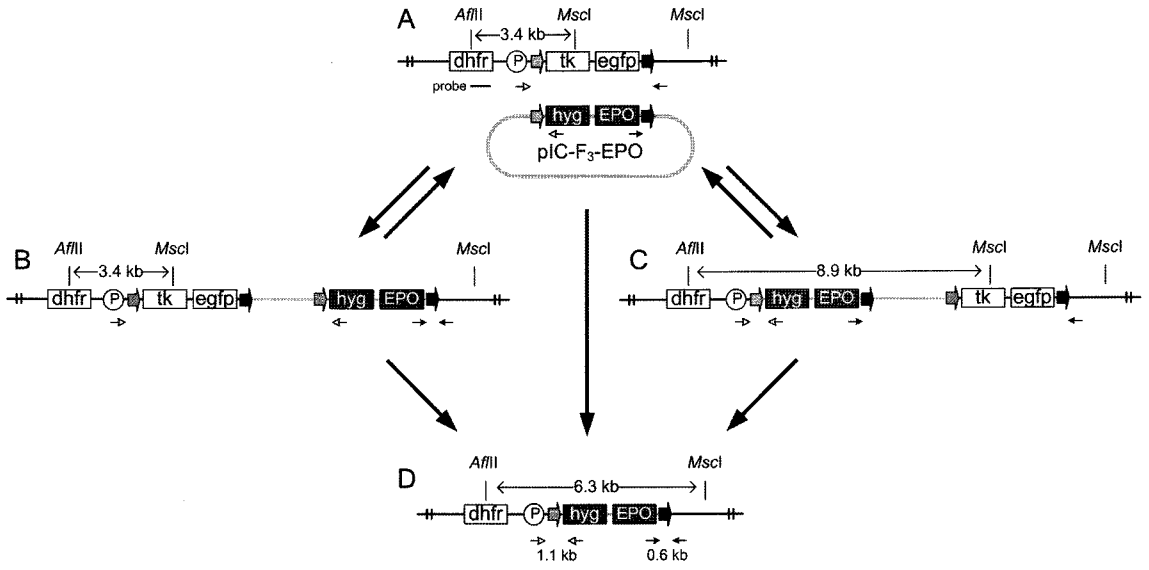

Fig. 4. Strategy of Flp-mediated cassette exchange.
Four kinds of genomic DNA structures are possible after introduction of pIC-F_3-EPO: no exchange by promoter-trap insertion of pIC-F_3-EPO (**A**), partial exchange by only one reciprocal crossover between *FRT* sequences (**B**) or F_3 sequences (**C**), and complete cassette exchange (**D**). Negative selection using the HSV-TK gene and positive selection using the promoterless hygromycin gene were adopted for the selection of correctly targeted clones. The exchange cassettes of pEGFP-F_3 and pIC-F_3-EPO are flanked by the F_3 mutant sequence (▨) and *FRT* sequence (■). The probe used for Southern blot analysis shown in (**A**) has an exact homology with the outside of the exchange unit of pEGFP-F_3. Two combinations of PCR primers are indicated with close and open arrows. Ⓟ, CMV promoter; tk, herpes simplex virus thymidine kinase gene; hyg, promoterless hygromycin gene.

