# Granuloma-Specific Expression of Mycobacterium Virulence Proteins from the Glycine-Rich PE-PGRS Family

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**Pathogenic mycobacteria, including the agent of tuberculosis, *Mycobacterium tuberculosis*, must replicate in macrophages for long-term persistence within their niche during chronic infection: organized collections of macrophages and lymphocytes called granulomas. We identified several genes preferentially expressed when *Mycobacterium marinum*, the cause of fish and amphibian tuberculosis, resides in host granulomas and/or macrophages. Two were homologs of *M. tuberculosis* PE/PE-PGRS genes, a family encoding numerous repetitive glycine-rich proteins of unknown function. Mutation of two PE-PGRS genes produced *M. marinum* strains incapable of replication in macrophages and with decreased persistence in granulomas. Our results establish a direct role in virulence for some PE-PGRS proteins.**

Pathogenic mycobacteria initiate long-term infection by entering host macrophages where they cause extensive remodeling of their vacuolar environment to prevent vacuolar acidification and lysosomal fusion (*1*, *2*). Replication in macrophages plays a crucial role in persistence in vivo, distinguishing pathogenic from nonpathogenic mycobacteria (*3*, *4*).

Because mycobacteria are facultative intracellular pathogens, we hypothesized that they selectively express specific genes in host macrophages to facilitate replication and persistence in vivo. Consequently, we used differential fluorescence induction (DFI), a fluorescence-activated cell sorter (FACS)–based method (*5*–*7*), to search for *M. marinum* genes activated in macrophages. *M. marinum*, which grows relatively rapidly at 25° to 35°C, causes a systemic tuberculosis-like disease in fish, frogs, and other cold-blooded animals, which are its natural hosts, and a peripheral disease in warm-blooded animals (*8*–*11*), including a peripheral granulomatous disease in immunocompetent humans (*12*). The clinically silent phase of infection in leopard frogs (*Rana pipiens*) is characterized by granulomas consisting largely of modified (epithelioid) macrophages and containing only a few *M. marinum* (*10*). The general pattern of infection is similar to that of clinically silent (latent) human tuberculosis. *M. marinum* can be adapted easily to rapid growth at 37°C and can cause systemic infection of lungs and spleens of mice that protects against a subsequent challenge with virulent *M. tuberculosis* (*13*). *M. marinum* is closely related to *M. tuberculosis* genomically (*14*). Both organisms also occupy identical vacuolar niches in cultured mammalian macrophages (*1*, *2*). Yet unlike *M. tuberculosis*, *M. marinum* is neither an aerosol nor serious (biosafety level 3) human pathogen.

DFI was adapted to enrich for *M. marinum* sequences with enhanced expression in macrophages (Fig. 1A) (*7*, *15*, *16*). Eight macrophage-activated promoters (*map*s) were identified from three promoter-trap libraries of *M. marinum* DNA fragments that represented about one-fourth of the *M. marinum* genome (Table 1 and Fig. 1A). We found very highly activated macrophage-induced mycobacterial promoters compared with earlier studies (*5*, *6*), perhaps as a result of our modification of DFI, where bacteria were subjected to both positive and negative cell sorting. All the identified *mag*s (macrophage-activated genes) are homologs of *M. tuberculosis* genes (Table 1) (*17*). Intracellular activation of the *map*s was confirmed by confocal microscopy, showing that many of them were expressed weakly or not at all outside macrophages (Fig. 1B). All six *map*s tested were also active in granulomas of frogs for several months (Fig. 1C and Table 1), suggesting that the bacteria are sequestered in an intracellular niche during persistent infection.

