3. RESULTS

3.1 The construction of stable clones in Flp-In system

3.1.1 The presence of the FRT site in the host cell line genome

The FRT sequence used for the subsequent targeting strategy located just downstream of the ATG initiation codon of the lacZ-Zeocin fusion gene, so its existence in the Flp-In 293 cell line genome could be verified indirectly by expression of the lacZ gene (Fig. 7; Invitrogen Co., The Netherlands). The lacZ gene product, ß-glactosidase, can be visualized by X-gal staining of the cell. Before transfection, Flp-In 293 cells, grown under Zeocin selection, were stained by X-gal to indirectly test the presence of the FRT site in the host genome. After X-gal staining, every single cell took on blue color (Fig. 7). This indicated active lacZ expression, and consequently the presence of the FRT site in the Flp-In 293 cell genomes.

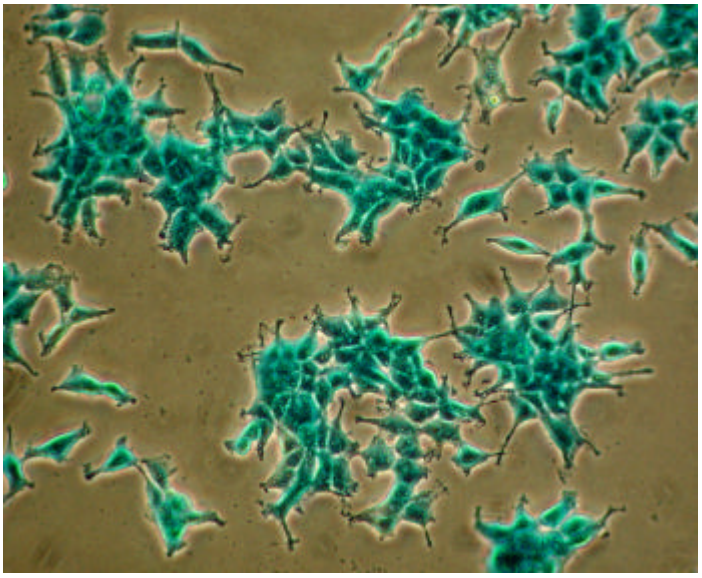


Fig. 7. The presence of the FRT site in the host cell line (Flp-In 293 cells) genome.

Flp-In 293 cells in a 6-well plate were cultured for at least two weeks in high glucose DMEM containing 100 µg/ml of Zeocin. Before the transfection assay, cells were stained with X-gal as described in Methods and photographed under bright field microscopy with a 10X objective. The uniform staining of cells by the X-gal indicated the presence of the FRT site in the host cell genome.

3.1.2 Acquisition of positive stable clones with Flp-In system

I used four expression plasmids, pFRT-CMV-EGFP^{WT}, pFRT-CMV-EGFP^{CpG-}, pFRT-EF-EGFP^{WT} and pFRT-EF-EGFP^{CpG-} (see Methods), to generate stable cell lines with different promoter and transgene combinations. Flp-In 293 cells were cotransfected with each of the above expression vectors and the plasmid pOG44 at a ratio of 9:1 (w/w) by means of calcium phosphate precipitation as described in the Methods section. 24 hours after transfection, one third of the transfected cells were subjected to FACS analysis; the rest were plated onto 10cm petri dishes for stable clone selection with 100µg/ml of hygromycin. FACS analysis showed commensurate expression levels between all four constructs at the transient transfection stage (data not shown).

When the size of the cell colonies formed on the petri dish reached about 3 mm in diameter (about two weeks after plating), a number of EGFP expressing clones were picked for each construct, after pre-screening with fluorescent microscopy. Single copy integration of the transgene at the FRT site in these clones was confirmed by Southern blotting. A representative scheme of restriction enzyme digestion, the probe, and the Southern blot is shown in Fig. 8. A single band of 1.34 kb (s.c) detected on the blot indicates the single integration event at the FRT site in the corresponding clones (Fig. 8B, lanes 1-5, 7 and 8 for clones derived from the pFRT-EF-EGFP^{CpG-} construct; lanes 1-9 for clones derived from the pFRT-CMV-EGFP^{CpG-} construct). The multiple copy integration was signified by the band (m.c) at the same molecular weight as the plasmid besides the 1.34 kb band. Among the pFRT-EF-EGFP^{CpG-} clones, clone No. 6 has multiple copies (m.c) of the transgene at the FRT site (Fig. 8B, lane 6 and P). While clone No. 10 derived from the pFRT-CMV-EGFP^{CpG-} construct has a random integration of the transgene marked by the band (random) different than the s.c or m.c in size (Fig. 8, lane 10). The lane H, the host cell genome included as negative control, did not hybridize with the EGFP^{CpG-} fragment as expected (Fig. 8B, lane H).

Altogether, I picked 64 clones derived from the four expression constructs. Clones were recorded with the copy number of the transgene, and the expression status of the transgene at the time point of clone picking (Table 5).

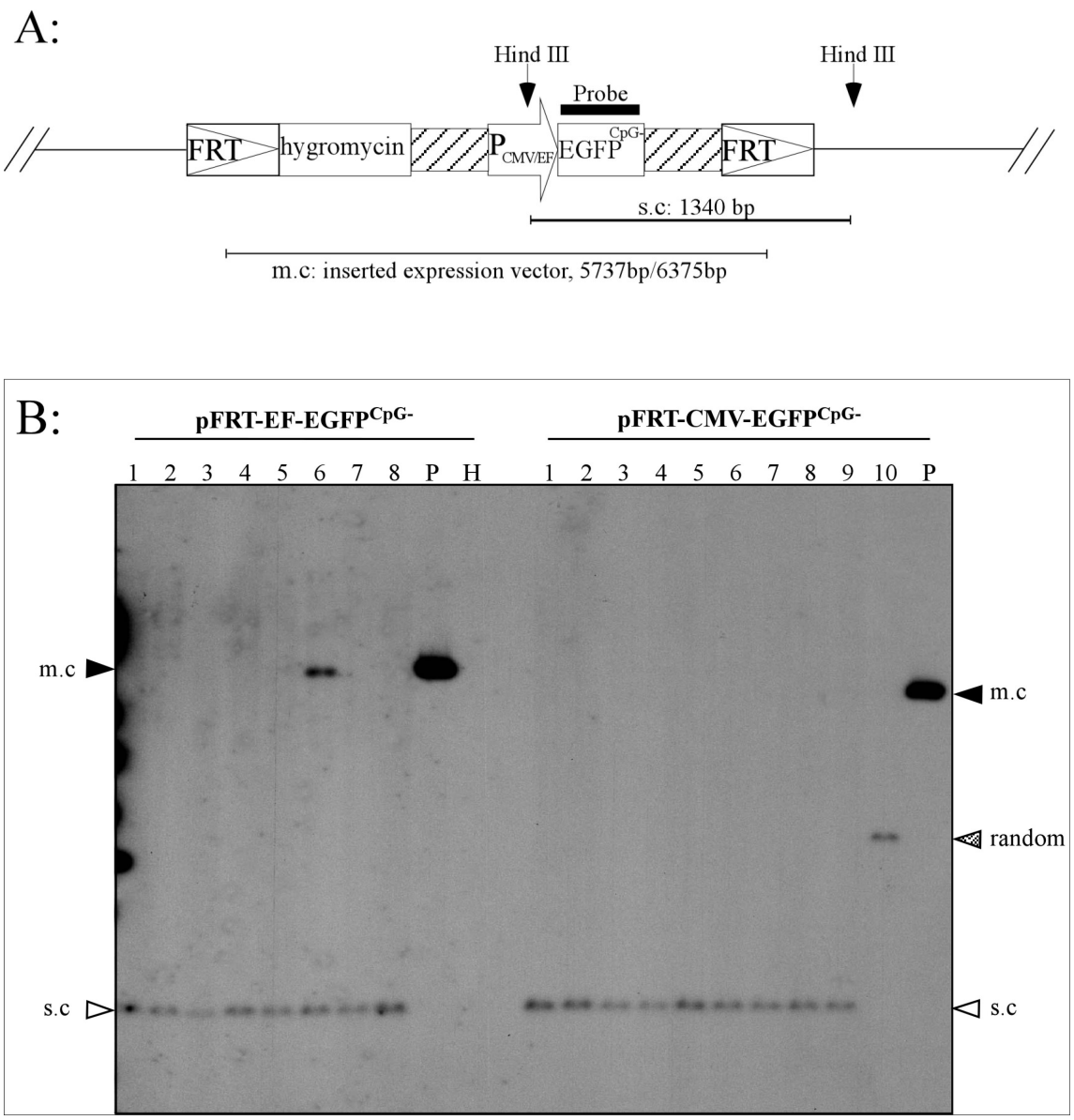


Fig. 8. The copy number of the transgene at FRT site examined by Southern blotting.

