Integration site-specific transcriptional reporter gene analysis using Flp recombinase targeted cell lines

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While high-throughput genome-wide approaches are useful to identify important regulatory regions, traditional reporter gene methodologies still represent the ultimate steps in fine structure analysis of transcriptional control elements. However, there are still several inherent limitations in the currently available transient and stable transfection systems often leading to aberrant function of specific cis *elements. In this study we overcome these problems and have developed a novel and widely applicable system that permits the comparison of transcriptional reporter gene activities following site-specific genomic integration. By using Flp recombinase-mediated integration, the system allows the integration and expression of a series of reporter gene constructs at exactly the same genomic location and orientation in all cells of any one culture. The resulting reporter gene lines carry a single reporter gene, which is incorporated within a measurably active chromatinized setting, thus more closely reflecting the endogenous gene environment.*

INTRODUCTION

The mechanisms controlling gene transcription are complex, and understanding these processes requires the identification and characterization of gene regulatory regions. While recent advances in high-throughput genome-wide approaches to identifying gene regulatory elements are informative (1,2), reporter gene methodologies still represent the end game of any gene-specific analysis of transcriptional regulation (3). As such, reporter gene analysis has provided remarkable insights into the mechanisms governing gene expression. However, there are still several inherent limitations in the currently available systems whose ultimate objective is mimicking the expression pattern of the endogenous gene and testing the effects of mutations or changes in *cis* elements in the promoter of an introduced reporter gene.

The most common functional assay used to map *cis*-acting regulatory regions is the transient transfection assay in which a reporter is expressed under the control of a regulatory region of the gene to be analyzed. In this assay, hundreds or even thousands of copies of the reporter gene construct can enter the cell (3). Since most transcription factors are present at low concentration, only a fraction of the constructs entering the cell receive the full complement of proteins needed for the proper function of the control region (3), often leading to aberrant function of specific control elements (4,5).

Many gene regulatory regions have been shown to function inappropriately in transient transfection assays. In addition to the concentration effects outlined above, aberrant activity may also be due to the lack of an appropriate chromatin conformation (5). More specifically, many long-range enhancer and silencer elements do not function unless integrated within the genome using either stable transfection assays or transgenic mouse models (6–8). As chromatin context is likely to effect how transcription factors interact with their cognate *cis* elements (9,10), the transient transfection assay, while a reasonable indicator of important regulatory regions, is not a completely appropriate model to assess the full influence of regulatory regions on the expression of a particular gene.

The use of stable transfection assays, where a reporter gene expression cassette is integrated into the genome of a cell lines, has extended our knowledge of the function of context-critical *cis*-acting elements (3). However, stable transfections have additional problems such as integration site concerns as well as an inability to directly compare between wild-type and mutant reporters due to the random nature of the integration.

In this study, we overcome these problems and have developed a novel and widely applicable system, which permits the comparison of transcriptional reporter gene activities following site-specific genomic integration. By using site-specific Flp recombinase-

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mediated integration (11), the system allows the integration and expression of a series of reporter gene constructs at exactly the same genomic location and orientation in all cells of any one culture. The resulting reporter gene lines, which are isogenic and of single-gene copy, are incorporated within a measurably active chromatinized setting, thus more closely reflecting the endogenous gene environment.

MATERIALS AND METHODS

Cell Lines

Cell lines Flp-In™ -Jurkat T (Invitrogen, Carlsbad, CA, USA) and K562 (ATTC, Manassas, VA, USA) and its Flp recombinase target (FRT) derivatives, were cultured in RPMI-1640 (Trace Biosciences, Castle Hill, NSW, Australia) supplemented with 100 µg/mL each of penicillin and streptomycin (Trace Biosciences) and 10% fetal bovine serum (FBS; Thermo Trace Ltd, Noble Park, VIC, Australia) at 37°C in 5% CO_2. FRT-K562 cells were generated by transfection with the FRT vector, pFRTLacZeo, exactly as described by the manufacturer (Invitrogen). Single site integration of the FRT sequence was checked by Southern hybridization using a probe obtained by use of the PCR primers SV40-F and LacZeo-R (see below) and pFRTLacZeo plasmid DNA (Figure 1).

Construction of Promoter-Green Fluorescent Protein Reporter Plasmids

A 1110-bp *Kpn*I and blunted *Hin*dIII fragment containing -993 to +110 of the tumor necrosis factor (TNF) promoter (12) was cloned into the *Kpn*I and blunted *Bam*HI multiple cloning sites (MCS) of the pd2EGFP-N1 (Clontech Laboratories, Mountain View, CA, USA) expression vector. The TNF promoter-green fluorescent protein (GFP) expression cassette was then excised using *Bgl*II, which occurred just upstream of the *Kpn*I site in the MCS of pd2EGFP-N1, and *Not*I, which occurred downstream of the GFP sequence of pd2EGFP-N1, and subcloned into *Kpn*1 and *Not*1 sites in the MCS of the pcDNA5/FRT expression vector (Invitrogen), which had 967 bp of the cytomegalovirus (CMV) promoter and regulatory sequences removed.