To determine if genes in addition to the *mag*s were activated in host granulomas, we used DFI to screen directly for genes induced in chronically infected frogs (*10*, *15*, *16*). Seven independent promoter sequences activated within granulomas were identified from one promoter library. *map* 25, *map* 85, and *map* 86 were reisolated, confirming that these genes are expressed in granulomas of infected animals, as well as in cultured macrophages. In addition, we identified a second class of promoters activated specifically in host granulomas but not in cultured macrophages. Their downstream genes are homologs of *M. tuberculosis* Rv0321 (*dcd*), encoding a deoxycytidine triphosphate deaminase; Rv1106c, encoding a probable cholesterol dehydrogenase; Rv0631c (*recC*), encoding RecC; and Rv1205, encoding a protein of unknown function (*17*).

Two genes selectively expressed in both macrophages and granulomas, *mag* 24 and *mag* 85, are homologous to members of the large *M. tuberculosis* PE/PE-PGRS protein family, which comprises roughly 5% of the coding DNA of *M. tuberculosis* (Table 1 and Fig. 1) (*17*). The genes of this family are scattered throughout the genome of *M. tuberculosis* and other closely related mycobacteria (*14*, *17*, *18*). This family, characterized by a relatively conserved ~110–amino acid $NH_2$-terminus, is subdivided into the PE and PE-PGRS subfamilies (*17*). In the PE-PGRS proteins, the conserved $NH_2$-terminus is followed by a region of irregularly spaced glycine dipeptide repeats spanning 100 to >500 residues. Most terminate just after the glycine-rich region, while a few have unique COOH-terminal extensions beyond. The function of these proteins is unknown and has been the subject of considerable speculation (*17*, *19*). The PE-PGRS genes vary among clinical isolates of *M. tuberculosis* (*18*), leading to the hypothesis that they represent a source of antigenic diversity or that their glycine repeats inhibit host major histocompatibility complex class I processing, akin to the glycine repeats of the Epstein-Barr virus EBNA-1 protein (*17*).

Sequence analysis of the *M. marinum* genome flanking *map* 24 revealed a PE-PGRS gene (*mag* 24-1) (Fig. 2 A) (*17*, *20*). However, we found two additional open reading frames (*mag* 24-2 and *mag* 24-3) downstream of *mag* 24-1, both encoding PE-PGRS proteins homologous to MAG 24-1 (Fig. 2, A and C). All three MAG 24 proteins have the unique COOH-terminal extensions homologous only to those of the *M. tuberculosis* Rv1651c and Rv3812 proteins, which are, in turn, homologous to each other (Fig. 2C) (*17*).

To dissect the roles of the three MAG 24 proteins, we constructed *M. marinum* strains with insertion mutations in each of the three *mag* 24 genes by homologous recombination (Fig. 2, A and B) (*21*). All three mutants grew at rates similar to those of wild-type *M. marinum* in minimal medium (*22*, *23*), indicating that they are not auxotrophic mutants and the proteins do not play a role in bacterial replication outside host cells.

The *mag* 24-1 mutant, L1D, and the *mag* 24-3 mutant, L2D, did not replicate in macrophages (Fig. 3A). The *mag* 24-2 mutant, P59D, was not attenuated for macrophage

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replication (Fig. 3A), consistent with the finding that a transcriptional fusion of the region upstream of *mag* 24-2 (*map* 24-2 in Fig. 2A) to *gfp* was not expressed either constitutively or in macrophages (*22*). The intergenic distances between the three *mag* 24 genes (Fig. 2A), coupled with the results of the mutational analysis, indicated that each of the *mag* 24 genes was transcribed independently. MAG 24-1 and MAG 24-3 may be functionally redundant because macrophage persistence was not completely abrogated in either of these mutants (Fig. 3A). To confirm the role of MAG 24-1 in macrophage replication, we introduced the wild-type *mag* 24 genes into L1D by homologous recombination (Fig. 2, A and B). The resulting partial merodiploid strain, L1D-T, was restored for macrophage replication (Fig. 3A).

