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In situ detection of transcriptionally active chromatin and genetic regulatory elements in individual viable mammalian cells

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SUMMARY

Using a newly developed FACS method for quantifying the expression of the *Escherischia coli lacZ* reporter gene in viable mammalian cells, we have obtained cloned cell lines in which the expression of *lacZ* is under the control of native endogenous transcription elements. We infected the murine pre-B cell 70Z/3 with transcriptionally disabled retroviruses containing *lacZ* and employed the FACS–FDG technique to detect and sort rare *lacZ*$^+$ cells in which we expect integration is near such endogenous transcription elements. After two rounds of enrichment we obtained a population of cells that was 80–90% positive for *lacZ* activity. Clones derived from the *lacZ*$^+$ pool differ from each other with respect to their overall level of *lacZ* activity as well as in the pattern of *lacZ* expression among cells within an individual clone. Treatment of these *lacZ*$^+$ 70Z/3 clones with lipopolysacharide (LPS; which is known to stimulate differentiation of 70Z/3 from a pre-B cell to an IgM-expressing B cell) greatly decreased *lacZ* expression in one clone, 7e17. *lacZ* expression in this clone was 50–100 times lower within 24 hr of LPS addition and coincided with the acquisition of IgMκ on the surface of 7e17. This suggests that a transcriptionally active domain of chromatin that harbors the *lacZ* construct is down-regulated during the transition induced by LPS stimulation.

INTRODUCTION

Previously we have developed a method which allows fluorescence-activated cell analysis and sorting of viable mammalian cells based on their expression of recombinant *E. coli lacZ* (Nolan, Fiering & Herzenberg, 1988). This method, termed FACS–FDG, provides individual cell quantification of enzymatic activity in a heterogeneous cell population in concert with any of the standard fluorescence-activated cell sorter (FACS) features (analysis, sorting, cloning, etc.) The method relies on the rapid hypotonic loading of cells at 37° with the non-fluorescent beta-galactosidase substrate fluorescein-di-galactoside (FDG). FDG loading is terminated by returning the cells to isotonicity by dilution into 10 or 20 volumes of ice-cold medium. Cells expressing *lacZ* cleave FDG to produce free fluorescein, which is retained in viable cells and is detectable by FACS. We have shown that the amount of fluorescein produced within each cell is a function of time and the enzymatic activity present in that cell (Nolan *et al.*, 1988; our unpublished results).

Infection of BW5147 T cells with a retrovirus in which *lacZ* is driven by an internal promoter (SV40) yielded positive clones with different average levels of β-galactosidase activity (Nolan *et al.*, 1988; our unpublished results). These data, and that of others (O'Kane & Gehring, 1987; Allen *et al.*, 1988), suggest that sites of integration could have a profound effect on gene expression, presumably due to the proximity of nearby regulatory elements.

To examine *lacZ* expression that is under the control of endogenous regulatory elements, we inserted *lacZ* into two different transcriptionally disabled self-inactivating (SIN) retroviruses which contain deletions in the 3′ U3 region of the LTR. As a consequence of retroviral biology, infection of target cells with these constructs leads to transfer of the 3′ LTR U3 deletions to the 5′ LTR U3 region, thus inactivating transcription from the integrated virus. We made *lacZ*-encoding retroviral constructs in which: (i) the enhancer elements of the Moloney LTR were deleted leaving the CAAT and TATA boxes intact for endogenous enhancer searching (pEnhsr1); or (ii) all LTR promoter and enhancer elements were deleted (CAAT and TATA boxes as well as 72 bp repeats deleted) for endogenous promoter searching (pProsr1).

After infection of 70Z/3 cells with these recombinant retroviruses we have been able to enrich for rare β-galactosidase-expressing (*lacZ*$^+$) target cells using the FACS–FDG technique. Clones which express *lacZ* were derived from the sorted *lacZ*$^+$ cells by FACS cloning after FDG staining. Different but reproducible patterns of *lacZ* expression were found among the clones. Expression patterns of a panel of clones were analysed for responsiveness to LPS. One clone, 7e17, is exquisitely responsive to LPS, while expression of *lacZ* in other clones remains stable or is only partially affected.