A: Schematic diagram of restriction enzyme digestion and probing of the integrated expression vector structure at the FRT site. Genomic DNA was cut by Hind III and probed with the EGFP^{CpG-} fragment. Single copy integration is shown by the 1340 bp band (s.c), and multiple copy integration by the corresponding vector length (m.c). B: Southern blot hybridization for detecting the integration copy number. Genomic DNA from both pFRT-EF-EGFP^{CpG-} and pFRT-CMV-EGFP^{CpG-} clones along with host cell line was digested with Hind III and probed with the EGFP^{CpG-} fragment digested from the pFRT-EF-EGFP^{CpG-} plasmid. Linearized plasmids were included as positive control (lane P), host 293 cell line as negative control (lane H). Clones 1-5, 7 and 8 of the pFRT-EF-EGFP^{CpG-} construct have a single copy of the transgene at the FRT site as indicated by

the single band at the s.c position, whereas clone 6 has multiple copies of the transgene, explained by the m.c in addition to the s.c band; Clones 1-9 of the pFRT-CMV-EGFP^{CpG-} construct have single copies of the transgene at the FRT site, whereas clone 10 contains a random integrated EGFP^{CpG-} transgene shown by the band with a size different than the plasmid itself and the s.c band.

Clones that were further analyzed were tested and confirmed for negative lacZ expression and Zeocin sensitivity. In principle, all the clones containing single integrants of the expression constructs are isogenic cell lines (i.e., they are genetically identical). Clones used in the time course experiment were chosen according to the phenotypic criteria of homogeneous EGFP expression, lacZ inactivity, and Zeocin sensitivity. Thus, by using the Flp-In system in Flp-In 293 cells, I successfully created isogenic cell lines with a single, stably integrated transgene at a defined locus in the genome.

Table 5. Statistics of clones derived from each construct with different features.

Construct		pFRT-CMV-EGFP^{WT}	pFRT-CMV-EGFP^{CpG-}	pFRT-EF-EGFP^{WT}	pFRT-EF-EGFP^{CpG-}
No. of clones obtained		8	18	21	17
lacZ negative§		2	2	2	3
Zeocin sensitive§		2	2	2	3
No. of clones analyzed with Southern blotting		8	18	21	17
No. of clones with single copy of EGFP		3	12	17	16
No. of clones with multiple copies of EGFP		5	5*	4	1
No. of clones with random integration of EGFP		0	2*	0	0
No. of clones with EGFP status of	Homogeneous	4	14	15	12
	Heterogeneous	4	4	4	4
	Negative	0	0	2	1

*: one clone has both multiple copies and random integration of the transgene.

§: only tested for clones used for time course experiments.

3.2 The time course of transgene expression in the Flp-In system

3.2.1 Transgene expression under antibiotic selection over time

First, I decided to test if expression of the EGFP transgene is stable over prolonged time periods when the cells were cultured in selective medium. To do so, I chose cell clones according to the aforementioned criteria and analyzed the expression level of EGFP by flow cytometry (FACS) on a weekly basis for 9 weeks. A 3D view of the overlaid FACS profiles showed relatively stable expression of EGFP over time (Fig. 9). Among the cell lines investigated, both the $EGFP^{WT}$ and $EGFP^{CpG-}$ transgenic cell lines showed stable EGFP expression during the time course, when driven by the CMV promoter. About 99% of cells in the clonal population still maintain the initial high level of EGFP expression for more than two months. In comparison, when driven by the EF promoter, the $EGFP^{CpG-}$ transgenic cell lines showed relatively stable EGFP expression in the majority of cells during the time course. EGFP expression in a tiny amount of cells was silenced. Expression of the transgene stayed stable for about 50 days. At the end of the time course, about 6.9% of cells in the population stopped expressing the $EGFP^{CpG-}$. The silencing process was in a very slow rate. For the transgene $EGFP^{WT}$ driven by the EF promoter, suppression of EGFP expression started in a very small amount of cells after only three-week culture. Similarly, the silencing rate was also rather slow. 3.7% of silenced cells detected at day 21 increased to only 6.4% till the end of the time course. In general, the rate of silencing process in both the pFRT-EF-$EGFP^{WT}$ and pFRT-EF-$EGFP^{CpG-}$ clones was low and alike. In conclusion, the whole cell population has relatively stable expression of the transgene under hygromycin selection, especially when the EGFP transgene was driven by the CMV promoter; however, suppression of the transgene expression seemed existing as well even under selective culture conditions, but was a slow process.

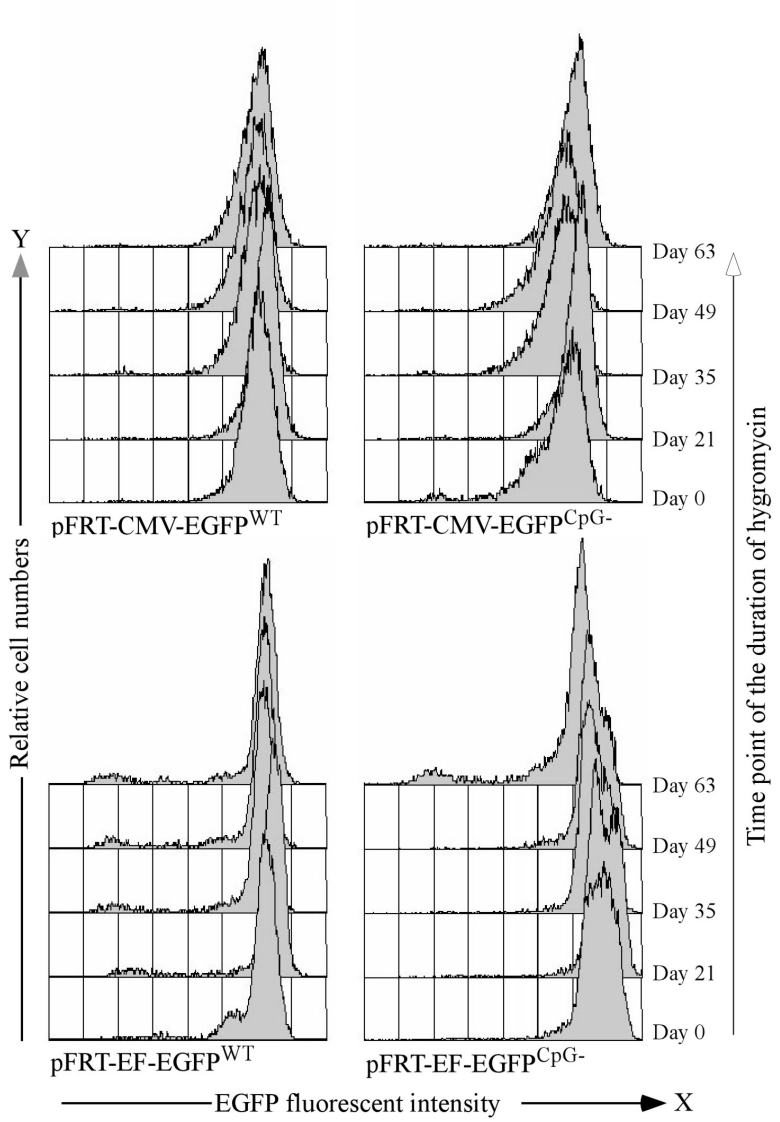


Fig. 9. Stability of the transgene expression under antibiotic selection.