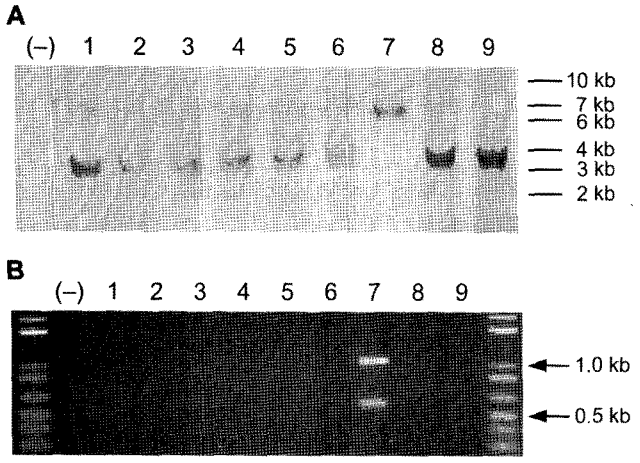

Fig. 5. Investigation of rearranged DNA structures after targeting of pIC-F_3-EPO into the FC28 parental clone.
(–), DG44 as a negative control; Lanes 1–9, FC28T1-FC28T9. Only in the case of FC28T7, a 6.3 kb DNA band was detected by Southern blot analysis (**A**) and both of the bands approaching 1.1 kb and 0.6 kb were detected by PCR analysis (**B**).

insertion of pIC-F_3-EPO (Fig. 4A), partial exchange by only one reciprocal crossover between *FRT* sequences (Fig. 4B) or F_3 mutant sequences (Fig. 4C), and complete cassette exchange (Fig. 4D). The band of about 6.3 kb, in the case of complete cassette exchange, can be distinguished from those of the non-exchanged form (3.4 kb) or intermediates (3.4 kb or 8.9 kb) by Southern blot analysis. The Southern blot result of the 9 representative targeted clones (FC28T1-FC28T9) is shown in Fig. 5A. FC28T7 shows the 6.3 kb fragment, which implies that this clone was established by complete cassette exchange in a site-specific manner. On the other hand, all the other clones had the 3.4 kb fragment, which implies that these are either non-exchanged clones (Fig. 4A) or partially exchanged clones obtained by only one reciprocal crossover between the *FRT* sequences (Fig. 4B).

With our design of Southern blot, the DNA structures displayed in Fig. 4A and Fig. 4B cannot be distinguished. For the more precise investigation of genomic DNA structures and the convenience of analysis, a PCR test was performed. The PCR analysis result of the 9 representative targeted clones is indicated in Fig. 5B. Only FC28T7 showed both the 1.1 kb and 0.6 kb DNA bands, which is in good agreement with the Southern blot result. No bands were detected in the other 8 clones, which means that these clones have no intermediate forms and only non-exchanged forms.

Among a total of 31 clones, one clone (FC28T7) was proven to be obtained by complete cassette exchange. Therefore, the recombination efficiency of the FC28 parental clone, which is defined as the ratio of correctly targeted clones out of the total recovered clones, was 3%.

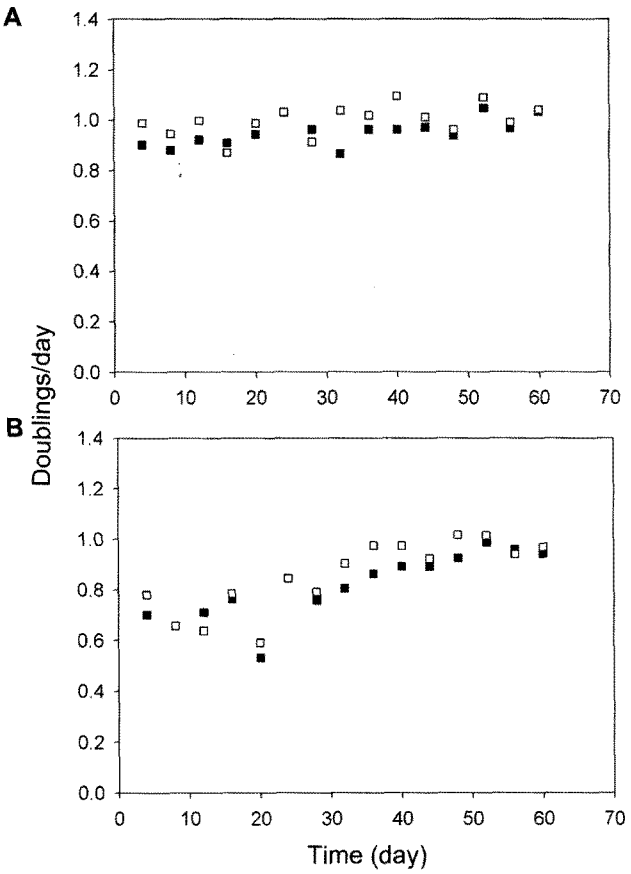

Fig. 6. Growth characteristics of FC28T7 (**A**) and EPO 05-8 (**B**) during long-term culture in the presence (■) and absence (□) of corresponding selection pressure.