Abbreviations: FACS, fluorescence-activated cell sorter; FDG, fluorescein-di-galacoside; LPS, lipopolysaccharide; LTR, long terminal repeat; PETG, phenyl-ethel-thio-galactoside; SIN, self-inactivating.

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MATERIALS AND METHODS

Construction of pProsr1 and pEnhsr1

The *lacZ*-containing plasmid, pON405 (a kind gift of Dr Bill Manning and Ed Mocarski, Stanford University) was digested with DraI, and a 3·5 kb fragment containing *lacZ* was isolated. BamHI linkers were ligated onto the 3·5 kb DraI fragment and the ligation digested with BamHI and SalI. The digestion was separated on a 1% low melting agarose gel and the approximately 3·2 kb Bam-Sal fragment isolated. This *lacZ* containing Bam-Sal fragment was ligated to a purified 6·5 kb XhoI-BamHI fragment generated by digestion of either plasmid, pJrEnh$^-$ or pJrPro$^-$ (a kind gift of Heidi Stuehlmann, Stanford University and Brad Guild, MIT). After transformation into JM109 on X-gal-containing LB plates, blue colonies were selected for further analysis to confirm the correct orientation of *lacZ*.

Transfection of pProsr1 and pEnhsr1 into ψ-2 cells and infection of 70Z13

The plasmids pProsr1 (10 μg) and pEnhsr1 (10 μg) were separately co-transfected with 10 μg of pSV2-neo into 3×10^6 ψ-2 cells by electroporation in the presence of 0·5 μg of carrier DNA (calf thymus). Cells were plated after electroporation into DMEM with 5% calf serum, 5% horse serum, glutamine and penicillin/streptomycin, and after 24 hr G418 was added to a concentration of 1000 μg/ml. After selection in G418 for 2 weeks, the surviving ψ-2 cells were loaded with FDG as below and enzymatic cleavage of FDG halted after 5 min with 1 mM final concentration of the competitive inhibitor phenyl-ethyl-thio-galactoside (PETG) (G. Nolan, S. Fiering and L. Herzenberg, unpublished data). *lacZ*$^+$ cells were then sorted and expanded in culture. $0{\cdot}5 \times 10^6$ 70Z/3 cells were added to a confluent monolayer of the sorted, *lacZ*$^+$, ψ-2 cells in a 100 × 15 mm LUX dish in the presence of Polybrene (2 μg/ml). After 16 hr the non-adherent 70Z/3 cells were removed, replated and removed three times over a period of 48 hr to remove contaminating (adherent) ψ-2 cells.

Growth of 70Z/3 cells and LPS inductions

70Z/3 cells were grown in RPMI with glutamine, 5% fetal calf serum, 5% horse serum, penicillin/streptomycin and 50 μM 2-mercaptoethanol. LPS at a concentration of 10 μg/ml (Difco, *S. typhosa* 0901) was used to induce 70Z/3 cells (Paige, Kincade & Ralph, 1978).

FDG and immunofluorescent staining of cells

FDG staining was carried out as described by Nolan *et al.* (1988). For double-staining of cells with FDG and anti-κ, cells were first stained by FACS–FDG, pelleted at 4° and resuspended in 100 μl of cold staining media containing 10 μg/ml of biotinylated monoclonal anti-Igκ. After incubation on ice for 15 min, the cells were washed three times with 2 ml ice-cold staining media and pelleted at 4°. After the third pelleting, cells were resuspended in 100 μl of Texas Red (Molecular Probes, Junction City, OR)-complexed Avidin (Vector Laboratories, Burlingame, CA) and incubated on ice for 15 min. Cells were washed three times as before and resuspended in 100 μl of staining media containing 1 μg/ml propidium iodide.

