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IN SITU DETECTION OF STAGE-SPECIFIC GENES AND ENHANCERS IN B CELL DIFFERENTIATION VIA GENE-SEARCH RETROVIRUSES

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We demonstrate that infection of an LPS-responsive pre-B cell line with transcriptionally-defective retroviruses containing a reporter gene (*lacZ*) can result in viral integrations where expression of *lacZ* is differentiation stage-dependent. Because expression of *lacZ* is dependent upon flanking cellular sequences these retroviral integrations represent *in situ* gene fusions with cellular enhancers (Enhsr1) and genes (Gensr1) which are either induced or repressed during LPS-stimulated differentiation. One of the well-documented effects of LPS upon pre-B cells is the induction of κ light chain transcription via NF-κB. The identification of LPS-stimulated gene repression during B cell differentiation indicates that LPS has multiple effects upon gene expression during the pre-B to B cell transition. The identification of cellular enhancers and genes which are downregulated during the transition from the pre-B to the B cell stage indicates that other transcription factors, in addition to NF-κB, are required for this step in differentiation. Finally, we present some initial experiments which indicate the gene-search retroviruses can introduce expression of *lacZ* into normal hematopoietic cells *in vitro* and *in vivo*.

INTRODUCTION

The differentiation of hematopoietic stem cells to committed B cell progenitors and then to immunoglobulin-secreting plasma cells occurs in a step-wise fashion.[1] These stages of differentiation represent discrete points where intrinsic (e.g., Ig rearrangement), or extrinsic (e.g., stromal cells, T cells) mechanisms can influence the maturation of B-lineage cells. Because B-lineage cells must satisfy different requirements at different stages in their maturation, the expression of stage-specific gene products presumably allows B-lineage cells to respond to the intrinsic or extrinsic signals controlling their differentiation.

In Figure 1 we show a schematic depicting the step-wise differentiation of B-lineage cells and indicate some of the known genes or gene products which are expressed in a differentiation stage-specific fashion. It is difficult to know if any of these genes are involved in deciding whether a cell should commit to the next step in differentiation. Some gene products may influence the decision by providing information relevant to the status of the cell (e.g., pseudo-light chain proteins)[2] or others may be involved in implementing the differentiation decision (e.g., J-chain).[3] The gene products responsible for decision-making during differentiation of B cells have yet to be identified. Although B cell differentiation is one of the best understood mammalian differentiation pathways, we probably know only a fraction of the proteins which are necessary for their ordered maturation.

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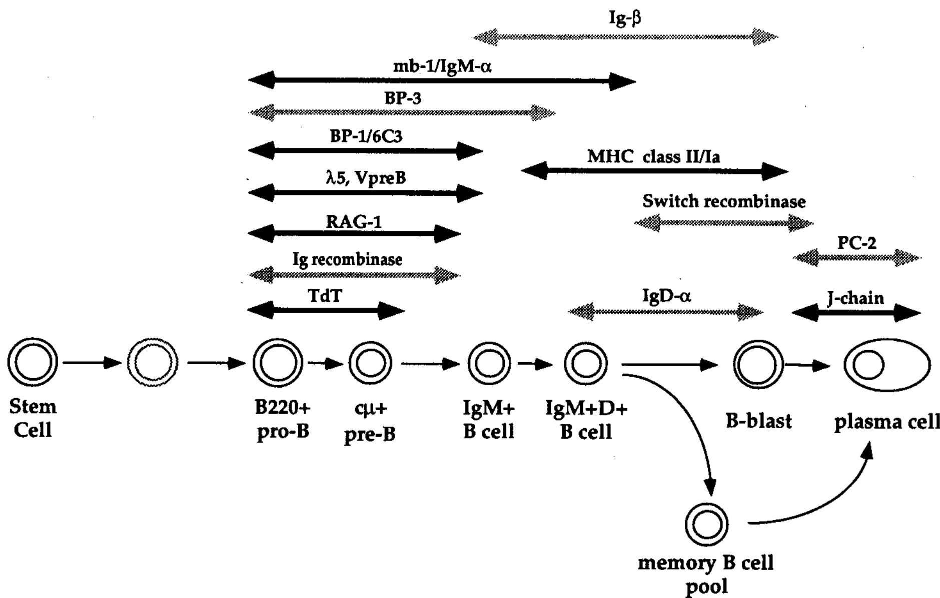

Figure 1. Stage-specific gene expression during murine B cell differentiation. This schematic depicts the step-wise maturation of B-lineage cells with the order of differentiation indicated by thin black arrows. Above specific stages in B-lineage differentiation we indicate genes (thick black arrows) or gene products (thick gray arrows) which are expressed in these specific stages of B cell differentiation. Gene products are distinguished from genes because coding sequences for these proteins have not yet been identified and consequently the evidence for their stage-specific expression is based on biochemical or immunochemical data. This figure represents a compilation of data from references listed below in addition to interpretation by the author. For references concerning expression of: BP-1/6C3, BP-3, Ia, PC-2 see [1]; switch recombination see [25]; Ig recombination see 6; TdT see 7; RAG-1 see [28]; J-chain see [3]; λ5, VpreB, and mb-1 see [17]; IgM-α, IgD-α, and Ig-β see [29-31].