Four clones, one clone for each expression construct, were selected according to the criteria of positive EGFP expression, hygromycin resistant and Zeocin sensitive, and kept in selective medium for more then two months. EGFP expression was analyzed with a Becton Dickinson FACScalibur workstation with consistent instrumental settings on a weekly basis. FACS profile overlays revealed EGFP expression under the selection of hygromycin (100µg/ml).

3.2.2 Unstable transgene expression under nonselective conditions over time

I also wanted to know how stable the transgene with only one copy integrated into cell genomes could maintain over time without antibiotic selection. In order to address this question, I chose the same clones as used in section 3.2.1. The clones were kept in medium without hygromycin selection, and were followed the expression level of the transgenes, $EGFP^{WT}$ and $EGFP^{CpG-}$, over a long period of time by FACS analysis. Unstable transgene expression was displayed in a more dynamic way than clones kept under selection. Cells started suppressing the expression of $EGFP^{WT}$ and $EGFP^{CpG-}$ transgenes after two weeks, as shown in the overlaid FACS time course profiles (Fig. 10). The CMV promoter could support expression of the $EGFP^{WT}$ or $EGFP^{CpG-}$ transgene for about 30 days; in contrast, the transgenes driven by the EF promoter supported homogenous expression for barely 20 days. In addition, the silencing rate observed without antibiotic selection was much faster than under hygromycin selection. About 46% of cells in both clones with the CMV promoter decreased the EGFP expression level after 70 days in nonselective culture. This transgene suppression upon withdrawal of the selective pressure was highlighted when compared to the same clones cultured with hygromycin selection, in which more than 99% of cells still maintain the initial high level of EGFP expression. For both the pFRT-EF-$EGFP^{WT}$ and pFRT-EF-$EGFP^{CpG-}$ clones, 21.3% and 3.5% of silenced cells detected at day 21 raised to 64.6% and 66.9% as measured at day 70, respectively. Nonetheless, the final extinction of transgenic expression of $EGFP^{WT}$ or $EGFP^{CpG-}$ occurred regardless of the promoter used (experimental observation, W. Liu.)

Interestingly, two different silencing patterns, resembling the rheostat and on/off modes, were observed during the time course analysis. After withdrawal of the hygromycin, both $EGFP^{WT}$ and $EGFP^{CpG-}$ transgenes exhibited a gradual decline of the expression level when driven by the CMV promoter. Suppression of the transgene expression was regulated in a rheostat mode. A great number of cells within the clonal population gradually decreased the transgene expression rate, and consequently the whole population was constituted by cells with various expression levels, ranging from high to moderate, and to almost zero level (Fig.

10; upper panel). In contrast, when the EGFP^{WT} or EGFP^{CpG-} transgene was driven by the EF promoter, suppression of the transgene was controlled in an on/off mode. After removal of the hygromycin from the culture, cells within the clonal population either maintained the initial high expression level of the transgene over time, or stopped the transgene expression completely. When the population was detected at the single cell level, a mosaic expression pattern was observed. The population was formed by two types of cells, i.e., either the EGFP positive or negative cell. They formed two distinct peaks when analyzed by FACS (Fig. 10; lower panel).

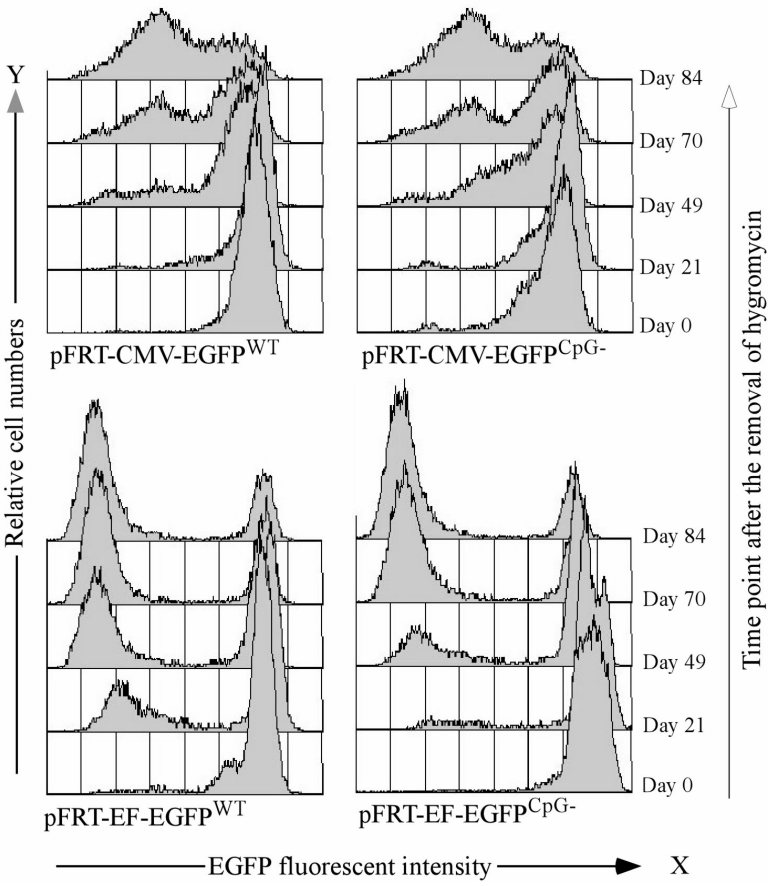


Fig. 10. Unstable expression of the transgene under nonselective conditions.

Four clones, one for each expression construct, were kept in nonselective medium for more than two months. EGFP expression was analyzed on a Becton Dickinson FACScalibur workstation with consistent instrumental settings on a weekly basis. FACS profile overlays revealed unstable EGFP expression over time.

3.2.3 The transcriptional instability of the FRT locus in the host cell line genome

The FRT site was tested and claimed by the system provider to be integrated into a locus in the genome that is very transcriptionally active. However, time course experiments showed an unstable expression of either transgene $EGFP^{WT}$ or $EGFP^{CpG-}$ at the FRT site when driven by either the strong CMV or EF promoter under selection-free culturing conditions. Therefore, considering the direct relation between the expression stability of the transgenes and the permissivity of the FRT site, I tested if this integration site was transcriptionally permissive over time for transgenes under nonselective conditions. After being kept in medium without Zeocin selection for three weeks, the host cell line Flp-In 293 was then stained with X-gal. I found that some cells were indeed not stained blue, which meant these cells were no longer expressing the lacZ gene (Fig. 11). Accordingly, the FRT locus became transcriptionally incompetent after the cells were exposed to the nonselective medium after only five weeks.

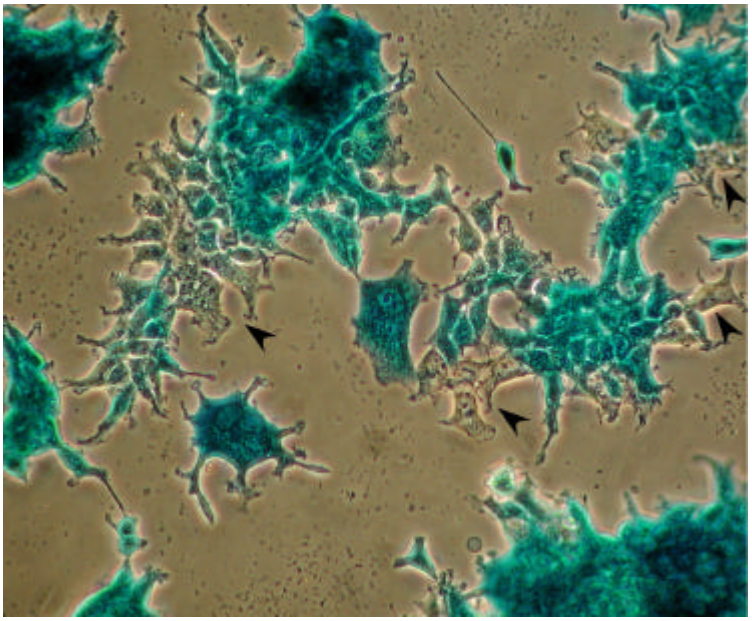


Fig. 11. Gradual loss of transcription at the FRT site.