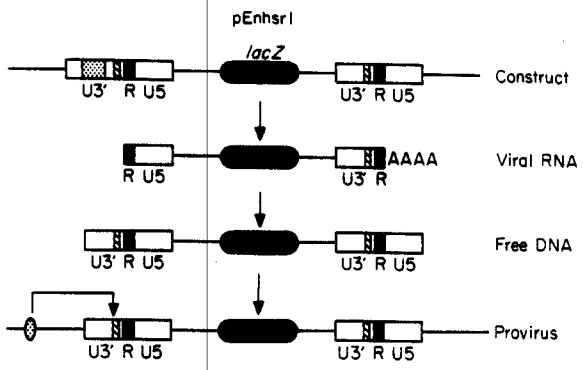

Figure 1. Schematic depiction of pEnhsr1 and model for *lacZ* expression. This figure depicts how a *lacZ*-encoding provirus which lacks the Moloney leukemia virus enhancer region (stippled box) would be generated from the pEnhsr1 construct. The provirus generated from pEnhsr1 will lack the viral enhancer region (stippled box) but still retain CAAT and TATA box motifs (hatched box). This defective proviral transcriptional unit can be activated by an enhancer element (stippled oval) in the flanking, endogenous chromatin. Thus, with pEnhsr1, viral integrations nearby endogenous enhancer elements will result in *lacZ* expression.

RESULTS

Transfection of pProsr1 and pEnhsr1 into ψ-2 and isolation of *lacZ*$^+$, recombinant retrovirus-producing ψ-2 cells

The self-inactivating (SIN) retrovirus constructs contain a deletion of the viral transcriptional control elements in the U3 region of the 3′ LTR. After infection of a target cell, retroviral biology dictates (Varmus, 1988) that the single-stranded viral RNA is reverse transcribed to double-stranded DNA in a manner that results in the U3 region of the 3′ LTR serving as the template for both the 5′ and 3′ LTR. Thus the recombinant retroviruses will synthesize viral RNA efficiently in the ψ-2 retrovirus helper line, since they have an intact 5′ LTR, but upon infection of a target cell the deleted U3 of the 3′ LTR will become the U3 of the 5′ LTR (Fig. 1)—leaving an integrated provirus that is transcriptionally inactive (Hawley *et al.*, 1987; Yu *et al.*, 1986; Yee *et al.*, 1987). Since our constructs contain no internal promoter or other known regulatory elements, *lacZ* expression is presumably dependent upon read-through transcription from an endogenous promoter with pProsr1 or cis-activation by a proximal enhancer element with pEnhsr1. In Fig. 1 we depict how pEnhsr1 would lead to integration and expression of *lacZ* in a target cell.

The pProsr1 and pEnhsr1 were constructed as detailed in the Materials and Methods. The plasmids were co-transfected with pSV2neo into ψ-2 cells by electroporation. After selecting for 2 weeks in G418, cells were stained with FDG and enzymatic cleavage of FDG halted with 1 mM of the competitive inhibitor, PETG. *lacZ*$^+$ cells were sorted under cold, conditions and then cultured in the presence of G418. The pools of *lacZ*$^+$, ψ-2 transfectants were considered to be producing *lacZ* recombinant retrovirus due to their high *lacZ* expression.

Infection and FACS enrichment of *lacZ*$^+$ cells

70Z/3 cells were infected by co-cultivation with the pooled *lacZ*$^+$ ψ-2 cells in the presence of polybrene for 16 hr. Non-