A *Nhe*I/*Xho*I fragment of the CR2 promoter containing -1250/+75 of the CR2 promoter (13) was cloned into a pGL3-basic vector (Promega, Madison, WI, USA) re-engineered to express GFP (derived from the pdEGFP-N1 vector) as the reporter gene. The CR2-GFP expression cassette was excised using *Kpn*I and *Sal*I and subcloned into the pcDNA5/FRT (Invitrogen) derivative vector, from which the CMV promoter had been removed.

Stable Transfection and Analysis of Reporter Gene Expression

K562 cells were transfected by electroporation (270 V and 960 µF capacitance), while Jurkat cells were transfected using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's directions. In both instances, 1×10^7 cells were cotransfected with 10 µg FRT/GFP promoter plasmid and 90 µg Flp recombinase-expressing plasmid, pOG44 (Invitrogen) (11). Following transfection, cells were allowed to recover for 24–48 h before propagation in 500 µg/mL hygromycin B. Cell populations were then analyzed 2–4 weeks after selection in hygromycin. Prior to flow cytometric analysis, cells were harvested and washed twice in 1× phosphate-buffered saline (PBS) before resuspension of the pellet in flow cytometric analysis buffer (PBS supplemented with 5% FBS and 0.2% sodium azide). Cells were analyzed using a EPICS XL™ flow cytometer (Beckman Coulter, Fullerton, CA, USA), and GFP fluorescence was detected at a wavelength of 515 nm on the FL3 channel of the instrument. Data analysis was performed using FlowJo software (TreeStar, Ashland, OR, USA). Percent GFP expression was determined by gating on the viable cell population using a forward versus side scatter plot, then analyzing GFP fluorescence of viable cells on a histogram. Mean fluorescence (MF) intensity was calculated based on the total cell population.

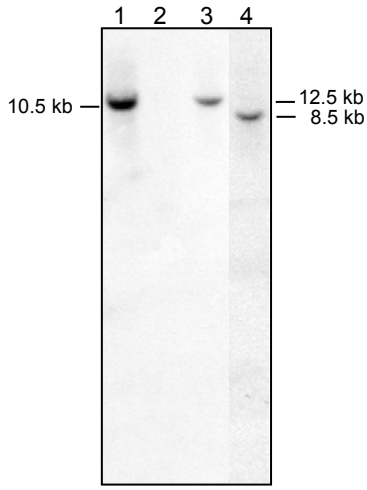

Figure 1. Analysis of Flp recombinase target (FRT) sequence copy number in K562 and Jurkat derivative cell lines. Genomic DNAs from three zeocin-resistant FRT-K562 clones and the commercially available Flp-In-Jurkat cell line were digested with *Hin*dIII and subjected to Southern analysis. The digests were hybridized with a probe that represented the simian virus 40 (SV40) promoter -LacZeo interval of the pFRT-LacZeo integration vector. Lanes 1–3, DNA from three zeocin-resistant K562 clones that had been stably transfected with pFRTLacZeo; lane 4, DNA from the Flp-In-Jurkat cell line. The lines shown in lanes 1, 3, and 4 contained integrated sequences that exceeded the total size of pFRT-LacZeo (8.2 kb) and so had integrated the entire sequence.

DNA Extraction and PCR

DNA from 2.5×10^6 cells, 2–3 weeks postselection, was extracted using the QIAamp® DNA blood mini kit (Qiagen, Valencia, CA, USA) as per the manufacturer's directions. Eluted DNA (1 µL) was used directly in a PCR (2 mM dNTP, 1× PCR buffer, 50 mM $MgCl_2$, 10 µM each oligonucleotide, and 2.5 U *Taq* DNA polymerase). The PCR was performed using the following oligonucleotides from Sigma Genosys (Castle Hill, NSW, Australia): GFP-F 5′-GCGCGATCACATGGTC CTGCTGGAGTT-3′; SV40-F 5′-GC ATGCATCTCAATTAGTCAGCAAC CA-3′ and LacZ-R 5′-GCATGCATC TCAATTAGTCAGCAACCA-3′. The PCRs proceeded at 95°C for 4 min for 1 cycle, then 95°C for 30 s, 61°C for 30 s, 72°C for 45 s, for 32 cycles, followed by a final extension of 72°C for 7 min. PCR products were analyzed on a 1% agarose gel.