Here we describe our continuing effort to detect and identify stage-specific genes in the differentiation of B-lineage cells. Our approach to this problem relies on forming *in situ* gene fusions with cellular genes via self-inactivating retroviruses containing *lacZ* reporter gene constructs.[4,5] Activation of the *lacZ* reporter gene contained within the transcrip tionally-active provirus requires integration in either trascriptionally-active chromatin (Enhsr1) or in an intron of a transcriptionally-active gene (Gensr1). Others have utilized reporter gene constructs containing *lacZ* to identify developmentally-regulated chromatin and genes in mammalian embryonic development.[6,7] Transposable elements containing *lacZ* have been used to detect developmentally regulated chromatin domains in *Drosophilia*.[8,9,10]

RESULTS

Models for Expression of LacZ *via the Gene-Search Retroviruses*

In Figures 2 and 3 we illustrate how the gene-search retroviruses self-inactivate their ability to drive transcription from their 5' LTR. This approach was originally described by Yu et al..[11] When copying viral RNA into DNA, reverse transcriptase uses the 3' U3 as the

Enhsr1

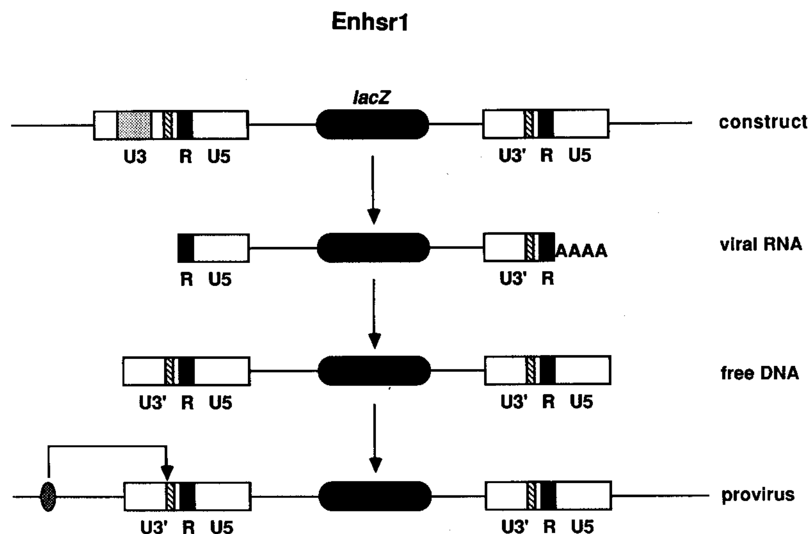

Figure 2. Schematic depiction of Enhsr1 and model for *lacZ* expression. This figure depicts how a *lacZ*-encoding provirus which lacks the Moloney leukemia virus enhancer region (stippled box) would be generated from the Enhsr1 construct. The provirus generated from Enhsr1 will lack the viral enhancer 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.

template for the U3 region in both the 5' and 3' viral LTR.[12] When a mutation or deletion is made in the 3' U3 of a retroviral construct, infection of target cells will generate proviruses which have a different transcriptional phenotype than the parent construct. The gene-search retroviruses exploit this feature of retroviral biology since they generate proviruses which are transcriptionally-incompetent relative to the parental construct.

In the case of Enhsr1, the retrovirus construct contains a deletion of the Moloney enhancer region in the 3' LTR (Figure 2). Following entry of Enhsr1 viral particles into target cells, a provirus will be generated which will lack the Moloney enhancer region in either LTR. The only transcription control elements remaining in the LTR will be the CAAT and TATA motifs of the Moloney promoter. Because the promoter elements are no longer associated with the viral enhancer, the *lacZ* gene must rely on transcription control ele ments in the flanking cellular chromatin for efficient expression. Thus *in situ* gene fusions with Enhsr1 represent a fusion between transcriptional regulatory elements, presumably enhancers, which are capable of activating transcription at the Moloney promoter. Therefore, *in situ* gene fusions with Enhsr1 represent a fusion of cellular transcriptional control elements and the Moloney promoter region.[4]

In *lacZ*$^+$ cells with integrations of the Gensr1 provirus a transcriptional and translational fusion between a cellular gene and *lacZ* is generated (Figure 3). To accomplish this we developed the *lacZ* reporter construct, AcLac.[5] In AcLac the translation initiation codon (ATG) of *lacZ* has been replaced by the splice-acceptor region of the Moloney *env* gene. Because the splice-acceptor sequences are in frame with *lacZ*, AcLac will form a translational fusion with a cellular gene if it is spliced to a coding exon. The splice-acceptor of *env* is predicted to have three separate splice acceptance points which each break the translational codon at one of three possible positions in the triplet.[13,14] Because of this feature, Gensr1 can potentially form a translational fusion with any cellular gene that contains an intron following a coding exon.[5]