Host Flp-In 293 cells were stained with X-gal after being kept in Zeocin free medium for five weeks. Black arrows highlight cells that were not stained blue along with some cells with light blue staining in this microscopic field. This implied the silencing, or suppressed expression of the lacZ gene, and consequently the decreased permissivity of transcription at the FRT site.

3.2.4 The irreversible expression pattern of subpopulations after FACSorting

Equilibrium or oscillation exists between transcriptionally active and inactive cells within the whole cell population (Kerr et al., 1989; Ko et al., 1990; Fiering et al., 1990; Reddy et al., 1991; Feng et al., 1999; Feng et al., 2001). Additionally, cell clones obtained through limited dilution could take after the initial

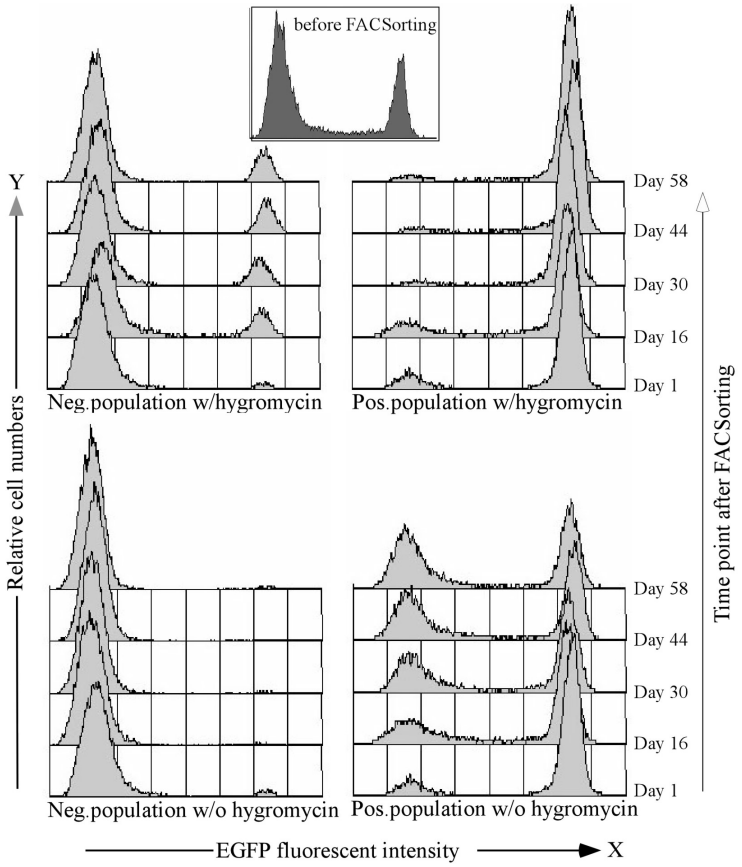


Fig. 12. The irreversible expression state of the transgene after FACSorting.

Negative and positive cell populations separated by FACSorting were kept in medium with or without hygromycin. Expression state of the transgene EGFP was followed by FACS and compared by the FACS profile overlays. With the presence of hygromycin in the medium, a small peak of positive, transgenic expressing cells reappeared over time, the sorted positive population stayed positive. In the absence of hygromycin, the sorted negative cells could not switch on the expression of the transgene EGFP, but more and more cells were turned off transgene expression among the initial sorted positive population. The inset is the FACS profile of the clone before FACSorting.

transgenic expression pattern of the clone from which they are derived, or the expression pattern is inheritable (Feng et al., 2001). Therefore, I decided to investigate the dynamic transgenic expression of the positive and negative subpopulations separated by FACSorting, and detecting whether these two cell subpopulations could regain the same expression pattern as of their parental cell line over time. To this end, I took two pFRT-EF-EGFP^{WT} clones and sorted each into two populations on a FACSorter (Becton Dickinson Inc.) according to their EGFP expression state, named as transgenic positive and transgenic negative expressing populations. Post-sorted cell populations were then maintained in medium with or without hygromycin selection for 6 weeks. Expression of the EGFP transgene was measured by flow cytometry at intervals of one week. FACS profiles of one clone were overlaid and compared (Fig. 12). When maintained under the pressure of hygromycin, a small proportion of the sorted negative population gained EGFP expression, as indicated by the increased size of the peak on the right side of the X axis on the FACS profiles; the sorted positive population still stayed positive after almost two months under selective conditions (Fig. 12, upper panel). By contrast, when the cells were kept in medium without hygromycin selection, cells from the sorted negative population did not switch back to the EGFP expression; instead, the whole population remained negative for EGFP expression. As expected from the previous observation, I found more and more cells from the sorted positive population tuned off the EGFP expression over time, and the whole population became heterogeneous under nonselective conditions (Fig. 12, lower panel).

In short, in the presence of selection, a small portion of cells from the sorted negative populations could regain expression of the EGFP transgene. However, no cells from the sorted positive populations turned off transgene expression. In contrast, when the sorted subpopulations were cultured in medium without hygromycin selection pressure, the switching tendency of transgene expression was always from positive to negative, no initially EGFP negative cells turned on the expression of the transgene. Taken together, the transgenic expression pattern of the sorted positive and negative populations could not, at least not completely, be reversed to the parental cell line expression pattern (shown as the inset FACS profile) as described by other studies (Kerr et al.,

1989; Ko et al., 1990; Fiering et al., 1990; Reddy et al., 1991; Feng et al., 1999; Feng et al., 2001).

3.2.5 The variance of the transgenic expression characteristics between clones

All positive clones I got from each expression plasmid are isogenic, i.e. they have the same genetic background. In principle, the expression pattern of the EGFP transgene should therefore be identical in every clone. In order to test this hypothesis, I picked another two positive clones each from the pFRT-CMV-EGFP^{CpG-} and pFRT-EF-EGFP^{CpG-} constructs and followed their transgenic expression over time under nonselective conditions. Overlaid time course FACS profiles revealed different rate of silencing between the two pFRT-EF-EGFP^{CpG-} clones. However, their initial EGFP expression level was commensurate and the tendency of transgenic suppression was the same under nonselective culture condition as well (Fig. 13, lower panel). For the two pFRT-CMV-EGFP^{CpG-} clones, the mean EGFP expression level of clone 2 was 1.6-fold higher than that of clone 1, but progressive silencing of the EGFP transgene in both clones had a similar rate (Fig. 13, upper panel). Compared to the two pFRT-EF-EGFP^{CpG-} clones, the rate of silencing of the EGFP transgene in these pFRT-CMV-EGFP^{CpG-} clones was slower. Expression of EGFP was stable for about 40 days.