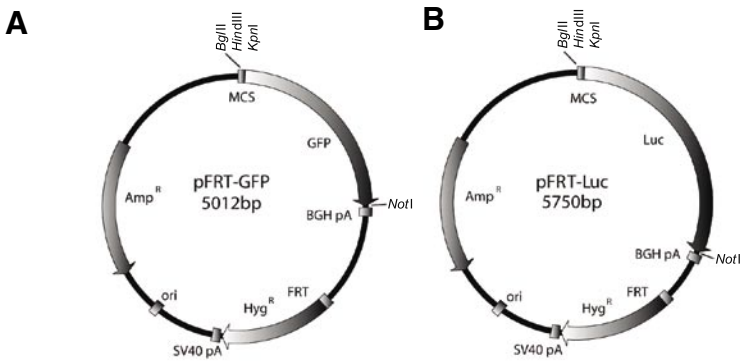

Figure 2. Physical maps of transcriptional reporter vectors pFRT-GFP and pFRT-Luc. The reporter plasmids (A) pFRT-GFP and (B) pFRT-Luc, including the multiple cloning site (MCS) that contains unique restriction sites for *Bgl*II, *Hin*dIII, and *Kpn*I are shown. Also shown are the Flp recombinase target (FRT) sequence, the bovine growth hormone, and simian virus 40 (SV40) poly(A) sites, the hygromycin and ampicillin drug resistance genes derived from the pcDNA5/FRT plasmid. The positions of the *Not*I and *Bgl*II sites, which define the junctions between pcDNA5/FRT sequences and (A) pd2EGFP or (B) pGL3-basic sequences are shown. GFP, green fluorescent protein.

RESULTS

Generation of Two Promoterless FRT/Reporter Gene Vectors Allows Integration of Various Regulatory Sequences

The transcriptional reporter system was based on the Flp-In expression system (Invitrogen). The pcDNA5/FRT vector containing the FRT sequence (Flp-In), was engineered to contain suitable transcriptional reporter genes. In order to adapt the FRT system as a site-specific stably integrating vector, promoter/reporter gene cassettes were prepared and transferred into pcDNA5/FRT from which the CMV promoter and enhancer sequences were removed. Specifically, promoters of interest introduced into the reporter vectors pd2EGFP-N1 or pGL3Basic were excised, along with the destabilized GFP or luciferase reporter sequences, and recloned into the pcDNA5/FRT vector from which the CMV promoter had been removed. To simplify this process, we also generated two new vectors (Figure 2) suitable for direct cloning of promoter fragments into the MCS. These vectors have unique restriction enzyme sites for *Bgl*II, *Hin*dIII, and *Kpn*I in the MCS suitable for the introduction of any promoter fragment or regulatory region and either the GFP (Figure 2A, pFRT-GFP) or luciferase genes (Figure 2B, pFRT-Luc).

Validation of the Flp-FRT Integrated Reporter System

To test the FRT system, a reporter vector was generated that comprised TNF promoter sequences driving GFP expression. Following transfection of the Jurkat T cell line, which contained a single FRT genomic sequence, with the pFRT-TNF/GFP construct together with the Flp recombinase expression vector pOG44, expression of the Flp recombinase resulted in site-specific integration of pFRT-TNF/GFP (Figure 3A). Integration resulted in the hygromcyin B resistance gene being in-frame with the simian virus 40 (SV40) promoter and ATG initiation codon of the LacZ-Zeocin fusion gene, enabling expression of hygromycin resistance, while simultaneously inactivating LacZ-Zeocin expression (see the Flp-In system manual). The recombinant pFRT-TNF/GFP cell line was selected by hygromycin treatment. Approximately 48 h following transfection, the cells displayed GFP fluorescence, indicating transient expression of the reporter. Upon addition of hygromycin B 2–5 days after transfection, transient GFP expression diminished leaving only a few stably expressing cells (0.01%). Following continual seeding in media supplemented with hygromycin for 2–3 weeks, visible aggregations of GFP-expressing cells was observed (Figure 3B), indicating that Flp recombination and integration had occurred. Upon outgrowth, the resulting cell line was checked for zeocin sensitivity and lack of β-galactosidase expression, indicating that