Stability Test During Long-Term Culture

As shown in Fig. 6 and Fig. 7, FC28T7 showed stable cell growth and q_{EPO} contrary to EPO 05-8, which indicated an increasing profile of cell growth and a decreasing pattern of q_{EPO} regardless of whether selection pressure existed or not. The q_{EPO} of EPO 05-8 in the absence of 5 nM MTX decreased substantially at the point of 32 days of cultivation. EPO 05-8 showed a prominent decrease also in the presence of 5 nM MTX, starting from 44 days after cultivation. q_{EPO} and doublings per day at the beginning and at the end of cultivation are summarized in Table 2. The q_{EPO} of EPO 05-8 in the absence of selection pressure after 60 days (≈51 generations) was 0.9 μg/10^6 cells/day, which is only 28% of the value obtained at the beginning of long-term culture (3.2 μg/10^6 cells/day). Similarly, the q_{EPO} of EPO 05-8 in the presence of selection pressure after 60 days (≈50 generations) was 1.0 μg/10^6 cells/day, which is approximately 31% of the value obtained at the beginning of long-term culture (3.3 μg/10^6 cells/day). On the other hand, the q_{EPO} values of FC28T7, both in the presence and absence of the selection pressure, were maintained almost constantly even after 60 days of cultivation (≈57 and 61 generations).

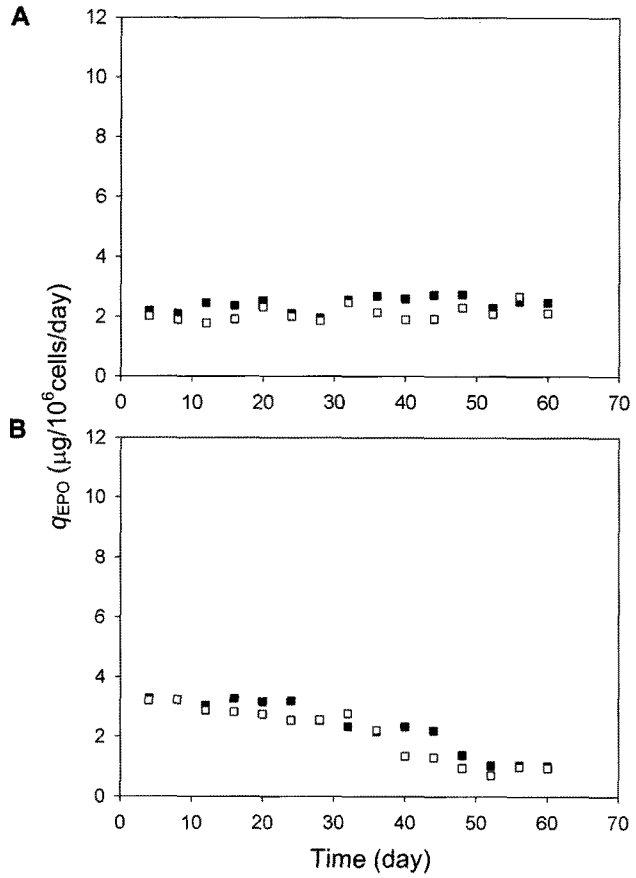

Fig. 7. EPO production characteristics of FC28T7 (**A**) and EPO 05-8 (**B**) during long-term culture in the presence (■) and absence (□) of corresponding selection pressure.