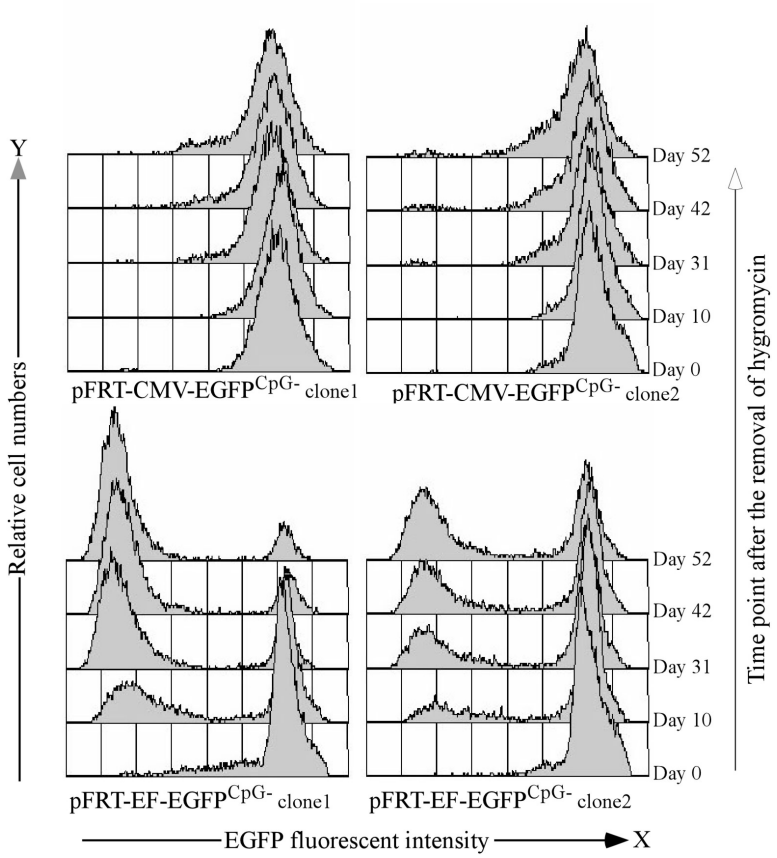


Fig. 13. The variance of transgenic expression between clones.

Two clones each from the pFRT-CMV-EGFP^{CpG-} and pFRT-EF-EGFP^{CpG-} constructs were analyzed for transgenic expression by flow cytometry over time under nonselective condition. Two pFRT-EF-EGFP^{CpG-} clones showed comparable initial EGFP expression level, but clone 2 had a slower silencing rate than clone 1. Two pFRT-CMV-EGFP^{CpG-} clones had a similar silencing rate, but different initial EGFP expression level, with clone 2 1.6-fold higher than clone 1.

3.2.6 Unstable, but higher transgene expression level with multiple copies

We expected to get cell lines with stable transgene expression through the Flp-In system. This was done by integrating a single copy of the EGFP transgene at the predefined FRT site in the cell genome, but the expression of the single copy transgene was not stable without antibiotic selection over prolonged time period. One clone I isolated from the pFRT-EF-EGFP^{CpG-} construct had multiple copies of the EGFP transgene at the FRT site. Therefore, I

studied this clone under the same genetic constellation as the single copy. The FACS profile of this clone showed a higher initial expression level (Fig. 14; day 0), with the mean fluorescent value 1.7-fold higher than that of its single copy counterparts (Fig. 10 and Fig. 13, FACS profiles of pFRT-EF-EGFP^{CpG-} clones at Day 0). Under selective pressure, the expression of EGFP was stable, whereas after maintenance in nonselective medium for less than 10 days, a small proportion of the cells had already decreased or even lost transgene expression. In addition, the silencing rate was similar to its counterparts with only a single

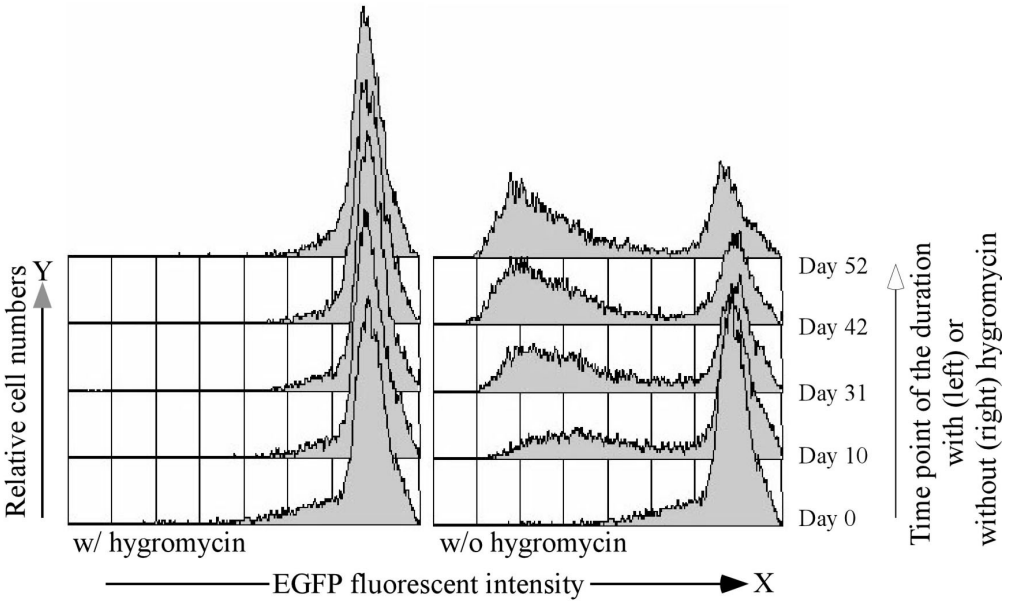


Fig. 14. Transgenic expression of the clone with multiple copies of the transgene.

One pFRT-EF-EGFPCpG- clone with multiple copies of the transgene EGFPCpG- was analyzed by FACScanning under selective and nonselective conditions over time. Upon derivation, this clone showed higher EGFP expression than the counterparts with single copy EGFP CpG- (Fig. 10 and Fig. 13, FACS profiles at Day 0). Over time, transgene expression stayed stable in the presence of hygromycin selection. However, the expression was decreased at a rate of similar to clones with single copy transgenes. Additionally, some cells showed a gradual decrease of the expression level and some a silenced EGFP expression. This is a different mode of silencing than the straightforward positive to negative switch exhibited in those clones containing a single copy of the transgene.

copy of the transgene. However, through FACS analysis I observed a different pattern in the silencing process related to the transgene copy number. In contrast to the single negative peak formed over time from the initially single positive peak for clones with a single transgene copy under nonselective conditions (Fig. 10), the multiple transgene copy clone displayed a slope on the FACS profile. The slope was formed from cells with a medium level of EGFP expression, and continued to the negative peak shaped by the cells lacking of EGFP expression (Fig. 14). The silencing process of transgenic expression indeed has a nexus with the transgene copy number.

3.3 Construction of stable clones by the "Sorting-Subcloning" approach

3.3.1 Stable clone construction by the "Sorting-Subcloning" approach

As the first attempt to obtain homogeneous and stable transgene expression was partially successful, I decided to try a different approach: the selection of stable transgene integration events by sorting positive cell populations. Variables for comparative analysis were again the promoter type, CpG content of the transgene and also the presence or absence of vector sequences. To this end, I isolated the transgene transcription cassette fragments from the four expression vector backbones and transfected these cassette fragments into HeLa cells by lipofection. The four cassettes are described in Fig. 15. Under this transfection condition, the classical approach to obtain stable clones by antibiotic selection was no longer feasible because no antibiotic

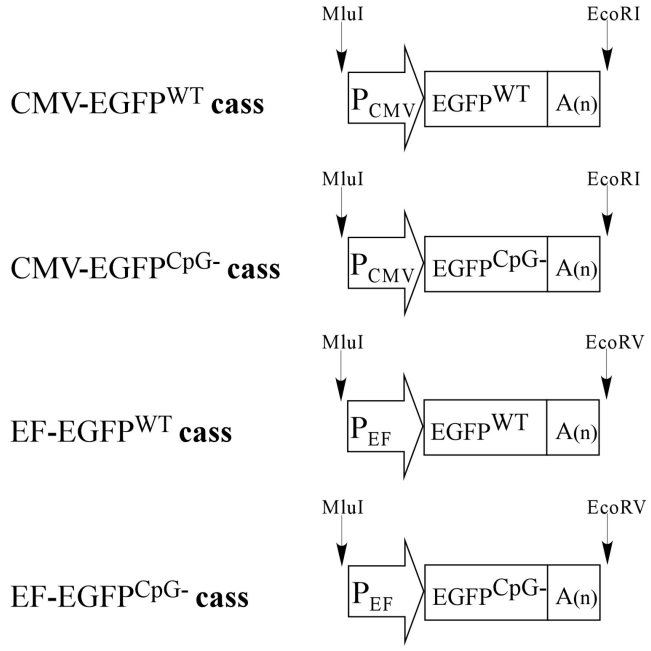


Fig. 15. Transgene cassettes used to generate stable clones with the "Sorting-Subcloning" approach.