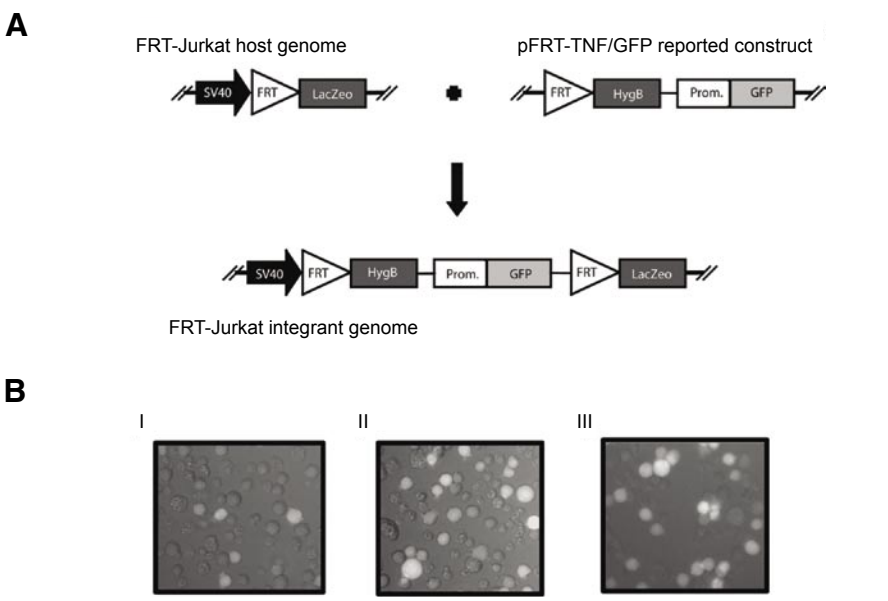

Figure 3. Derivation of Flp-FRT transcriptional reporter lines. (A) Genomic arrangement of the host FRT-Jurkat line and FRT-Jurkat integrant line containing the tumor necrosis factor (TNF) promoter-FRT reporter. (B) FRT-Jurkat cells were transfected with pFRT-TNF/green fluorescent protein (GFP) and grown for 3 days (I). Following 3 days of treatment with hygromycin (II) an increase in GFP-positive cells were seen (0.01%). After 2 weeks of hygromycin selections, all cells were GFP positive (III). Cells were observed at 20× magnification using fluorescence microscopy. FRT, Flp recombinase target; SV40, simian virus 40.

integration of the reporter construct was FRT site-specific and that second site integrations, which did not involve the FRT site, were not present. The presence of nonhomologous integrations are confirmed by re-expression of Flp recombinase (via retransfection with pOG44), which served to excise the FRT-GFP reporter at the FRT site. If such second site integrations are present, the cells will exhibit zeocin resistance, expression of β-galactosidase, but continue to express the GFP reporter gene.

As a further test to determine whether the TNF/GFP reporter cassette had integrated specifically at the FRT site, PCR was used to detect Flp recombinase-mediated integrants. Two 5′ PCR primers were designed within the SV40 (Figure 4A, SV40-F) and GFP (Figure 4A, GFP-F) sequences, together with a 3′ primer that annealed within the LacZ gene (Figure 4A, LacZ-R). DNA from both the Jurkat FRT host cell line and the TNF/GFP integrant were amplified with both combinations of primers (Figure 4B). PCR using DNA from the Jurkat FRT host cell line resulted in the presence of a 510-bp fragment corresponding to the SV40-LacZ fragment of DNA (Figure 4B, lane 1), whereas no detectable PCR product was observed with the primers that amplify the GFP-LacZ interval (Figure 4B, lane 2). In contrast, PCR using DNA from the putative TNF/GFP integrant, resulted in the presence of a 1020-bp fragment when primers GFP-F and LacZ-R were used (Figure 4B, lane 4), corresponding to the sequence between GFP of the reporter construct and the LacZeo sequence of the parental genomic DNA. This result confirmed the successful integration of the TNF-GFP cassette into the host cell in the correct orientation. Furthermore, the SV40-LacZ combination of primers failed to amplify the 510-bp fragment (Figure 4B, lane 3), demonstrating the absence of cells in which the reporter construct was not present at the FRT site.