The L1D mutant bacteria also had diminished persistence in chronic granulomas of leopard frogs (*10*), persisting at an ~25-fold lower level than wild-type bacteria in spleen (Fig. 3B) with similar results being obtained in liver (*22*). Histopathological examination of livers from the L1D-infected frogs revealed either a marked decrease in the gran-

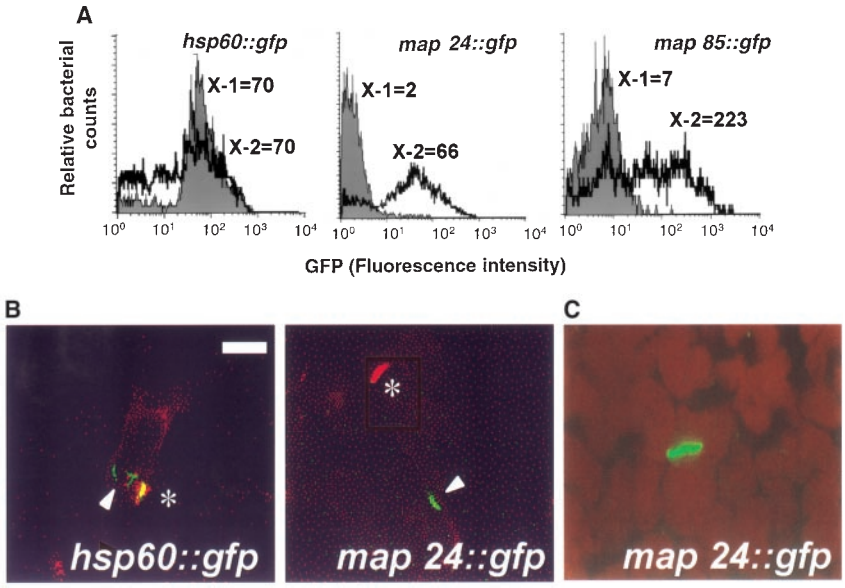



**Fig. 1.** Macrophage-dependent bacterial gene expression. (**A**) Independent *map*s exhibited varied levels of ex vivo and macrophage-dependent gene expression. J774 macrophages were infected for 24 hours with *M. marinum* bearing various promoter fusions (*23*). The geometric mean fluorescence of bacteria released from the macrophages (x-2) and bacteria grown ex vivo (x-1) (*15*) was analyzed and quantitated with a FACStar cytometer and CellQuest Software, respectively (Becton Dickinson). Histograms show relative fluorescence intensity of bacteria grown ex vivo (shaded) and those released from macrophages (solid line). The magnitude of macrophage-activated fluorescence (Table 1) was calculated by dividing x-2 by x-1. For *M. marinum* bearing the constitutively expressed promoter fusion *hsp* 60::*gfp* (*28*, *29*), x-1 and x-2 are identical as expected. GFP, green fluorescent protein. (**B**) J774 cells were grown on glass cover slips and infected with *M. marinum* bearing *hsp* 60::*gfp* or *map* 24::*gfp* for 24 hours and stained with polyclonal antisera to *M. marinum* followed by rhodamine-conjugated antibody to rabbit immunogloblin G (red), without permeabilizing the macrophage monolayer, so as to stain extracellular bacteria only. The cover slips were examined by laser confocal microscope with a dual [rhodamine and fluorescein isothiocyanate (for GFP)] filter. Intracellular bacteria are designated by an arrowhead, and extracellular bacteria by an asterisk. For *M. marinum hsp* 60::*gfp*, the intracellular bacteria are green fluorescent and the extracellular bacteria appear yellow, being both green and red fluorescent owing to GFP expression and antibody staining. For *M. marinum map* 24::*gfp*, promoter activation causes the intracellular bacteria to fluoresce green, but the extracellular bacteria are pure red, indicating no GFP expression outside the macrophage. (**C**) A fluorescent *M. marinum map* 24::*gfp* within a liver granuloma. Cryosections (10 μm) were prepared from livers and spleens of frogs infected with *M. marinum* bearing the different *map gfp* fusions so as to produce chronic granulomas (*10*). The frog cell nuclei were stained with propidium iodide. Laser confocal microscopy was used to capture images of serial 1-μm sections each under red and green filters, which were combined to build a single image showing the red nuclei of the granuloma cells and the green bacteria. Bar, 4 μm (B and C).