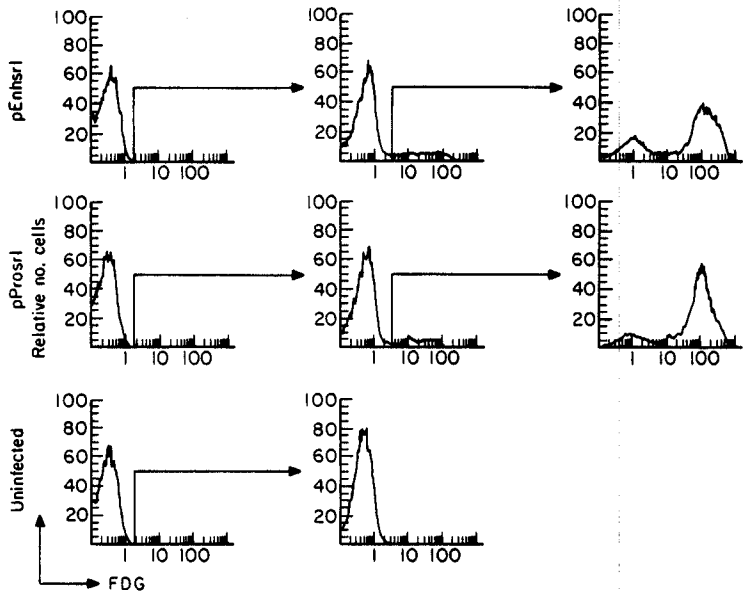

Figure 2. FACS enrichment of *lacZ*-expressing cells. Following co-cultivation of 70Z/3 cells with pProsr1- or pEnhsr1-transfected ψ-2 cells, 70Z/3 cells expressing *lacZ* were enriched by sorting based upon their fluorescence due to cleavage of FDG to fluorescein. To accomplish this, 70Z/3 cells were removed from the ψ-2 monolayer and replated on petri-dishes three times to remove residual ψ-2 cells. 70Z/3 cells were then stained by the FDG technique and enzymatic cleavage was allowed to proceed for 2 hr. Fluorescent cells were then sorted on the FACS and cultured. After expansion for 1 week, the cells were again stained by the FDG technique, re-analysed and again sorted for fluorescein-positive cells. This secondary sorted population was re-analysed after 1 week of expansion by FACS–FDG, as previously. Uninfected 70Z/3 cells were also stained by FACS–FDG. Cleavage was allowed to proceed for 2 hr, and 'rare bright' cells were sorted. After expansion for 1 week in culture, this sorted population of rare bright cells was analysed as previously. Rare bright cells in uninfected 70Z/3 cells were not enriched due to their phenotypic instability.

adherent 70Z/3 cells were separated from the adherent ψ-2 fibroblasts as described above. The 70Z/3 cells were then hypotonically loaded with FDG and incubated on ice for 2 hr. Cells positive for *lacZ* after this incubation were sorted under sterile conditions (Fig. 2) and cultured. After allowing sorted cells to recover and expand (approximately 1 week) they were again loaded with FDG. The reaction was allowed to proceed on ice for 2 hr and positive cells sorted as before (Fig. 2). This secondary sort was cultured and, after expansion, an aliquot was removed, loaded with FDG and analysed after a 2-hr incubation on ice. The analysis of the double-sorted populations from both infections is displayed in Fig. 2. This analysis revealed that approximately 70–90% of these doubly enriched cells were positive for *lacZ* activity. In Fig. 2 one can see that retroviruses, derived from both pProsr1 and pEnhsr1, can infect 70Z/3 cells, and positive cells can be enriched to nearly 90%.

We also have been able to infect the T-cell line BW5147 by co-cultivation with pooled, *lacZ*$^+$, ψ-2 cells transfected with either pProsr1 or pEnhsr1. We enriched for *lacZ*$^+$ cells in a manner similar to that shown for 70Z/3 (data not shown). Inducibility or constituitivity of these has not yet been shown.

We have also noted that a low frequency of cells stained by the FDG technique are transiently fluorescent. These 'rare bright' cells occur even though the cells possess no *E. coli lacZ* gene. Sorted rare brights from uninfected 70Z/3 cells are not stable (Fig. 2). At present we do not understand the cause of these rare, false positive cells.

Analysis of *lacZ* expression in individual cells

Clones derived from the infections of 70Z/3 and BW5147 with pProsr1 and pEnhsr1 have much lower activities than cloned cell lines infected with *lacZ*-encoding retroviruses in which *lacZ* is driven by an internal promoter (SV40) (data not shown). Further, the 70Z/3 and BW5147 clones derived by infection with the transcriptionally disabled viruses exhibit much lower activity than the ψ-2 retrovirus producer cells used to infect them (in these producer cells *lacZ* expression is controlled by a transcriptionally competent 5′ LTR).

In Fig. 3 we show some representative histograms of fluorescence per cell for different clones derived from infections with either pProsr1 and pEnhsr1. These patterns are reproducible after repeated reculture and reanalysis, suggesting they represent specific patterns of expression dictated by the transcriptional capacity of the locus where they are integrated. In some cases we found several clones that have nearly identical patterns of expression. They might be independent isolations of the same clone or may represent independent integrations at the same or a nearby locus, suggesting retroviral integration is non-random as has been proposed recently (Shih, Stoye & Coffin, 1988). Experiments are in progress to examine these questions.