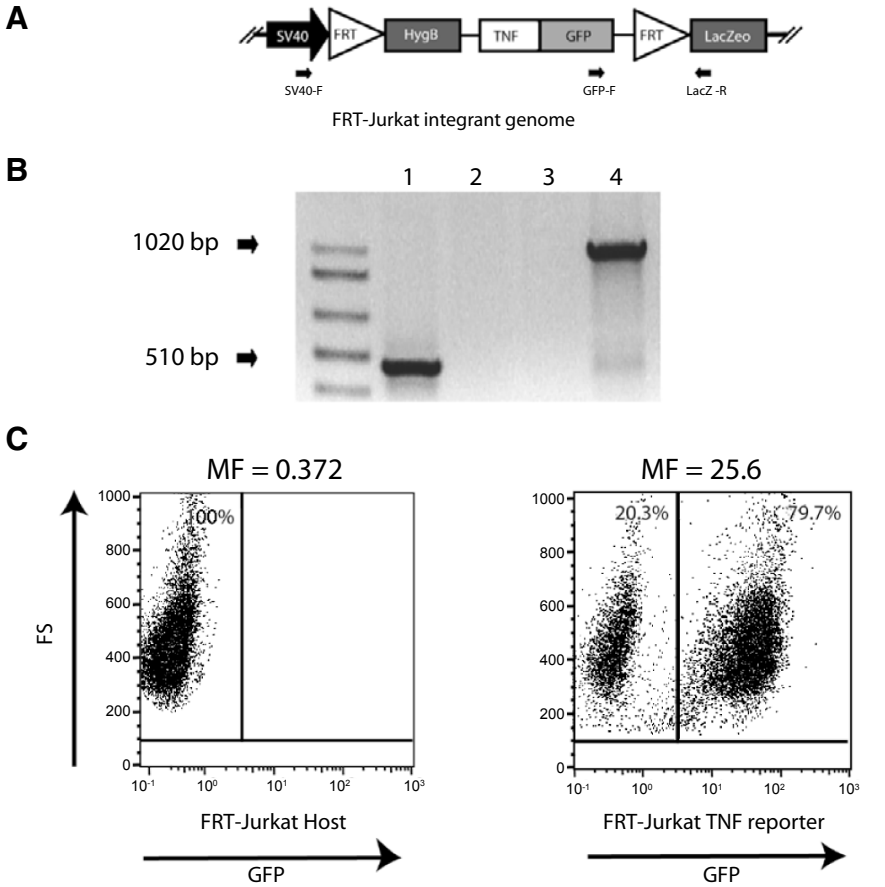

Figure 4. Validation of the Flp-FRT integrated reporter system. (A) Genome organization of the FRT-Jurkat tumor necrosis factor (TNF) reporter integrant line showing the positions of the 5′ simian virus 40 (SV40) and green fluorescent protein (GFP) PCR primers and the common 3′ LacZ primer used to check integration. (B) Flow cytometric analysis of the TNF promoter/GFP reporter indicated that 79.7% of cells were expressing high levels of GFP compared with the host FRT-Jurkat line (0%). (C) PCR analysis using the primers shown in panel A indicated that integration of the TNF/GFP reporter construct had occurred at the Flp recombinase target (FRT) site. Lanes 1 and 2 represent PCR products from parental genomic DNA using primers specific for SV40 and GFP, respectively. Lanes 3 and 4 represent the PCR products from the TNF-FRT integrant line using the same primers, respectively. The distance between the SV40 and LacZ sequences was predicted to be 510 bp, and the distance between the GFP and LacZ sequences was predicted to be 1020 bp. MF, mean fluorescence; FS, forward scatter.

Primarily due to concerns regarding the presence of bacterial sequences in the integrated reporters, the stability of FRT-Jurkat TNF/GFP reporter gene expression was assessed over a 24-day period following recovery from cryopreservation (Table 1). The results show that high level GFP expression was maintained during extended growth. Up to 9 days of growth, there was no significant change in either the MF or the proportion of cells that expressed the reporter. However after 24 days growth, there was an increase in the proportion of cells that expressed GFP. These changes may be due to extended cell culture per se or to specific changes in the methylation pattern across the inserted reporter region.

Table 1. Stability of Reporter Gene Expression

Days in Culture	GFP Status	Mean Fluorescence
5	GFP- 39.8%	0.35
	GFP+ 60.2%	48.2
9	GFP- 35.2%	0.37
	GFP+ 64.8%	43.8
24	GFP- 17.2%	0.36
	GFP+ 82.8%	42.8

GFP, green fluorescent protein.