**Table 1.** Characteristics of *map*s and *mag*s. Promoters with intracellular activation of fluorescence (Fig. 1A), trapped by differential fluorescence induction (*15*, *16*), were sequenced with a primer derived from the pFPV27 vector (*5*) and their sequences compared with the National Center for Biotechnology Information (NCBI) sequence databases. Putative functions were assigned based on those determined or postulated for close homologs (>50% amino acid identity). Percent amino acid identities are derived from the *mag* sequences contained in the promoter insert spanning lengths of 64 to 130 amino acids. ND, not determined.

| Promoter construct | Magnitude of macrophage activation* | Activity in granulomas† | *M. tuberculosis* homolog‡ (% amino acid identity) | Protein features or putative function | Possible role in mycobacterial persistence in vivo |
|---|---|---|---|---|---|
| *map* 15 | 4 | ND | – | – | – |
| *map* 24§ | 33 | Yes | PE-PGRS genes, Rv3812 and Rv1651c (Fig. 2C) | Family of glycine-rich proteins unique to some pathogenic mycobacteria (*17*) | Replication in host macrophages and persistence in granulomas (this study) |
| *map* 25 | 3 | Yes | Rv3416 (WhiB3) (96%) | Transcriptional activator of early sporulation in *Streptomyces coelicolor* (*27*) | Regulate sporulation-like features during long-term persistence |
| *map* 49 | 32 | Yes | Rv1200 (79%) | Transmembrane (metabolite) transport protein in *S. coelicolor*, *Escherichia coli*, *Hemophilus influenzae* (*17*) | Nutrient transport into vacuole of intracellular bacteria |
| *map* 62‖ | 3 | Yes | – | – | – |
| *map* 83 | 2 | ND | Rv0575c (77%) | Oxidoreductase in *S. coelicolor* | Counter oxygen radicals of macrophage |
| *map* 85 | 32 | Yes | PE/PE-PGRS gene (53%) | Mycobacterial glycine-rich protein family (*17*) | – |
| *map* 86‖ | 6 | Yes | – | – | – |

**map* activity inside cells was determined as described in Fig. 1A. †*map* activity in granulomas of infected frogs was determined as described in Fig. 1C. ‡Genes described by Cole *et al*. (*17*). §Although the beginning of *mag* 24 is contained within the *map* 24 insert, its homology became apparent only after sequencing of the region downstream of the insert in the genomic clone. ‖Inserts homologous to *M. tuberculosis* (*map* 86) or *Mycobacterium leprae* (*map* 62) genes but not adjacent to the *gfp* fusion.

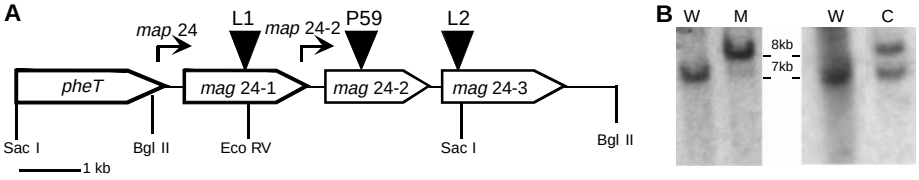
ulomatous response or a qualitative change in the host response to the infection (*15*, *22*). Whereas frogs infected with wild-type *M. marinum* have defined granulomas composed mainly of epithelioid macrophages, the L1D-infected animals had more diffuse lesions consisting primarily of lymphocytes (*10*, *15*).