Transgene cassettes (cass) are composed of transgene promoter, coding region and polyA tail. They were obtained by digesting the corresponding plasmid by MluI and EcoRI or EcoRV enzymes.

resistant gene was transfected along with the transgene into the cells. The "Sorting-Subcloning" approach was then established and used to enrich the EGFP positive cells. Clones of single cell origin were generated through limited dilution. During the first round of FACSorting, the EGFP positive cells were collected and comprised around 5% of the total cell population. The percentage of EGFP positive cells largely depended on the transfection efficiency. During the second round of FACSorting, 1~5% of EGFP positive cells were usually detected and collected. The percentage of EGFP positive cells was associated with transgene promoters used. When the EF promoter was used to direct the expression of the transgene, more EGFP positive cells were normally detected than when the CMV promoter used. The EGFP cells collected after the second round of sorting were subjected to limited dilution and plated in 96-well plates. Single cell seeding was examined by light microscopy on the day after plating. Expression patterns of clones were observed by fluorescent microscopy (Table 6). Using this approaching, about 2/3 of the resultant clones obtained were EGFP homogeneous, less than 10% of the clones were silenced. Isolated clones from single cell seeding were expanded and the EGFP expression was followed over a long period of time.

Table 6. Summary of clones with different expression patterns obtained by the "Sorting-Subcloning" approach.

	Cassette				**Linearized**	
	CMV-EGFP^{WT}	CMV-EGFP^{CpG-}	EF-EGFP^{WT}	EF-EGFP^{CpG-}	pCMV-EGFP^{WT}	pCMV-EGFP^{CpG-}
No. of Homogeneous clones	70 (59.8%)†	63 (70.8%)	48 (84.2%)	38 (88.4%)	50 (38.2%)	46 (43.4%)
No. of Heterogeneous clones	36 (30.8%)	17 (19.1%)	9 (15.8%)	3 (7.0%)	58 (44.3%)	52 (49.1%)
No. of Silenced clones	11 (9.4%)	9 (10.1%)	0 (0.0%)	2 (4.6%)	23 (17.5%)	8 (7.6%)
Total	117	89	57	43	131	106

(x%)†: percentage of specific expression pattern.

I successfully isolated CMV-EGFP^{WT} and CMV-EGFP^{CpG-} clones with uniform EGFP expression by the "Sorting-Subcloning" approach as judged by fluorescent microscopy. In contrast, those stable EGFP positive clones were unachievable with the traditional antibiotic selection protocol after HeLa cells were transfected with pCMV-EGFP^{WT} or pCMV-EGFP^{CpG-} plasmid (W. Liu, unpublished data), even though stable EGFP positive clones could be selected by G418 after HeLa cells were transfected with pEF-EGFP^{WT} and pEF-EGFP^{CpG-} plamids (W. Liu, unpublished data). Therefore, I asked whether or not the prokaryotic sequences on the transgene expression vector practically interfered with acquisition of stable CMV-EGFP^{WT} and CMV-EGFP^{CpG-} clones in this

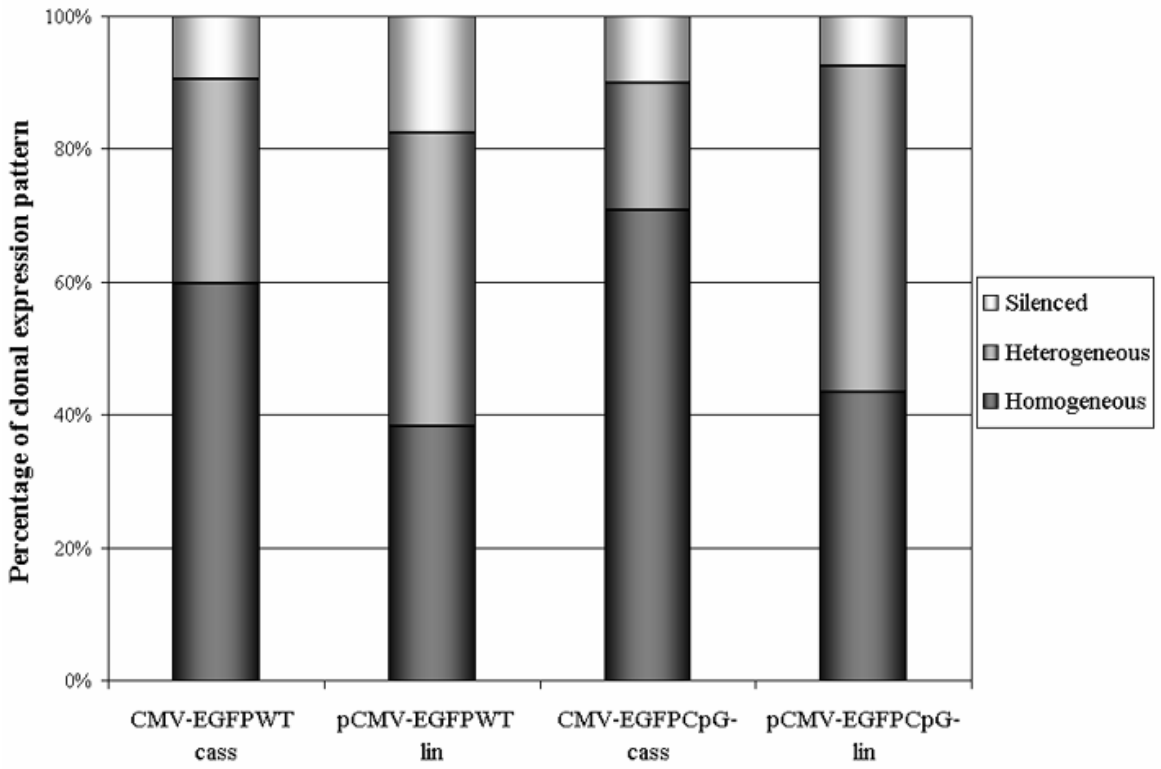


Fig. 16. Comparison of clonal expression patterns obtained by the "Sorting-Subcloning" approach.

Stable clones were obtained by the "Sorting-Subcloning" approach after transfection with CMV-EGFPWT cassette (cass), linearized pCMV-EGFPWT(lin), CMV-EGFPCpG- cassette (cass) and linearized pCMV-EGFPCpG- (lin) in HeLa cells. More than 50% of the clones derived from cassette transfection expressed EGFP homogeneously; on the contrary, about 50% of the clones derived from transfection with linearized expression plasmids were EGFP heterogeneous. Fewer clones showed silenced expression of EGFP derived from transfection with cassette (cass) than transfection with the linearized plasmids (lin). Absolute clone numbers are shown in Table 6.

"Sorting-Subcloning" approach. To do so, I transfected HeLa cells with the whole transgene expression vectors, pCMV-EGFP^{WT} and pCMV-EGFP^{CpG-}, in parallel with the corresponding transgene cassettes, CMV-EGFP^{WT} and CMV-EGFP^{CpG-}. Surprisingly, stable clones were successfully isolated by the "Sorting-Subcloning" approach, with the traditional antibiotic selection method bypassed. The clonal expression patterns were then recorded (Table 6, column 'Linearized'). About 40% of clones obtained showed homogeneous EGFP expression at high levels, while the rest of clones showed either heterogeneous EGFP expression or were completely silenced. However, a comparison of these two different transfection settings showed that almost 2-fold more homogenous clones were generated from transfection with transgene cassettes, CMV-EGFP^{WT} and CMV-EGFP^{CpG-}, than from transfection with the linearized expression plasmids, pCMV-EGFP^{WT} and CMV-EGFP^{CpG-}. In turn, more heterogeneous or silenced clones were obtained from transfection with the linearized expression plasmids compared to transfection with the transgene cassettes (Fig. 16; "lin" vs. "cass"). Taken together, I could drew a conclusion that the prokaryotic sequences on the transgene expression vectors contributed to transgene suppression. However, this effect can not entirely explain the observed gene suppression. Other unknown factors or mechanisms likely exist.