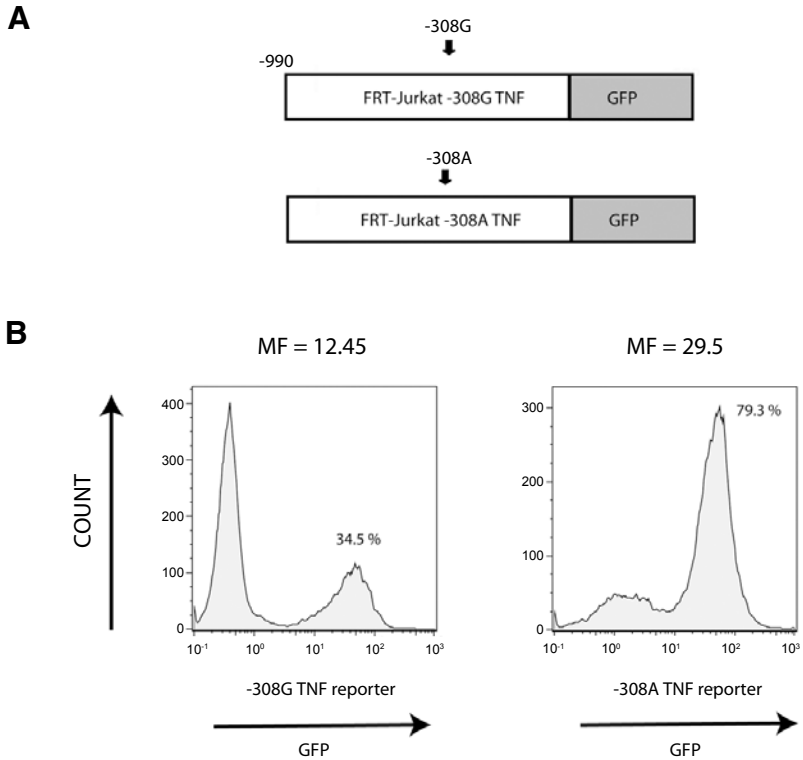

Figure 5. Comparative analysis of polymorphic -308 tumor necrosis factor (TNF) promoter sequences. (A) Representation of both the -308G TNF/green fluorescent protein (GFP) and -308A TNF allelic promoter-GFP reporters. (B) Both reporters were integrated into the Flp recombinase target (FRT) site of the FRT-Jurkat line, and GFP expression was assessed by flow cytometry. The -308G reporter induces 34.5% of cells to express GFP, whereas the -308A reporter induces 79.3% of cells to express GFP. MF, mean fluorescence.

Applications of the Flp-FRT System

FRT Jurkat cells expressing TNF promoter/GFP reporter allows quantitative analysis of transcriptional activity at a single cell level. To evaluate the utility of the FRT integrated reporter system for quantitative promoter analysis, the TNF/GFP integrant was analyzed for GFP expression by flow cytometry. The TNF/GFP-containing cell line showed approximately 80% positivity for GFP (Figure 4C, FRT-TNF/GFP), whereas the parent FRT-Jurkat cell line showed no detectable GFP expression (Figure 4C, Jurkat FRT). The results show that the FRT reporter gene system provides a powerful way to assay transcriptional activity on an individual cell basis. The fact that only a percentage of the total number of cells express at any one time is the result of the stochastic nature of transcriptional enhancers, which serve to increase the probability of transcription initiation rather than increasing the level of expression (14,15). This is a little-studied transcriptional phenomenon due to the lack of easily manipulable in vitro systems, but the use of the Flp-FRT reporter system, as described here, makes such studies much simpler and easier to perform. Also, the use of flow cytometry to measure transcriptional activity not only allows the quantification of the proportion of transcriptionally active cells, but also enables the determination of the MF of the GFP positive cells as a quantitative measure of transcriptional activity (16,17). For instance, the TNF/GFP-containing cell line showed a MF of 25.6 for green fluorescence (Figure 4C, FRT-TNF/GFP), which is a 71.2-fold increase in activity over the parent FRT-Jurkat cell line (Figure 4C, MF = 0.372).

The Flp-FRT reporter system allows the study of mutant or polymorphic forms of a promoter/regulatory region. Most gene promoter analyses using reporter gene constructs rely on the comparison of activities of different truncated or mutated forms of the promoter sequence following transient transfection.

To test our system, we chose to compare constructs of the TNF promoter that differed by a single nucleotide (Figure 5). The G/A polymorphism at -308 in the TNF promoter is associated with elevated TNF levels. We have shown, in conventional transient transfection assays using a luciferase reporter system, a 2.5-fold difference in transcriptional activity between the two alleles (12). The two TNF-GFP reporters were integrated into the FRT-Jurkat cell line, and the hygromycin-resistant populations were analyzed by flow cytometry. The line carrying the -308G construct showed a MF 12.45, and 34.5% of cells expressed GFP (Figure 4B, -308GTNF). In contrast, the line carrying the -308A variant showed a >2-fold increase in both MF (29.5) and in the number of GFP expressing cells (79.3%) compared with -308G (Figure 5B, -308ATNF). Quantitative real-time PCR using primers specific for the TNF/GFP reporter demonstrated that each cell line had 1 genome equivalent of the respective construct, and so the differences in GFP expression were not due to loss of the TNF/GFP expression construct (data not shown).

The Flp-FRT reporter system allows the study of chromatin context-dependant regulatory regions. We have previously shown that the cell type-specific and developmentally restricted pattern of human complement receptor 2 (CR2/CD21) expression is controlled by proximal promoter sequences together with an intronic transcriptional silencer (designated CRS for CR2 silencer) (7). Using reporter constructs containing the CR2 proximal promoter with and without the CRS, we have shown that the CRS represses transcription in CR2 nonexpressing cells in stable transfection experiments. This repression is not observed using transient transfection assays, but strong repression is seen when reporter constructs containing these elements are stably integrated into the genome.