The *mag* 24-1 gene first induced at 7 to 20 hours after entry into cultured macrophages (*22*) was expressed indefinitely in granulomas of infected frogs (Fig. 1C). Consistently, disruption of *mag* 24-1 diminished *M. marinum* replication in macrophages as well as persistence in granulomas. Our results show an immediate role in pathogenesis for some PE-PGRS proteins by facilitating macrophage replication, similar to the effect of the secreted Erp protein of *M. tuberculosis* (*24*). Their contribution to bacterial replication in cultured macrophages, where antigen presentation does not play a role in bacterial survival, indicates that they affect persistence in granulomas independent of, or in addition to, an effect on antigen presentation. A recent screen of macrophage-induced *M. tuberculosis* promoters expressed in the attenuated *Mycobacterium bovis* BCG (bacillus Calmette-Guerin) strain has also revealed a PE-PGRS gene (*6*).

There is precedence for virulence determinants to be interspersed in highly variable regions in other bacteria, e.g., the *Neisseria gonorrhea* pili and the streptococcal M proteins (*25*). Although the subcellular localization of the PE-PGRS proteins is not known, their amino acid composition predicts them to be surface proteins like the M proteins and pili (*17*, *22*).

*M. marinum* and *Mycobacterium ulcerans* are phylogenetically the most closely related mycobacteria to the *M. tuberculosis* complex (*14*). This is not to say that *M. marinum* is a surrogate for *M. tuberculosis*, but that these two pathogenic mycobacteria likely share a common ancestor (*14*) and employ indistinguishable strategies to replicate in macrophages and persist in granulomas. Perhaps the most telling similarity is that at the histological level, *M. marinum* infection of the human dermis is virtually indistinguishable from human dermal tuberculosis (*12*, *22*).

The hallmark of chronic mycobacterioses, whether in the mammalian or amphibian host, is the maintenance of lifelong asymptomatic infection. The metabolic and replicative state of the mycobacteria in granulomas is largely unknown owing to the experimental intractability of investigating such lesions (*26*). Our ability now to identify mycobacterium gene classes specifically induced in different phases of chronic granulomas provides new avenues to better understand central questions of mycobacterial infection and disease.



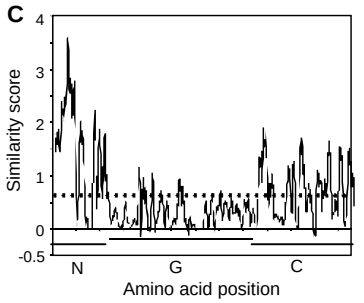




**Fig. 2.** Identification, homologies, and disruption of the *mag* 24 genes. (**A**) Overlapping Sac I and Bgl II fragments flanking *map* 24 of the *M. marinum* genome were isolated by genomic cloning and sequenced. The 440–base pair (bp) *map* 24 insert included 186 bp of the *pheT* gene and 47 bp of *mag* 24-1. In *M. tuberculosis* also Rv1651c and *pheT* are adjacent but in the opposite orientation to each other. The kanamycin insertion mutations L1, P59, and L2 were made in the three *mag* 24 genes, respectively, in either the Sac I or Bgl II fragment (*21*). The resultant suicide plasmids were used to create the *M. marinum* gene disruption mutants L1D (*mag* 24-1), P59D (*mag* 24-2), and L2D (*mag* 24-3) by sucrose counterselection (*21*). The complemented mutant L1D-T was created by reintroducing the Bgl II fragment into L1D with selection for the apramycin resistance gene in the plasmid (*21*, *30*). (**B**) Southern blot analysis of Bgl II–digested chromosomal DNA from the wild-type (lanes W), L1D mutant (lane M), and the complemented mutant L1D-T (lane C) probed with the *map* 24 insert probe (Fig. 1A), showing the 1.2-kb upward shift of the L1D Bgl II fragment and the presence of both wild-type and mutant alleles in L1D-T. (**C**) Protein similarity profile for MAG24-1, MAG24-2, MAG24-3, Rv1651c, and Rv3812. Their predicted protein sequences were aligned with the GCG program *pileup* with a zero gap extension penalty, and the average similarity of the alignment (dotted line) calculated in the GCG *plotsimilarity* program with the blosum62 scoring matrix. The five proteins are similar at their $NH_2$- (N) and COOH- (C) termini (50% identity or greater) but not in their intervening glycine-rich region (G), whereas the other *M. tuberculosis* PE-PGRS proteins have homology only to the $NH_2$-terminus of these proteins.