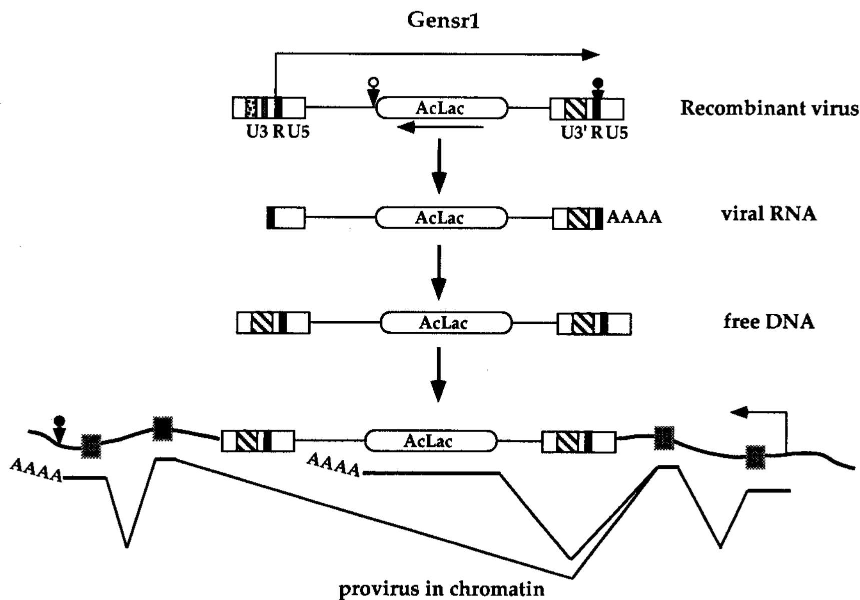

Figure 3. Model for Gensr1. A flow chart which depicts the generation of Gensr1 viral RNA, reverse transcription to a free DNA intermediate and subsequent proviral integration in an intron of a transcriptionally-active gene. A striped rectangle in the viral LTR indicates that the LTR contains a deletion of both the Moloney enhancer and promoter regions which results in a pro-virus which is transcriptionally-inert. Horizontal arrows indicate transcriptional orientation, note that transcriptional orientation of AcLac is opposite that of the retroviral construct and consequently we have inserted a unidirectional polyA site downstream of AcLac (vertical arrow with empty circle) which will function as the polyA cleavage and addition site for the *in situ* fusion with a cellular gene.

Enhsr1 Integrations Identify Cellular Transcription Control Elements That Confer Differentiation Stage-Specific Expression on LacZ

By infecting the pre-B cell line, 70Z/3, in either the uninduced or the induced state, we have derived a series of 70Z/3-Enhsr1 clones where *lacZ* expression is differentiation stage-specific.[4,5] We found that expression of *lacZ* is repressed in three clones (Figure 4)

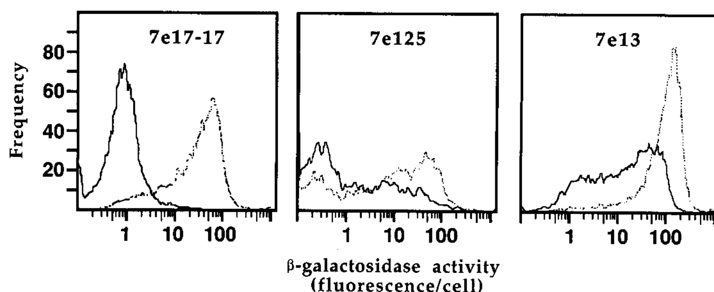

Figure 4. 70Z/3-Enhsr1 $lacZ^{+}$ integrants which are repressed during LPS-stimulation. Analysis of cells cultured in LPS is represented as a solid line while cells cultured in normal medium are represented as a broken line. See [32] for details on FACS-GAL analysis and [5] for details concerning analysis of 70Z/3-Enhsr 1 clones.

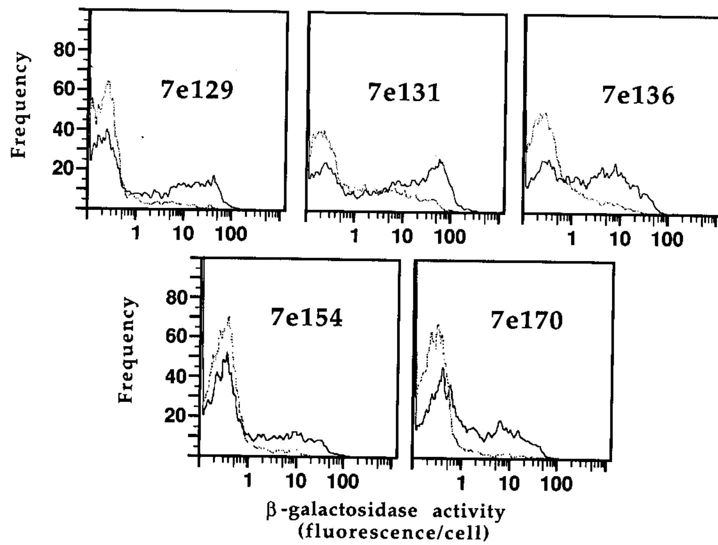

Figure 5. 70Z/3-Enhsr1 integrants which are induced by LPS stimulation. As in Figure 4, solid line represents LPS analysis and broken line normal medium analysis.