To determine whether the Flp-FRT system would be useful in the study of such context-dependant regulatory elements that apparently require genomic integration, we have generated two CR2-FRT constructs. The first construct contained only the CR2 promoter driving GFP expression (Figure 6A, -1250GFP), while the second FRT construct contained the CR2 promoter, the GFP reporter gene and the CRS (Figure 6A, -1250GFPCRS). The constructs shown in Figure 6A were transfected together with the Flp recombinase expression vector into K562 cells (a CR2 nonexpressing cell line) into which had previously been integrated a single FRT sequence (Figure 1, lane 1). Hygromycin-resistant populations that arose following the transfections were analyzed by flow cytometry. In comparison to the K562-FRT host cell line (Figure 6B, K562FRT), which contained only the FRT sequence (0.01% GFP+ cells, MF = 4), 91.5% of K562 cells (MF = 82), which had integrated the -1250 GFP construct, expressed GFP (Figure 6B, -1250GFP). In contrast, only 8% of cells (MF = 12) were GFP positive in K562-FRT cells that had integrated the construct containing the silencer (Figure 6B, -1250GFPCRS). When levels of GFP expression were evaluated, the -1250 GFP-containing cells expressed the GFP reporter gene, while cells transfected with the -1250 GFPCRS construct (containing the silencer) expressed extremely low levels of GFP (Figure 6C). These results indicate that the Flp-FRT transcriptional reporter system is extremely useful for the study of long range regulatory regions that are sensitive to chromatin or chromosomal context for appropriate function.

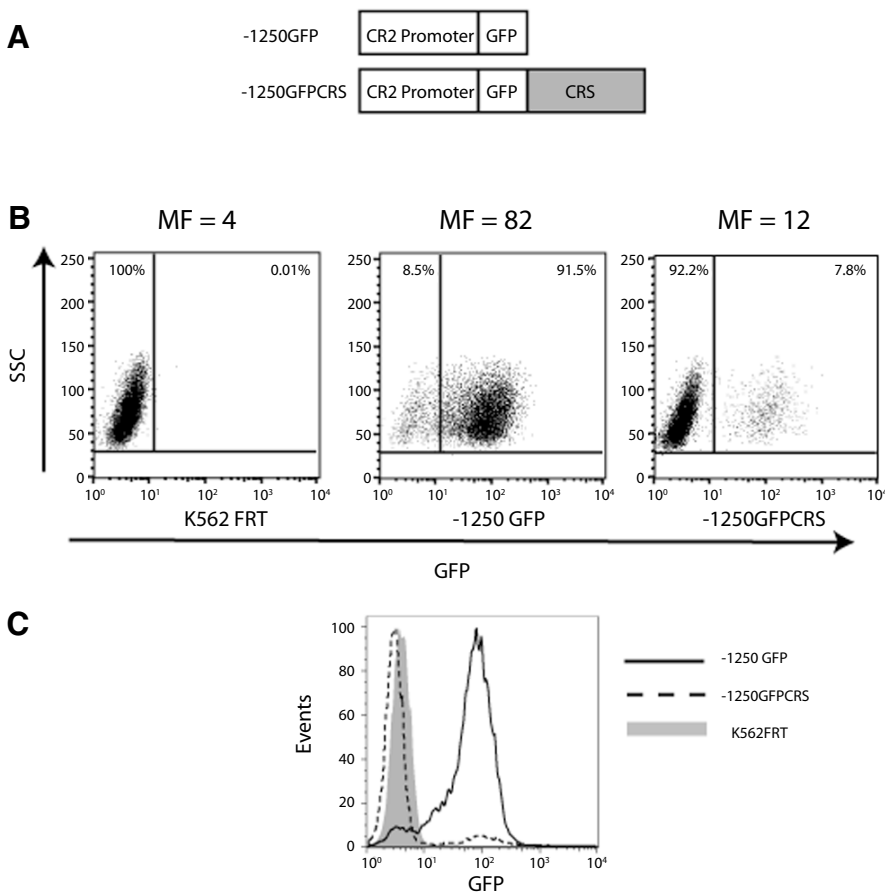

Figure 6. Effect of complement receptor 2 (CR2) silencer on the CR2 promoter activity in CR2 nonexpressing cells. (A) Representation of the CR2 promoter driving the green fluorescent protein (GFP) reporter (-1250GFP) and the reporter containing the CR2 silencer (CRS) downstream of the GFP sequence (-1250GFPCRS). (B) Flow cytometric analysis showing a marked decrease in GFP expression when the silencer was present (7.8% GFP positive cells) compared with the parent cell line -1250GFP (91.5% GFP positive cells). (C) The silencing effect is highlighted in an overlay of the GFP expression profiles of the three cell lines. FRT, Flp recombinase target; MF, mean fluorescence; SSC, side scatter.