Analysis of positive clones for responsiveness to LPS

To determine if *lacZ* can be integrated into developmentally regulated chromatin or transcriptional units and thus serve as a

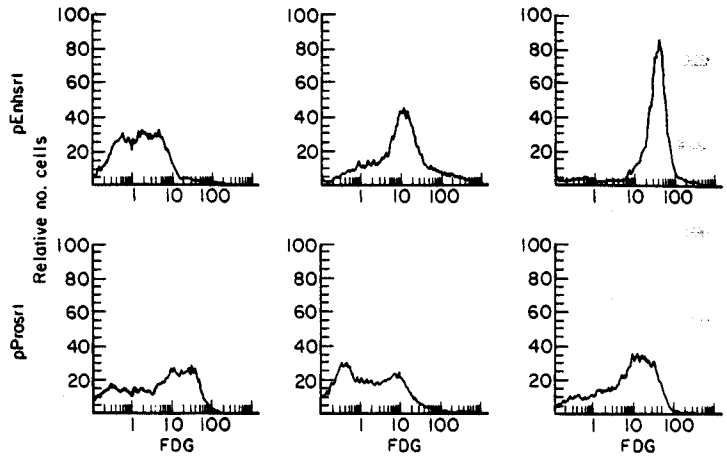

Figure 3. *lacZ*-expressing clones exhibit patterns of activity. The enriched, *lacZ*-expressing populations in Fig. 2 were loaded with FDG and enzymatic cleavage was allowed to proceed for 2 hr. Individual *lacZ*$^+$ 70Z/3 cells were deposited aspetically into 96-well trays by a FACS single cell cloning procedure (Parks & Herzenberg, 1984). These cells were expanded for 3 weeks and re-analysed by FACS–FDG. Enzymatic cleavage was allowed to proceed for 2 hr and cells were analysed at that time.

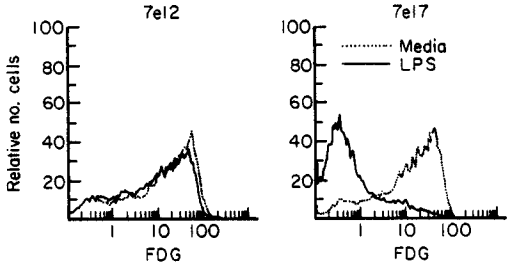

Figure 4. Identification of an LPS-responsive *lacZ*-expressing clone. *lacZ*$^+$ 70Z/3 clones with similar patterns of activity were pooled and analysed by FACS–FDG for a change in *lacZ* expression after 24 hr culture in the presence of LPS (10 μg/ml) relative to a media-only control. One pool showed a significant shift of *lacZ* activity relative to the media-only cells, and when members of this pool were analysed individually, the clone 7e17 was found to show a drastic reduction of *lacZ* activity after only 24 hr in the presence of LPS. A clone with a similar pattern of activity, 7e12, however, exhibits constitutive *lacZ* expression in the presence of LPS.

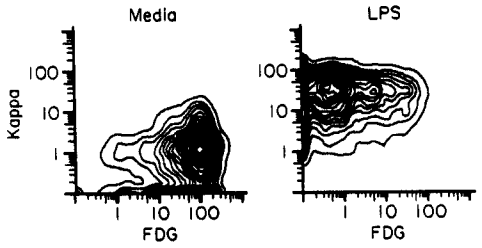

Figure 5. Reciprocal regulation of *lacZ* and κ light chain expression following LPS-induction of 70Z/3 cells. 7e17 cells were loaded with FDG and pelleted. The pellet was resuspended in ice-cold staining media with 0·1% NaN_3 and surface staining for κ light chains was begun by addition of 100 μl of anti-κ-biotin, followed by three washes, and finally a Texas Red-Avidin second layer stain was done. Cells were allowed to proceed with FDG cleavage for 2 hr, during which time staining for κ light chains was done. PETG was added to 1 mM at 2 hr to halt FDG cleavage. Cells were then analysed on the FACS. 7e17 cells that had been treated with 10 μg/ml of LPS for 24 hr were compared to 7e17 cells that were kept in normal media at an equivalent cell density.