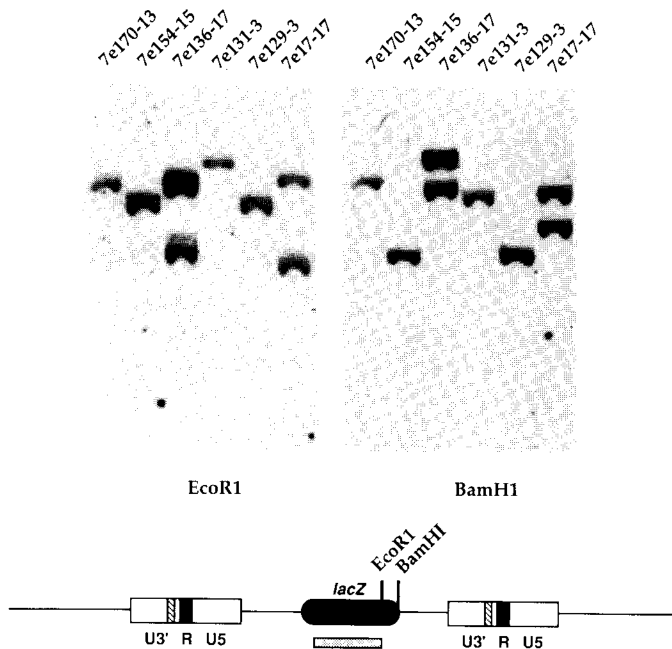

Figure 6. LPS-inducible Enhsr1 integrations represent independent proviral integrations. Southern blot analysis of genomic DNA from 70Z-Enhsr1 clones digested with either EcoRI or BamHI and hybridized with a *lacZ* probe (gray rectangle below provirus).

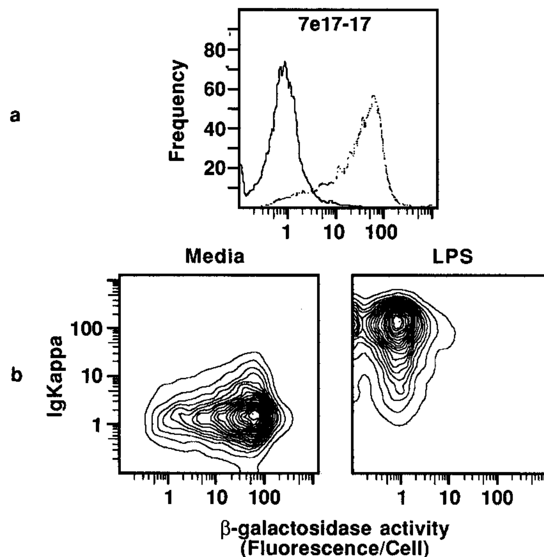

Figure 7. Regulation of *lacZ* and κ light chain expression following LPS-induction of the Enhsr1 clone, 7e17-17 cells. Equal numbers of 7e17-17 cells were either cultured in normal media or in media containing 10 μg/ml of LPS for 24 hours. The cells were then stained as described above and analyzed on the FACS. (a) Histograms representing β-galactosidase activity of 7e17-17 cells after 24 hours in LPS-containing media (solid line) or in normal media (broken line). (b) Dual parameter FACS analysis of κ light chain expression (Texas Red) vs. β-galactosidase activity (Fluorescein) in 7e17-17 cells cultured in normal media or in LPS-containing media. For a more detailed description of combining surface staining with the FACS-GAL technique see [5].

induced in five clones (Figure 5) during LPS-stimulated differentiation of 70Z/3 cells. These eight clones were derived from a total of 411 $lacZ^+$ 70Z/3-Enhsr1 clones and were the only clones found to undergo significant changes in expression during LPS-stimulated differentiation.

Because the five LPS-inducible clones shared a similar pattern of β-galactosidase expression upon LPS induction we felt that they may represent retroviral integrations in an identical site. Recent work has demonstrated that retroviral integration can be non-random and in some cases independent proviruses can integrate repetitively at an identical location.[15] We performed Southern blot analysis to examine the integration diversity among the five LPS-inducible clones (Figure 6). Because the restriction enzymes EcoRI and BamHI are only present once within the proviral DNA, these enzymes will generate *lacZ*-hybridizing restriction fragments unique to each proviral integration. Because the restriction fragments hybridizing with *lacZ* in Figure 6 are not of identical size in the five LPS- inducible clones (7e129-3, 7e131-3, 7e136, 7e154-15, 7e170), we conclude that they represent independent integrations of Enhsr1. Although the integrations are not identical by conventional Southern blotting, we cannot exclude the possibility that they have integrated within the same chromosomal locus.