Estimation of the EPO Gene Copy Number

The EPO gene copy numbers of FC28T7 and EPO 05-8 at the beginning and at the end of long-term culture were estimated using Southern blot analysis to elucidate the relationship between the stability of EPO production and the EPO gene copy number. Fig. 8 shows the result of the Southern blot. EPO gene copy numbers of EPO 05-8, in the presence and in the absence of selection pressure, at the 3rd passage were all 6 copies. These values were reduced to 5 and 3 copies at the 14th passage, respectively. On the other hand, a single copy of the EPO gene in FC28T7 was

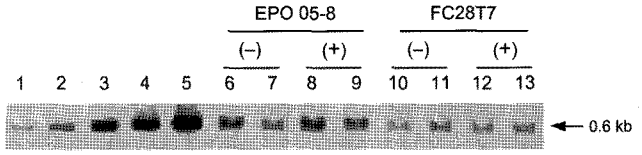

Fig. 8. Southern blot analysis for the investigation of the EPO gene copy number of FC28T7 and EPO 05-8 during long-term cultures in the presence (+) and absence (-) of selection pressure. Lanes 1-5, plasmid standards with different amounts corresponding to 1, 5, 10, 20, 50 copies per cell, respectively; Lane 6, 8, 10, and 12, cells were harvested and genomic DNAs were isolated after the 3rd passage; Lanes 7, 9, 11, and 13, cell were harvested and genomic DNAs were isolated after the 14th passage.

maintained during long-term culture regardless of the existence of selection pressure.

Full Batch Culture of FC28T7

After long-term cultivation for 60 days, the FC28T7 targeted clone was cultured in a batch mode for 12 days to estimate cell growth and EPO production in detail. Cells were seeded at a density of 10^5 cells/ml in 25-cm^2 T-flasks and one flask was sacrificed every 24 h for determination of viable cell concentration and estimation of EPO production level. As shown in Fig. 9A, cells reached the maximum cell concentration ($2.08\pm0.14\times10^6$ cells/ml) at day 6. The specific growth rate (μ), which was calculated using the data from day 1 to day 5, was 0.65 ± 0.02 day^{-1}. Fig. 9B shows the EPO production profile of FC28T7. The maximum titer and the specific EPO productivity (q_{EPO}) were 18.2 ± 2.2 μg/ml and 2.0 ± 0.1 μg/10^6 cells/day, respectively. This production level of FC28T7 was much higher than that of the EPO parental pool, which also contains non-amplified cells obtained in the course of developing an EPO-producing cell line by the traditional method described in the Materials and Methods section. The EPO concentration after 4-day cultivation in a batch mode was only 0.4 μg/ml and the q_{EPO} was 0.2 μg/10^6 cells/day.

Discussion

To achieve high-level production of therapeutic proteins, recent studies focus mainly on the optimization of downstream

Table 2. Specific EPO productivity and doublings per day of FC28T7 and EPO 05-8 at the beginning and end of cultivation.

		Specific productivity (μg/10^6 cells/day)		Doublings per day	
		Value at the 1st passage	Value at the 15th passage	Value at the 1st passage	Value at the 15th passage
FC28T7	(+)	2.2	2.5	0.90	1.03
	(-)	2.0	2.1	0.99	1.04
EPO 05-8	(+)	3.3	1.0	0.70	0.94
	(-)	3.2	0.9	0.78	0.97

(+), values obtained in the presence of selection pressure; (-), values obtained in the absence of selection pressure.