means for their isolation and identification, we pooled *lacZ*$^+$, 70Z/3 clones which had similar expression patterns, and induced the cells to undergo differentiation from the pre-B to B-cell stage with LPS (Paige *et al.*, 1978) and analysed the pools after 24 hr. One pool showed a substantial decrease in *lacZ* activity of a significant portion of the population. Analysis of individual members of this pool reveals that in one clone, 7e17, *lacZ* expression is greatly decreased relative to cells cultured without LPS. The clone 7e12 is shown for comparison since it has essentially the same expression pattern as uninduced 7e17; however, the *lacZ* expression in clone 7e12 is unaffected by LPS induction (Fig. 4). To determine if the abrogation of *lacZ* expression in 7e17 coincides with the progression of 70Z/3 to a B cell (as determined by the acquisition of IgMκ on the cell membrane) we stained 7e17 cells with anti-κ, developed fluorescein fluorescence by the FDG technique, and analysed by FACS. Figure 5 shows that the surface appearance of kappa chains coincides with a decrease of *lacZ* expression in these cells. Therefore the chromatin region of the integration site of pEnhsr1 in clone 7e17 negatively regulates expression of the construct during the induced differentiation of 70Z/3. This suggests the provirus construct is integrated in the vicinity of a gene whose expression is LPS modulated.

DISCUSSION

We have demonstrated that the genome of a mammalian cell can be seeded successfully with transcriptionally incompetent *lacZ*-encoding retroviruses to search for developmentally regulated chromatin. A similar approach was pioneered in prokaryotic systems by Casadaban, Chou & Cohen (1980). Here we extend this basic concept to eukaryotes where, using FACS and *lacZ* retrovirus constructs, one can isolate and analyse *in situ* regulatory elements and genes in their native chromatin state. Our analysis of *lacZ* expression in cloned cell lines suggests that integration of these viruses can detect endogenous transcription elements with distinct abilities to control expression at discrete rates. The causes of discrete as well as continuous distributions of β-galactosidase activity in individual clones are probably

many and complex but may reflect the transcriptional activity in the region of chromatin where *lacZ* is integrated. If, for instance, *lacZ* is integrated near a gene whose transcription rate is tightly controlled then *lacZ* activity in these cells might reflect this regulation by exhibiting a discrete distribution of activity. However, if *lacZ* is inserted near a gene whose transcription rate is sensitive to some parameter of cell growth then one could expect analysis of the population distribution to reveal non-discrete patterns of *lacZ* expression. Perhaps some of the unusual distributions (e.g. bimodal) of activity we find in our clonal lines represent such regulatory patterns.

As mentioned previously we have found that the clones derived from infection with the transcriptionally incompetent retroviruses, pProsr1 and pEnhsr1, have significantly less β-galactosidase than those where *lacZ* transcription is driven by the internal (SV40) promoter or with a fully functional Moloney LTR promoter. Although viral promoters are particularly efficient at initiating and maintaining high transcription rates, it seems reasonable that some endogenous transcription control elements could achieve the same rates of transcription as viral promoters. The lower activities seen with these *lacZ* retroviruses could be due to one or more of the following: (i) inefficient polyadenylation—the U3 deletion may remove sequences necessary for efficient polyadenylation of the *lacZ* transcript; (ii) inefficient translation—with pProsr1 the transcript should initiate 5′ of the LTR in the host cell genome, which possibly creates an ungainly 5′ end from which translation does not initiate efficiently; (iii) inefficient initiation of transcription—with pEnhsr1, an upstream enhancer element may not be able to interact with the CAAT and TATA elements of the LTR to give high transcription rates; (iv) none of the above. In comparison to SV40-driven *lacZ*-expressing clones, the pProsr1 and pEnhsr1 clones have the enzymatic activity of less than 100 β-galactosidase tetramers (M. Roederer, unpublished results). Thus FACS–FDG rapidly detects levels of expression less than those required for drug selection or surface marker expression. The exquisite sensitivity of the FACS–FDG assay should permit these or other transcriptionally incompetent retroviruses to reveal transcriptional units expressed at very low levels.