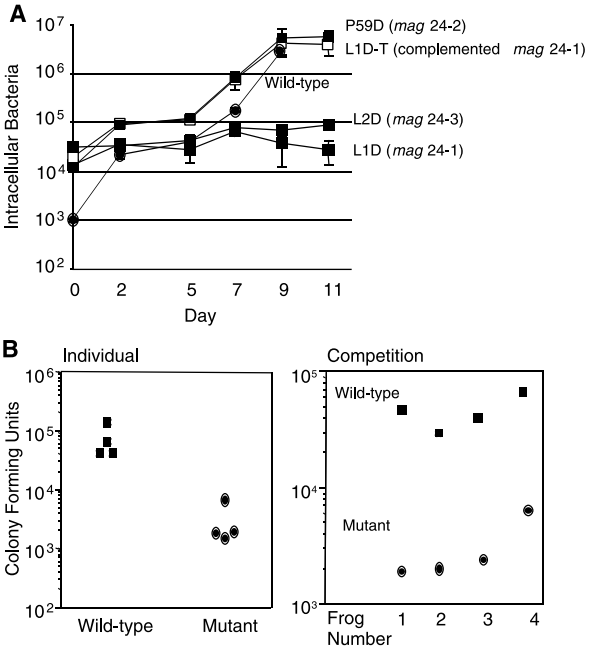



**Fig. 3.** Effect of the *mag* 24 mutations on replication of *M. marinum* in J774 macrophages and frogs. (**A**) J774 macrophages were infected with the wild-type, L1D, L1D-T, P59D, and L2D *M. marinum* strains (*23*) with the substitution of streptomycin for amikacin. Intracellular counts obtained periodically (*23*) are represented as the means of triplicate samples. Error bars represent standard errors of the means. The 0 time point is 5 hours after infection. The infecting inocula were virtually the same for all strains except that of the wild-type strain, which was about sevenfold lower. The plateau in growth of P59D and L1D-T at day 11 is due to a cytopathic effect on the monolayer, leading to the release of the intracellular bacteria, which are not counted in the assay (*23*). (**B**) Frogs were injected intraperitoneally with wild-type ($3.8 \times 10^5$ bacteria per frog) or L1D mutant ($7.0 \times 10^5$ bacteria per frog) for the individual assay, and with a mixture of $3.8 \times 10^5$ wild-type and $7.0 \times 10^5$ L1D *M. marinum* for the competition assay. Bacterial counts were obtained from spleens and livers at 8 weeks after infection (*10*). For the competition assay, the infected tissue homogenates were plated on solid bacterial medium in the presence and absence of kanamycin to distinguish wild-type (kanamycin-sensitive) from mutant (kanamycin-resistant) bacteria. The mutant bacteria were out-competed by the wild-type bacteria by 10- to 24-fold.

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# Regulation of B Lymphocyte and Macrophage Development by Graded Expression of PU.1

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**The ets family transcription factor PU.1 is required for the development of multiple lineages of the immune system. Using retroviral transduction of *PU.1* complementary DNA into mutant hematopoietic progenitors, we demonstrate that differing concentrations of the protein regulate the development of B lymphocytes as compared with macrophages. A low concentration of PU.1 protein induces the B cell fate, whereas a high concentration promotes macrophage differentiation and blocks B cell development. Conversely, a transcriptionally weakened mutant protein preferentially induces B cell generation. Our results suggest that graded expression of a transcription factor can be used to specify distinct cell fates in the hematopoietic system.**

The ets family transcription factor PU.1 represents a unique transcriptional regulator within the hematopoietic system (*1*). It is required for the proper generation of both myeloid lineages (macrophages and neutrophils) and lymphoid lineages (B and T lymphocytes) (*2*). The blocks to B cell and macrophage development caused by the loss of *PU.1* function are more severe than defects in neutrophil (*3, 4*) and T cell (*5*) development. *PU.1* is essential for regulating the proliferation and differentiation of macrophage and B lineage progenitors (*3, 6*). PU.1 regulates the expansion of such progenitors by controlling the expression of the *c-fms* and *IL-7R*α genes, which encode receptors for macrophage colony-stimulating factor (M-CSF) and interleukin 7 (IL-7), respectively (*3, 7*). Macrophage- and B lineage–specific gene expression programs are also severely affected by the *PU.1* mutation (*2, 3, 8*).