3.3.2 Stable expression of clones constructed with transcription cassettes by the "Sorting-Subcloning" approach

I wanted to test if the clones obtained from transfection with the transcription cassette could maintain EGFP transgene expression over an extended period of time in culture. To do so, I picked three homogeneous clones each from CMV-EGFP^{CpG-} cass, EF-EGFP^{WT}cass and EF-EGFP^{CpG-}cass transfection, and followed EGFP expression over time by FACS analysis. Homogeneous and steady EGFP expression was observed for more than 100 days under normal culture conditions (Fig. 17). Time course was initially carried out only for three CMV-EGFP^{CpG-} cassette clones. Interestingly, the single EGFP positive peak for clone 3 even slightly shifted towards the higher intensity direction. Clone 2 first showed a marginal higher EGFP intensity shift, and then returned to the original intensity. Clone 1 showed relatively stable EGFP intensity

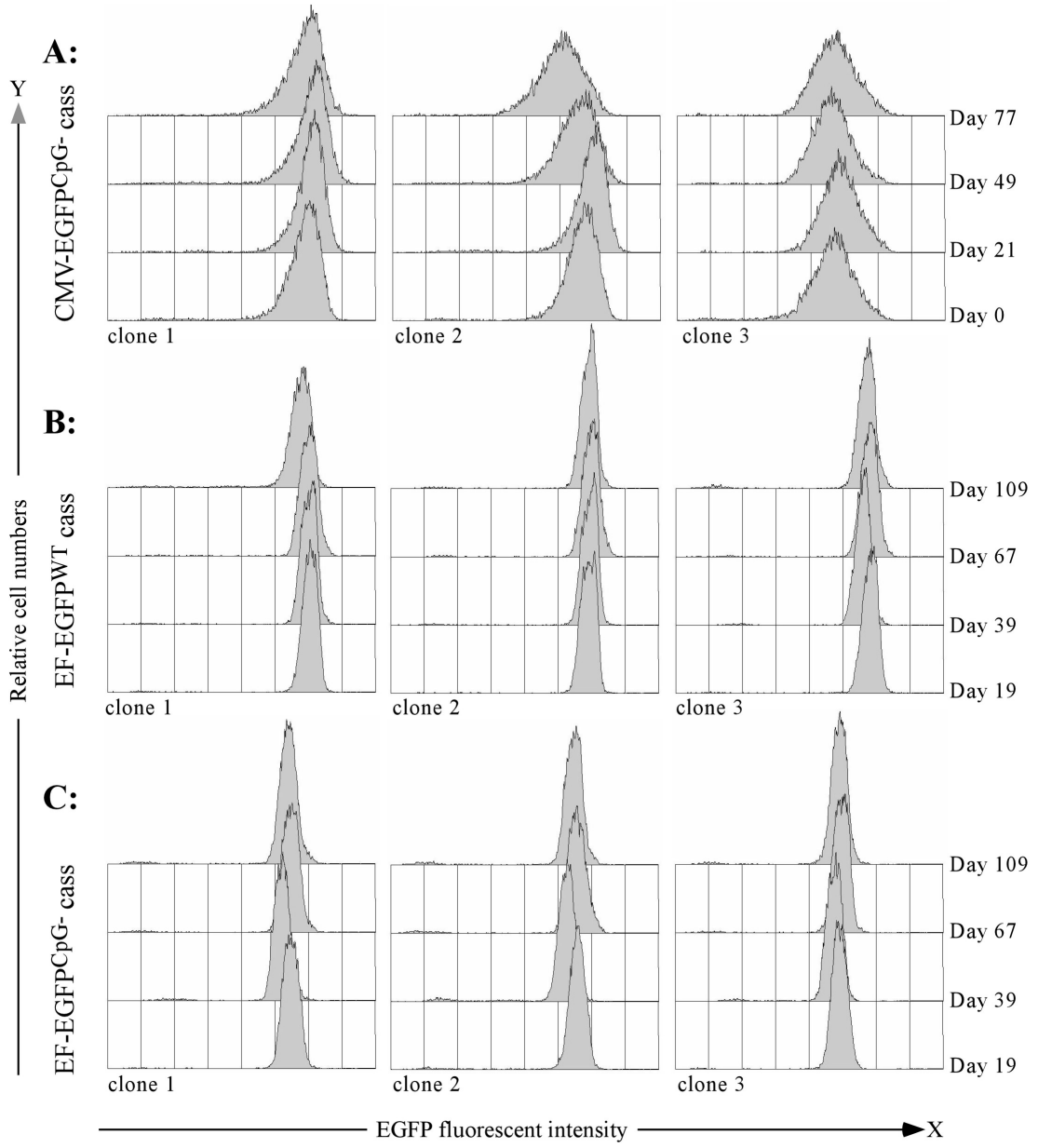


Fig. 17. Stable expression of clones derived from transfection with the "cass" by the "Sorting-Subcloning" approach.

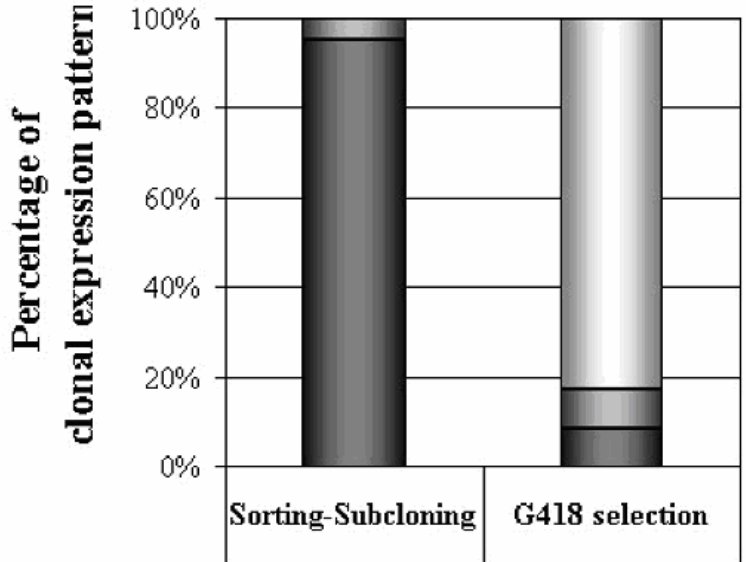
Three clones each were chosen after transfection with CMV-EGFP^{CpG-} cassette, EF-EGFP^{WT} cassette and EF-EGFP^{CpG-} cassette, and isolated by "Sorting-Subcloning". Time course was carried out for EGFP expression by FACScanning for 79 days, 109 days and 109 days, respectively. Homogeneous and sustained EGFP expression was displayed by the single EGFP positive peak at each time point on the overlaid FACS profiles.

during the whole time course (Fig. 17, panel A). Three clones each from transfection with EF-EGFP^{WT}cass and EF-EGFP^{CpG-}cass were followed upon isolation. Amazingly, all 6 clones displayed homogeneous and stable EGFP expression over a period of three months (Fig. 17, panel B and C). Due to the different timing of clone isolation, CMV-EGFP^{CpG-}cass clones were followed only 79 days; no CMV-EGFP^{WT}cass clones were available to be followed long enough to be included in this thesis, but are in future experimental consideration. The broad EGFP positive peak observed by FACS analysis is a general phenomenon for the CMV promoter, in comparison, the EGFP positive peak associated with the EF promoter is sharper. The broad peak observed might result from the existence of the enhancer in the CMV promoter. The transcription machinery formed on the CMV promoter likely has variable stabilities or processivities among cells due to the real-time availability of the enhancer.