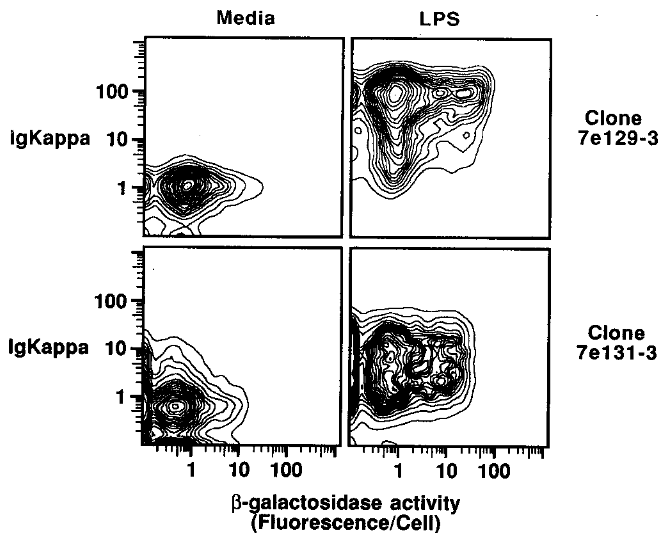

Figure 8. B-cell stage specific expression of *lacZ* in the Enhsrl clones, 7e129-3 and 7e131-3. Cells were cultured, stained and analyzed in a similar fashion to 7e17-17 cells in Figure 7.

To confirm that the Enhsrl cells were differentiating in response to LPS we examined the expression of the *lacZ* via FACS-GAL in combination with immunofluorescent staining for surface IgMκ. This dual parameter FACS analysis revealed that the Enhsrl clones were differentiating in response to LPS by expressing surface IgMκ while either repressing *lacZ* expression (7e17-17; Figure 7) or inducing *lacZ* expression (7e129-3, 73131-3; Figure 8). It is clear from this analysis that expression of *lacZ* in the repressible clone, 7e17-17, is reciprocally regulated with respect to differentiation of 70Z/3 cells. *LacZ* is expressed by the 7e17-17 cells in the absence of LPS when the cells are $IgM\kappa^-$ (the pre-B phenotype); however, after culture in LPS for 24 hr the 7e17-17 cells acquire the B cell phenotype ($IgM\kappa^+$) and became $lacZ^-$. This analysis confirms the differentiation stage-specific expression of *lacZ* in this Enhsrl clone. In this situation we presume that the Enhsrl provirus has integrated near a pre-B specific enhancer which controls the expression of a gene(s) that is shut off when the cell becomes a B cell. This explanation assumes the loss of positive regulation during LPS stimulation; however, active negative regulation, such as a silencer element,[16] is an equally plausible explanation.

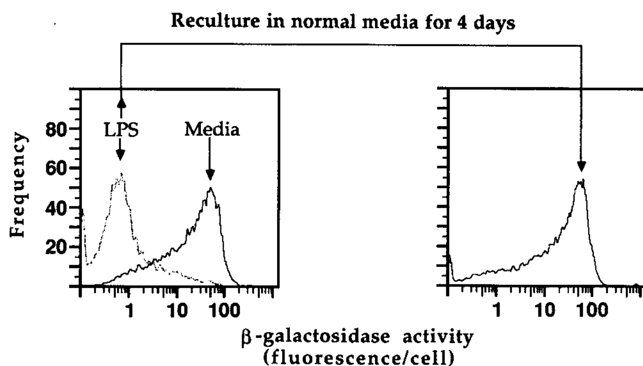

Figure 9. Repression of *lacZ* in 7e17-17 cells is relieved when LPS is removed. Cells which had been cultured for 24 hours in LPS were washed two times, recultured in normal medium and analyzed by FACS-GAL as before.

Dual parameter FACS analysis of the LPS-inducible Enhsr1 clones revealed that there are two distinct phenotypes of *lacZ* induction when *lacZ* expression and differentiation are analyzed simultaneously (Figure 8). In some clones (e.g., 7e129-3) the *lacZ*$^+$ cells tend to be found among the differentiated cells (IgMκ^+) within the clone while in another clone the *lacZ*$^+$ cells are equally represented in the IgMκ^+ and IgMκ^- portion of the clone (7e131-3).

Presumably the cellular enhancers which are controlling expression of *lacZ* in Enhsr1 clones also control expression of a cellular gene. Because multi-parameter FACS analysis allows us to determine the phenotype of the *lacZ*$^+$ cells within these clones we can make some predictions about the role of the associated cellular gene. Since differentiated cells IgMκ^+) are enriched among the *lacZ*$^+$ cells in 7e129-3 while there is no enrichment of IgMκ^+ cells among the *lacZ*$^+$ cells in 7e131-3, we would propose that expression of the cellular gene associated with *lacZ*$^+$ expression in 7e129-3 is related to the differentiation of 70Z/3 cells. The cellular enhancer controlling *lacZ* in 7e131-3 may control a gene whose expression is responsive to the mitogenic effects of LPS.