To analyze the function of PU.1 in B cell and macrophage development, we established a system for efficient retroviral transduction of murine fetal liver hematopoietic progenitors. On embryonic day 14.5, $PU.1^{+/-}$ or $PU.1^{-/-}$ progenitors were enriched using a lineage-depletion protocol (*9*), then infected by coculture with retroviral packaging cells, which stably produce high titers of murine stem cell virus (MSCV) vectors (*10*). $PU.1^{+/-}$ lineage-depleted ($Lin^-$) progenitors infected with a control virus (MSCV-EGFP) proliferated and differentiated on S17 stromal cells (*10, 11*) into pro-B cells ($CD19^+$, $B220^+$, $CD43^+$, and c-$kit^+$) and macrophages (Mac-$1^+$) in 10 to 14 days. The majority of cells were pro-B (86 ± 4%, $n = 7$); a minor fraction were macrophages (7 ± 3%, $n = 7$). $PU.1^{-/-}$ $Lin^-$ progenitors could not be productively infected with the control virus, because they failed to proliferate and died during the initial coculture. In contrast, $PU.1^{-/-}$ progenitors infected with the PU.1 virus (MSCV-EGFP-PU.1) and cultured on S17 survived and proliferated in response to IL-7. We found it intriguing that after 10 to 14 days these cultures contained many more macrophages (56 ± 4%, $n = 6$) than pro-B cells (19 ± 5%, $n = 6$). Identical results were obtained using M-CSF–deficient OP9 stromal cells (*12*), suggesting that the increased proportion of macrophages observed with PU.1-transduced mutant progenitors was not simply due to increased M-CSF–dependent proliferation. As did their heterozygous counterparts, PU.1-rescued pro-B cells expressed CD19, B220, CD43, and c-kit; the B-lineage–specific genes *mb-1*, *B29*, λ*5*, and *VpreB*; and they underwent V(D)J recombination at the *IgH* locus. Further analysis of the flow cytometry data from these experiments revealed that most macrophages expressed high levels of green fluorescent protein (GFP), whereas the pro-B cells were low or lacking in GFP (Fig. 1). If one assumes, in our vector system, that expression of PU.1 from the viral promoter is correlated with expression of GFP from an internal promoter (*10*), these results suggest that differing concentrations of PU.1 protein are required to promote development of B cells or macrophages.

To analyze PU.1 expression, rescued cells were expanded under conditions that select for pro-B cells or macrophages (*13*). After expansion, rescued pro-B cells no longer expressed GFP as seen by flow cytometry (Fig. 2A) or Western blotting. However, these cells expressed detectable PU.1 transcripts and protein but at substantially lower levels than their macrophage counterparts (one-fifth to one-seventh as much) (Fig. 2B) (*14*). We suggest that the two distinct levels of *PU.1* gene expression observed in the rescued pro-B cells versus macrophages are due to selection imposed on the differential activity of the retroviral long terminal repeat (LTR), which is caused by integration in distinct chromatin environments (*15*). It is noteworthy that wild-type macrophages express higher levels of PU.1 protein than their pro-B counterparts (Fig. 2C) (*16*). These results agree with earlier reports that *PU.1* RNA is expressed at higher levels in myeloid than in B lymphoid cell lines (*17*). Because the levels of PU.1 in wild-type and rescued macrophages are equivalent (Fig. 2, B and C), it is unlikely that the higher levels of PU.1 in rescued macrophages compared with pro-B cells are a consequence of retroviral expres-

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