3.3.3 Bypassing antibiotic selection to obtain stable clones is critical for long term stable transgene expression

I was able to efficiently generate EGFP homogeneous clones with both transcription cassettes and linearized plasmids with the “Sorting-Subcloning” approach. Time course experiments clearly showed that clones derived from transfection with CMV-EGFP^{CpG-}cassette, EF-EGFP^{WT}cassette and EF-EGFP^{CpG-}cassette could maintain homogeneous EGFP expression over a long period of time. I did not include the EGFP homogeneous clones that were isolated with the “Sorting-Subcloning” approach after transfecting HeLa cells with the whole linearized plasmids in the experiment showed in section 3.3.2. Thus, at this stage I was unable to conclude if the sustained EGFP expression observed in the time course owed to exclusion of the prokaryotic sequences that are included on the transgene expression plasmids and integrated into the host cell genome along with the transgene insertion. What is worth mentioning here again is that I successfully obtained homogeneous EGFP clones from transfection with linearized pCMV-EGFP^{WT} and pCMV-EGFP^{CpG-} plasmids by the “Sorting-Subcloning” approach. However, clones with homogeneous EGFP expression were unachievable by the traditional antibiotic selection protocol in HeLa cells (W. Liu, unpublished data). Therefore, the alternative possibility should not be ruled

out that the approach applied to prevent prokaryotic sequence integration to generate homogeneous EGFP clones is the key for long term transgenic expression. This "Sorting-Subcloning" approach used here bypasses antibiotic selection. This factor is important because antibiotic selection could expose cells to an abnormal environment, in which cells are forced to express the transgene along with the antibiotic resistant gene in order to survive during the stable clone selection process. After withdrawal of antibiotics cells would adapt to the new environment and subsequently might stop expressing these non-essential genes or keep the expression at a very low level.



	Sorting-Subcloning	G418 selection
No. of Silen. Clone	0	58
No. of Hete. Clone	3	6
No. of Homo. Clone	61	6
Total number	64	70

Fig. 18. Expression pattern comparison of clones isolated by "Sorting-Subcloning" and G418 selection.

HeLa cells were transfected with the linearized pEF-EGFP^{WT} plasmid. Stable clones were isolated by either "Sorting-Subcloning" or G418 selection approach. Clones with different EGFP expression states were observed by fluorescent microscopy and recorded. 61 out of 64 clones obtained by the "Sorting-Subcloning" approach had homogeneous EGFP expression, whereas only 6 out of 70 clones selected by G418 were EGFP homogenous.

In order to distinguish between these two possible factors underlying the sustained EGFP transgene expression, I used the “Sorting-Subcloning” approach and traditional antibiotic selection protocol in parallel to generate stable and EGFP homogeneous clones after transfection with the linearized EGFP expression plasmid pEF-EGFP^{WT} in HeLa cells. The pCMV-EGFP^{WT} expression vector was not suitable for this experiment because no EGFP positive clones were able to be obtained with G418 selection. HeLa cells were transfected with the linearized pEF-EGFP^{WT} plasmid by lipofection, and then split into two portions. One portion was subject to “Sorting-Subcloning” to obtain homogeneous EGFP clones; the other portion underwent G418 selection for 2 weeks to obtain stable EGFP homogeneous clones. The number of clones and their expression states for all the clones formed from both approaches were recorded. As obviously shown in Fig. 18, much more homogeneous EGFP clones were isolated by the “Sorting-Subcloning” approach than by the G418 selection protocol under identical transfection conditions. 61 out of 64 clones obtained by the “Sorting-Subcloning” approach had homogeneous EGFP expression, whereas only 6 homogeneous EGFP clones were isolated out of the 70 foci formed on the petri dish after G418 selection.

Stability of the EGFP^{WT} transgene expression was analyzed with 2 clones isolated by each approach. Cells were cultured in medium without G418 selection over time. FACS analysis was done to detect the EGFP expression every two weeks. Overlaid FACS profiles revealed stable and homogeneous EGFP expression throughout the whole time course for those clones derived from the “Sorting-Subconing” approach (Fig. 19, panel A). The single EGFP positive peak on the FACS profiles stayed unchanged till the end of the time course, a total of 84 days. For clones isolated with G418 selection, EGFP expression stayed stable for about 2 months. However, in the later stage of the time course a small amount of cells became silenced within the cell populations (Fig. 19, panel B). Unfortunately, I could not follow these four clones for a longer period of time due to culture contaminations, but due to experimental experience I suspect that the clones would soon stop expressing EGFP entirely after a small EGFP negative peak appeared on the FACS profiles. As a result, clones derived by bypassing antibiotic selection could maintain sustained and homogenous EGFP expression

over a long period of time, while clones selected by antibiotics would likely end up with suppression over time.

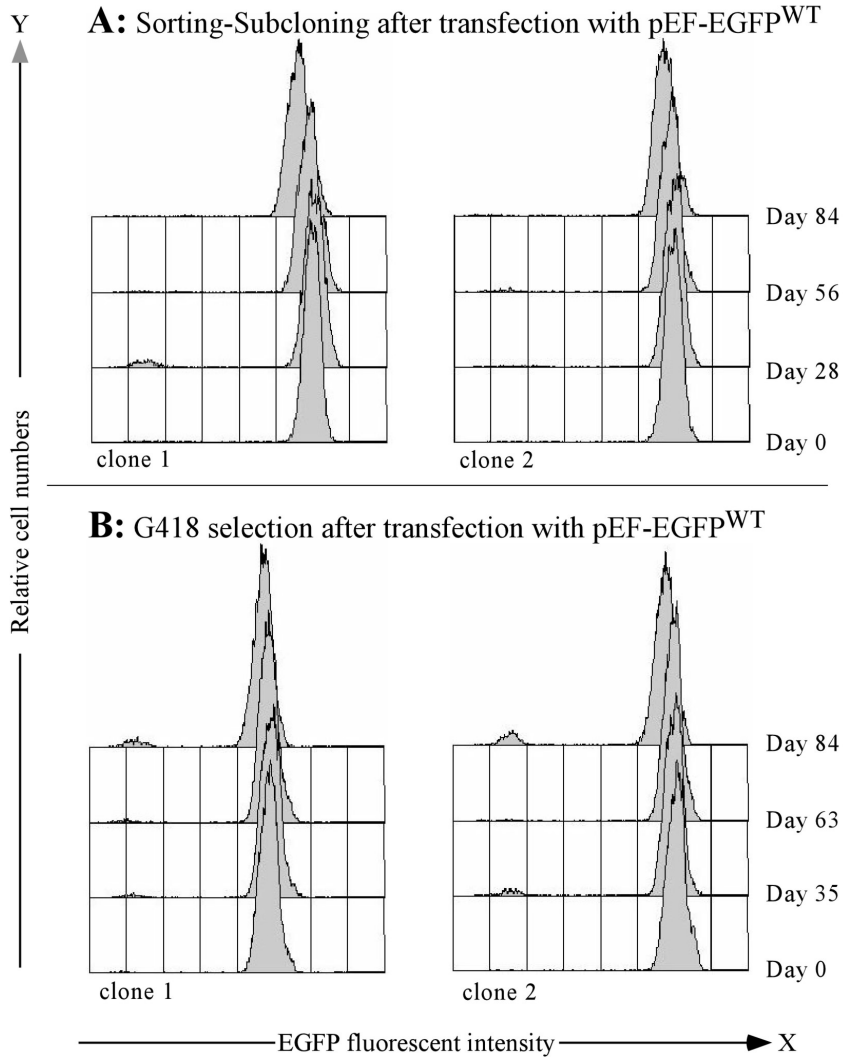


Fig. 19. Expression stability analysis of clones isolated by either the "Sorting-Subcloning" approach or G418 selection.

HeLa cells were transfected with the linearized pEF-EGFP^{WT}, and stable clones were isolated by either the "Sorting-Subcloning" approach or G418 selection side by side. Two clones each from both approaches were followed over time without G418 selection, and EGFP expression was measured by FACS at an interval of 2 weeks. FACS profiles at different time points were overlaid to compare the EGFP expression state over time. Panel A: 2 clones obtained by the "Sorting-Subcloning" approach showed very stable EGFP expression throughout the time course of 84 days. Panel B: 2 clones isolated from G418 selection first stayed EGFP active for the early 2 months or so, but a small amount of cells became silenced in the populations by the end of the time course.