To test the linkage between LPS-induced repression of *lacZ* and differentiation in 7e17-17 cells we asked whether the repression could be relieved by removal of LPS. In Figure 9 we show *lacZ* expression is recovered by LPS-treated 7e17-17 cells when they are cultured in the absence of LPS. The repression of *lacZ* is completely reversed since β-galactosidase β-gal) activity in the post-LPS 7e17-17 cells is equivalent to that of 7e17-17 cells which were never treated with LPS. It is interesting that the recovery of expression requires a period of 4 days while repression requires only 20-24 hr. The repression of *lacZ* and its reversibility in 7e17-17 indicate that LPS can also mediate repression of genes in pre-B cells. This would suggest that although LPS can mediate activation of genes in lymphoid[17] and myeloid[18] cells it may also have a repressive effect upon gene expression in B cell differentiation. One of the well-documented effects of LPS in pre-B cells is the activation of κ light chain transcription via the transcription factor, NF-κB.[19,20] Since NF-κB is thought to be responsible for the induction of gene expression, it presumably is incapable of mediating gene repression in LPS-treated pre-B cells as is seen with repression of *lacZ* in 7e17-17 cells. If NF-κB is incapable of mediating repression then multiple regulatory pathways must be triggered during LPS-stimulated differentiation of pre-B cells to B cells.

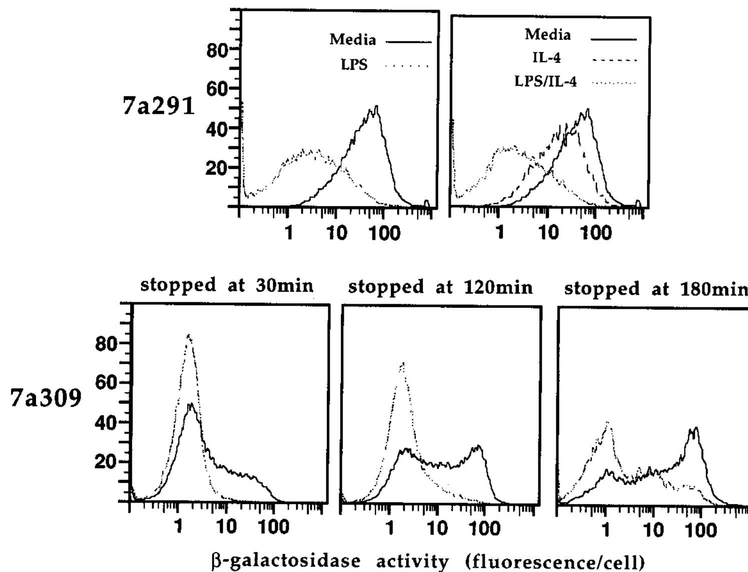

Figure 10. Gensr1 integrants are differentially-regulated during LPS stimulation of 70Z/3 cells. For 7a309, solid line is FACS-GAL analysis of LPS-stimulated cells and broken line is FACS-GAL analysis of cells in normal medium.

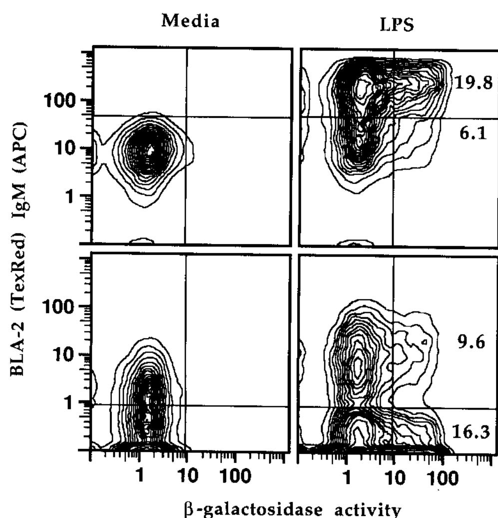

Figure 11. LPS stimulation of 7a309 cells results in $lacZ^+$ cells which are enriched for "differentiated" cells ($IgM\kappa^+$) rather than "activated" cells ($BLA\text{-}2^+$). Simultaneous analysis of BLA-2 (Texas Red), IgM (Allophycocyanin) and β-galactosidase activity (Fluorescein) in 7a309 cells that were cultured for 24 hours with or without 10 μg/ml LPS.

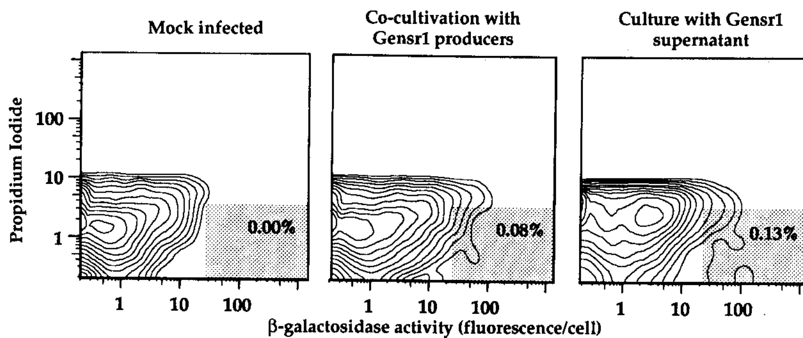

Figure 12. Transduction of *lacZ* expression into hematopoietic progenitor cells via Gensr1. Day 14 fetal liver cells were plated at 5 x 106 cells/ml either on an irradiated monolayer of Gensr1 producers (Ψ2/A8) or in Gensr1 supernatant with 4μg/ml polybrene for 24 hours. The mock-infected cells were cultured at an equivalent cell density in polybrene for an equal amount of time. The cells were then analyzed by FACS-GAL. The propidium iodide positive cells were hardware gated and thus excluded from the data collection. Data were collected on over 200,000 cells via Electric Desk run on a DEC 3600 computer. The percentage of β-gal positive cells within the gates (grayed rectangle) was estimated by Electric Desk run on a DEC 6310 computer. The percentage of β-gal^+ cells is indicated within the gray rectangles of each logarithmic 50% plot.