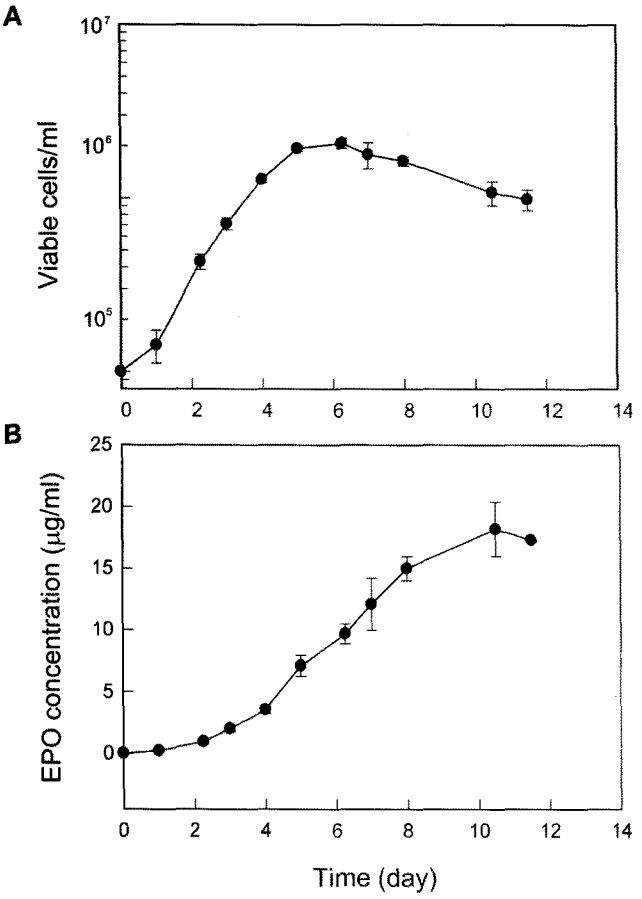

Fig. 9. Cell growth (**A**) and EPO production (**B**) during the batch culture of FC28T7.
Error bars represent the standard deviation calculated from data obtained in duplicate experiments.

processes and media development [21, 30]. However, epigenetic approaches on the enhancement of transgenes' expression levels and stabilities are also important for the efficient production of therapeutic proteins [21]. The epigenetic approaches can provide promising candidates, which can be optimized in the downstream processes to obtain stable high-level producers.

In this study, an epigenetic approach, site-specific recombination, was employed to establish a CHO cell line stably producing EPO. Koyama and his colleagues [20] reported successful achievement of the stable expression of a heterogeneous gene. They employed homologous recombination to target the heterogeneous gene into the locus of the hypoxanthine phosphoribosyl transferase (HPRT) gene, which was constitutively active. Our strategy was based on the selection of a stable parental clone, which has properties mediating stable and relatively high expression of any transgene, and the subsequent targeting of an EPO targeting vector using Flp-mediated cassette exchange. It was postulated that the parental clone's properties mediating a stable and relatively high expression of EGFP can be maintained after targeting of the EPO targeting vector using Flp-mediated cassette exchange, which will result in a stable EPO producer. This postulation was supported, in part, by the observation representing that recombinase-mediated cassette exchange did not alter the characteristic expression level of a chromosomal locus [5].

After random integration of pEGFP-F_3, a total of 50 parental clones, which had high fluorescence levels, were tested by Southern blot analysis to investigate copy number. Screening of parental clones bearing a single copy of the insertion vector construct was performed to confer stability of transgene expression with time. Among the 50 clones, 5 clones had a single copy of the pEGFP-F_3 vector (Fig. 2B). Since multiple-copy integration can occur in the process of traditional transfection, and clones with only a high-level of fluorescence were selected among various clones, most of the selected clones had more than 2 copies of the vector construct. There are several reports on the successful use of retroviral vectors for the single-copy integration of a vector construct [5, 29], and therefore, it is worthy to consider the use of this retroviral vector to improve the problem of multiple-copy integration caused by traditional transfection methods. Finally, 5 parental clones were selected based on their plasmid copy number. These 5 selected clones had a single copy of the pEGFP-F_3 vector construct within the loci, which conveyed a relatively high expression of the EGFP gene.

Subsequent long-term culture was conducted to estimate the production stability of these 5 parental clones. Although all 5 clones had a single copy of the insertion vector construct, the production stability should be monitored during prolonged culture since the chromosomal loci, into which the vector construct was integrated, as well as the copy number of the vector construct are critical to the production stability. Silencing of a transgene can occur by interactions between the transgene and adjacent chromosomal genes such as transcriptional interference and promoter suppression [7]. As shown in Fig. 3 and Table 1, FC28 had a stable level of green fluorescence even after 60 days of cultivation. Therefore, the FC28 clone was selected as the stable parental clone.