DISCUSSION

Studies aimed at identifying transcriptional response elements that influence gene expression are typically carried out using transient reporter gene assays (3). Variability in transient transfection assays can result from many sources, including the purity and amount of reporter DNA introduced into the cells, squelching due to cotransfection with a transfection control plasmid, and changes in culture conditions of the host cells (3). Also, the lack of appropriate chromatin interactions can influence promoter accessibility and potentially result in important *cis*-acting regulatory elements being overlooked. In this report we have successfully established a novel system to allow the study of gene regulatory regions in a chromatinized setting that more closely mimics the endogenous gene environment. Although a number of systems have been developed to site-specifically integrate transgenes into a single genomic location (18,19), none have been used as a tool to analyze the transcriptional elements that define the control regions of a gene. By adapting the Flp-FRT system, we can now efficiently integrate reporter gene constructs site-specifically into any cell line engineered to carry a single FRT sequence.

To ensure that this system is readily available for studying many different gene promoters and regulatory regions, two promoterless FRT/reporter gene vectors have been constructed. One vector carries a destabilized GFP as the reporter gene, allowing the quantitation of transcriptional activity on a per-cell basis or as MF intensity of the whole cell population. In some instances where interference from cellular autofluorescence is a concern or greater flexibility in assay design is required, the luciferase vector created can be utilized as an alternative, although we have not yet completely validated this reporter.

We have developed a novel system that can be readily used as a reporter gene assay. More specifically, this system showed detectable levels of

reporter gene expression from the stable integration of a single copy of the reporter gene expression cassette. This single-copy integration within the genome more closely mimics the endogenous gene arrangement when compared with traditional stable transfection methods (20). One of the greatest strengths of the system is the ability to directly compare activities of linker scanning mutants or single nucleotide polymorphisms (SNPs) with wild-type promoter sequences in the same chromatinized context when the activity differences are modest, without having to apply transfection efficiency normalizations. In transient reporter assays, such corrections are often larger than the activity differences being measured. For instance, in the TNF reporter experiments, we can reliably measure <2-fold differences in transcriptional activity. In addition, we have been able to quantitate changes in chromatin conformation following mutation using nuclease accessibility and chromatin immunoprecipitation (ChIP) assays (data not shown).

Another major advantage of the Flp-FRT reporter system is the ability to analyze regulatory regions that exert their effects via interactions with chromatin components. We have successfully shown that the intronic transcriptional silencer of the CR2 gene functions appropriately in our non-CR2-expressing cell line. Location-specific effects with randomly integrated stable reporters can often be a limitation when studying transcriptional silencers, as it can be difficult to determine whether silencing is due to specific integration of silencer regions or simply due to integration into regions of heterochromatin (21). This method simplifies the analysis of mutant constructs as all further constructs will be integrated into precisely the same genomic location as the parental plasmid. With traditional stables, analysis of wild-type and mutant constructs require extensive analysis of several polyclonal populations, analyzed on several occasions to obtain an indication of CR2 transcriptional activity in the bulk populations.

Although our FLP-FRT reporter system appears to be generally applicable to a wide range of studies, there are a number of potential limitations of the current system. The presence of bacterial sequences, which tend to be CpG-rich and hence susceptible to methylation in mammalian cells, may result in the reporter being silenced (22,23). However, our results indicate that during short-term growth of reporter lines, no significant silencing is apparent. This may be cell type-specific and would need to be assessed on a case-by-case basis. Another potential variable of the system is the chromatin environment surrounding the integration site that may influence expression from the reporter. Assessment of multiple cell lines, carrying FRT sequences in different genomic locations, may be necessary to obviate these potential differences. Also of potential concern is the presence of the SV40 minimal promoter upstream of the transcriptional reporter gene. However, as the SV40 sequences are approximately 3 kb upstream of the reporter gene and composed of multiple Sp1 binding sites (24), they are unlikely to have a profound effect on complex promoters, other than potentially having a general enhancing effect. This situation is similar to that found in transient reporter systems that carry the SV40 promoter.

In summary, the integration site-specific transcriptional reporter gene system we have developed is highly efficient, has a high degree of versatility, and has been proven useful in the study of different aspects of gene transcription. Due to the isogenic nature of the stable cell lines and the identical genomic location of the FRT integration site within each subline, direct comparisons can be made between polymorphic, mutated, or deleted regulatory sequences. The integration of a single-copy promoter of interest driving expression of a reporter gene, results in a valuable functional tool for the investigation of regulatory sequences influencing gene expression.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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