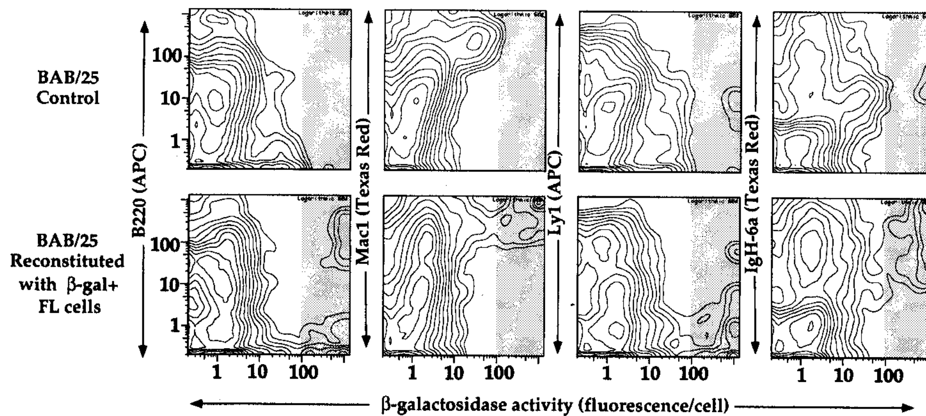

Figure 13. *In vivo* expression of *lacZ* following reconstitution with β-gal$^+$ fetal liver cells obtained by infection with the Enhsr1 retrovirus. Multiparameter FACS analysis of hematopoietic lineage markers (y-axis) versus β-gal activity (x-axis) of PerC cells from a BAB/25 host (IgH-6b) reconstituted with 5,000 β-gal$^+$ BALB/C (IgH6a) fetal liver cells following co-cultivation with Enhsr1 producer cells. There were 0.05% β-gal$^+$ cells in the control BAB/25 PerC that was examined on the same day while there were 0.63% β-gal$^+$ cells in the BAB/25 host reconstituted with *lacZ*$^+$ fetal liver cells. The stains were B220 (APC) vs. Mac1 (TexRed) vs. β-gal and Ly-1 (APC) vs. IgH6a (TexRed) vs. β-gal.

Gensr1 Integrations Identify Cellular Genes Which are Repressed or Induced During LPS-Induced Differentiation

The Enhsr1 integrations which were differentially regulated during LPS induction of 70Z/3 cells proved that *in situ* gene fusions could place a reporter gene (*lacZ*) under the control of endogenous transcription control elements.[4,5] Although isolation and characterization of the cellular gene associated with these native control elements is possible, this task could prove laborious when one considers the large distances over which enhancers can act. Therefore, to more readily identify genes associated with *lacZ* integrations that are differentially regulated we have developed the splice acceptor *lacZ* reporter construct, AcLac, as well as a retrovirus that contains AcLac, Gensr1.[5] Identification and characterization of the cellular gene associated with expression of *lacZ* in Gensr1 integrants is amenable to conventional cloning techniques for two reasons: (1) *lacZ* is fused to the transcript of the cellular gene, (2) β-galactosidase is fused to the product of the cellular gene. This permits the associated gene to be identified by probing cDNA libraries with Gensr1 sequences or oligonucleotides predicted from amino terminal sequencing of the β-galactosidase fusion protein.

In Figure 10 we illustrate two Gensr1 integrants which are regulated during LPS-stimulated differentiation of 70Z/3 cells. In 7a291, expression of *lacZ* is substantially repressed after exposure of the cells to LPS. In addition we find that IL-4 has a mildly repressive effect on expression of *lacZ* in 7a291. However, there appears to be no synergistic effect when 7a291 is treated with both LPS and IL-4. In 7a309 expression of *lacZ* is induced in a portion of the cells after exposure to LPS. We show the analysis of β-galactosidase activity at three different times of incubation. The longest incubation period (180 min) indicates that a majority of the cells in 7a309 have *lacZ* turned on by growth in LPS while in cells not treated with LPS, *lacZ* expression is inactive in the majority of the cells.

We then analyzed the induction of *lacZ* in 7a309 against IgMκ and BLA-2 expression (Figure 11). As with 7e129-3, 7a309 cells which are induced for *lacZ* expression are two-fold enriched for IgMκ expression. However, *lacZ*$^+$ cells show no enrichment for

BLA-2 expression relative to the *lac-Z*⁻ cells in 7a309. BLA-2 is a surface antigen found on actively dividing lymphoid and myeloid cells[21] whose surface expression is induced on 70Z/3 cells by LPS treatment (W. Kerr, unpublished). Because differentiated cells ($IgM\kappa^+$) are enriched among the $lacZ^+$ cells in 7a309 rather than activated cells ($BLA\text{-}2^+$) we would propose that the cellular gene product fused with β-galactosidase in 7a309 is linked to B-cell differentiation rather than activation.