Lastly, we are pleased with the possibility that these virus constructs can be used in combination with FACS–FDG to detect developmentally regulated chromatin and transcriptional units. We have demonstrated that in one clone of 70Z/3 cells, *lacZ* is integrated in a region of chromatin that is transcriptionally down-regulated during LPS-induced differentiation of 70Z/3 from a pre-B cell to a B cell. We believe this is the first demonstration of a reporter gene in mammalian cells that can be controlled by endogenous transcriptional elements in a differentiation-dependent manner.

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Discussion

F. Moddabec
In all the experiments that you showed in fact there was a down-regulation of β-galactosidase activity and, since when you stimulate the cells you enlarge the size, I wonder how much of that is due to dilution of the fluorescein within the cell that is now enlarged? Do you in any way correct for the size of the cell that is enlarged with the same amount of β-galactosidase expressed, the same amount of fluorescein produced but diluted in the larger area so that the signal is low?

L. Herzenberg
What we are measuring is the amount of fluorescein in a package, and whether that package is bigger or smaller, it is still the same amount of fluorescein, so you get the same amount of fluorescence. That is one thing. The differences between high and low is a factor of 100 or 1000, the difference in cell volume that you would have here would be quite small, a factor of 2 or 4 or something like that.

M. Collins
I would like to ask two questions. Firstly, if you clone out a high expressor from the population, how long does it take before you see some heterogeneity in expression again?

L. Herzenberg
Not long. If you set up the low ones and you set up the high ones, with the low ones you get a very narrow distribution around the region where you have sorted the cells of progeny clones. With the high ones, if you look about a week later, it is a fairly narrow distribution around the high ones but as time goes on you get more and more low ones. After about 2 months you still have not regenerated the original distribution.

M. Collins
Secondly, are you using a selectable marker in your virus?

L. Herzenberg
Not in this case, you do not need to because the FACS is a selectable marker. You just sort out one in a million cells or one in 10^8 which is fluorescent. We have done other experiments which might interest you which are maybe more puzzling. We have used episomal vectors with *lacZ* in them and there you do not have any particular integration site and they are fixed at between 50 and 100 copies per cell by having a hydromycin selection maintained. Still you have a very wide range of expression and again, if you sort the lows and sort the highs, they breed true. So you have a genetic or epigenetic control of the degree of expression.

M. Collins
It may depend on the system that you are using, because I have data using a surface marker, Tac antigen, with a constitutive promotor and I get quite tight patterns of expression that are actually maintained long term in culture.

L. Herzenberg
We see this too. We also see some which give rather broader distributions and part of the reason has to be with the promotor constructs that we used. We have now got much simpler promoter systems which do give narrower distributions, but still quite broad.

W. Gilbert
Could that broad distribution be due to selection against the β-galactosidase, could it be slightly inhibitory? You see an asymmetry in the recovery.

L. Herzenberg
Of course, it is possible. They do not obviously grow faster. In two different flasks, the populations are growing at about the same rate.

K. Rajewsky
Have you ever looked in synchronized cell populations?

L. Herzenberg
We have looked to see if it would have anything to do with the division cycle and cell cycle. It is not obviously related, not over this very large range. We have not looked at synchronized cells directly.

N. A. Mitchison
Len, could I ask you a very general question. This promoter search is going to pick up genes which have turned on or turned off in cell differentiation and in a general kind of way you can also look at that by subtraction libraries. What are the pros and cons?

L. Herzenberg
It is another way of doing it. That is a pro I believe. Another pro is that this is easier than subtraction libraries. By both methods you pick up unknown gene sequences. And then you have to characterize them. Maybe you could think of a way to precharacterize, that is to know which ones are more interesting than others. I do have some ideas on that. One is that you try to take the smallest step of differentiation that you can imagine, so you get the fewest number of inducible clones.

E. Clark
I may have missed it but how random is the integration into the genome? How much bias do you think you have?

L. Herzenberg
We really do not know but from the literature it is biased. You will be integrating into regions that are open for transcription, that is what the literature evidence clearly indicates. That means that you can not easily get it into a gene region which has not been committed. But you can find regions which are committed but are not yet expressed. So there are both advantages and disadvantages of that. And of course you can go in both directions here, which is very nice.