As shown in Fig. 5A, there was one completely exchanged clone (FC28T7) among the 31 candidates and the targeting efficiency was approximately 3%. The false-positive clones might have been obtained by promoter-trap events [3, 26]. It is likely that integration loci are not responsible for such a relatively low targeting efficiency since the targeting efficiencies of the other 4 parental clones were also similar (0–3%, data not shown). It is thought that improvement on the selection strategies needs to be considered.

The rearranged DNA structures were analyzed in detail using PCR analysis. Among the 31 clones after targeting and colony selection, neither of the DNA bands approaching

1.1 kb or 0.6 kb was detected except for in FC28T7 (Fig. 5B). In other words, false-positive clones have no intermediate forms, which is not consistent with the abundant existence of intermediate forms after site-specific cassette exchange by ϕC31 integrase in mouse ES cells [3]. Since the expression of Flp recombinase is not controlled in our experimental design, the intermediate forms that occurred in the process of recombination reactions may have been susceptible to additional reciprocal crossovers to complete cassette exchange (Fig. 4D) or return to the non-exchanged form (Fig. 4A). However, the incompatibility between F_3 and *FRT* recognition targets was retained and the fluorescence level of the FC28 parental clone was stably maintained during long-term culture (Fig. 3).

As shown in Fig. 6 and Fig. 7, FC28T7 maintained stable production of EPO even after 60 days of cultivation both in the absence and in the presence of selection pressure, which is consistent with our expectations. This implies that the FC28 parental clone's property, which confers stability of transgene expression during time, did not alter after cassette exchange with the EPO expression unit by Flp recombinase. On the other hand, the q_{EPO} of EPO 05-8 substantially decreased after 60 days of cultivation regardless of whether selection pressure existed or not. The reduction of the EPO gene copy number of EPO 05-8 with time was proven to be responsible for the decreased q_{EPO} by Southern blot analysis (Fig. 8). The EPO gene copy numbers of EPO 05-8 in the presence and absence of selection pressure at passage 14 were 83% and 50% of the values at passage 3, respectively. However, the q_{EPO} values of EPO 05-8 in the presence and absence of selection pressure after 60 days were only 31% and 28% of the values obtained at the beginning of long-term culture, respectively. Therefore, other phenomena besides the reduction of gene copy number might have a contribution in the substantial decrease of specific productivity. It is thought that tandem repeats of the amplification units the occurred in the course of gene amplification induced repeat-induced transgene silencing [6, 11], which might partially result in the decrease of specific productivity. Contrary to EPO 05-8, the stable and relatively high production of EPO in FC28T7 is supposed to be related, in part, to the function of DNA regulation elements such as the locus control region (LCR), or the scaffold/matrix-associated region (S/MAR) [21]. The effect of DNA elements may have enabled the clone harboring only a single copy of the EPO gene to produce EPO stably and at relatively high levels. The precise investigation of the chromosomal locus should be studied further.

In conclusion, the stable parental clone was screened based on the EGFP expression level and stability. Using Flp-mediated cassette exchange, the EPO expression unit could be correctly targeted into the stable parental clone, and FC28T7, a CHO cell line that produced EPO stably and relatively high, could be obtained without gene amplification. The EPO production level of FC28T7 was much higher than that of the non-amplified EPO pool and comparable to that of EPO 05-8, which was cloned from the EPO pool amplified at 5 nM MTX. Whereas EPO 05-8 lost its specific productivity up to 72% after long-term cultivation, FC28T7 maintained stable production of EPO. Although the targeting efficiency needs to be improved, the feasibility of Flp-mediated cassette exchange for establishing a CHO cell line stably producing EPO was verified. Since the Flp-mediated cassette exchange did not modify the parental clone's characteristics concerning the transgene's expression level and stability, stable production of any desired protein is expected to be accomplished by the targeting of transgene targeting vectors into the stable parental clone *via* Flp-mediated cassette exchange.

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