Gene-Search Retroviruses May Be Useful for Progenitor Cell and Gene Expression Studies in Normal Hematolymphoid Differentiation

In addition to studying B cell gene expression with transformed cell lines, we wish to apply the gene-search retroviruses to the study of normal hematopoietic differentiation both *in vivo* and *in vitro*. We have taken some of the first steps down this path. In Figure 12 we demonstrate that early hematopoietic progenitor cells (day 14 fetal liver cells) can express β-galactosidase following infection with Gensr1. Although the efficiency of this procedure is quite low, β-galactosidase$^+$ cells are readily discernible in the Gensr1 infections relative to the mock infected culture.

In another experiment we co-cultured day 14 fetal liver cells from a BALB/C fetus with irradiated Enhsr1 producer cells and sorted β-galactosidase$^+$ cells via FACS-GAL. We obtained approximately 5,000 cells by this procedure and used these cells for reconstitution of a lethally-irradiated BAB/25 allotype congenic host. Two months following irradiation, bone marrow, spleen and peritonal cavity (PerC) cells were analyzed for expression of β-galactosidase versus hematopoietic lineage-specific markers. We did not find a significant percentage of cells expressing β-galactosidase in the spleen and bone marrow of this animal; however, we did find a significant percentage (~0.6%) of cells in the PerC which express β-gal (Figure 13). The β-gal$^+$ cells in the PerC express the donor IgM allotype (IgH-6a) and Mac1. They are $Ly1^-$ and a portion of them express the 6B2 determinant, an epitope of the T200 antigen that is specific to B-lineage cells.[22] From the data in hand we cannot unambiguously assign the β-gal$^+$ cells to a hematopoietic lineage. However, this experiment indicates that the gene-search retroviruses may permit the study of gene expression in normal hematopoietic differentiation. Further experiments are required to determine if this approach is feasible for all arms of the hematopoietic system.

DISCUSSION

In this manuscript we have reviewed the use of gene-search retroviruses for the *in situ* detection of mammalian gene expression. We have utilized these retroviruses to identify genetic loci which respond to LPS-induced differentiation of a pre-B cell line. We have found that LPS mediates both repression and induction of gene expression during pre-B to B cell differentiation. Finally we present some preliminary experiments which indicate that the gene-search retroviruses may permit the study of gene expression during normal hematopoietic differentiation.

We have identified a total of ten gene-search retroviral integrations (both Enhsr1 and Gensr1) where expression of *lacZ* is differentially expressed during LPS stimulation of 70Z/3 cells. Four of these integrations (3-Enhsr1, 1-Gensr1) are repressed during LPS-induced differentiation of 70Z/3 cells while six (5-Enhsr1, 1-Gensr1) are LPS-inducible. Multi-parameter FACS analysis allowed expression of *lacZ* and surface antigens to be monitored simultaneously. This analysis demonstrated that the LPS-repressed Enhsr1 integration (7e17-17) is clearly restricted to the pre-B stage of differentiation. This analysis also indicated that *lacZ* expression in two LPS-inducible integrations (7e129-3, 7a309) is clearly associated with the differentiation of 70Z/3 cells to the B cell stage (surface $IgM\kappa^+$). From this analysis we predict that the cellular genes linked to these gene-search integrations are associated with a specific stage of B-lineage differentiation.

Our identification of multiple gene-search integrations which are either repressed or induced during the pre-B to B cell transition is not surprising. This transition is arguably the most dramatic step in B-lineage differentiation, because it represents the transition from antigen-independent to antigen-dependent differentiation. Upon becoming a mature B cell,

a B-lineage clone is no longer concerned with successfully rearranging the heavy and light chain genes under the restriction of maintaining allelic exclusion but is now ready to respond to antigen, T cells and macrophages in order to participate in an immune response to foreign antigen. Thus, the repression and induction of multiple genes during the transition from the pre-B to the B cell stage is probably a necessary requirement in B cell differentiation. In addition, our identification of loci which are repressed during LPS-induced differentiation of 70Z/3 cells argues for the participation of multiple regulatory circuits in the pre-B to B cell transition, some of which must not involve NF-κB. In support of this hypothesis, others have proposed a regulatory pathway that mediates the induction of κ light chain transcription which is independent of NF-κB.[23,24]

Finally, we are optimistic that the gene-search retroviruses will permit tracking of gene expression during normal lymphoid differentiation. Two different questions concerning lymphoid development can be approached by this technology depending upon the type of locus where the gene-search virus integrates. The first question is: What is the differentiation potential of hematopoietic progenitor cells? If we succeed in integrating a gene-search virus in a constitutively expressed locus (e.g., β-actin) of a hematopoietic stem cell, we can then analyze the differentiation potential of this pluripotent cell via surface staining for hematopoietic lineage markers in concert with FACS-GAL. Second, can we get integration of a gene-search virus in a lineage- or differentiation stage-specific locus? This would provide a molecular tag for this locus permitting its isolation and characterization. *In vitro* culture systems (Dexter or Witte-Whitlock cultures) and *in vivo* models (SCID, W/W^V) exist for the propagation of hematopoietic cells, making this approach technically feasible.

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