Modeling Early Epstein-Barr Virus Infection in *Drosophila melanogaster*: The BZLF1 Protein.

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Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis, and is associated with several forms of cancer, including lymphomas and nasopharyngeal carcinoma. The EBV immediate-early protein BZLF1 functions as a transcriptional activator of EBV early gene expression, and is essential for the viral transition between latent and lytic replication. In addition to its role in the EBV life cycle, BZLF1 (Z) also has profound effects upon the host cellular environment, including disruption of cell cycle regulation, signal transduction pathways, and transcription. In an effort to understand the nature of Z interactions with the host cellular environment, we have developed a Drosophila model of early EBV infection, where we have expressed Z in the Drosophila eye. Using this system, we have identified a highly conserved interaction between the Epstein-Barr virus Z protein and shaven, a Drosophila homolog of the human Pax2/5/8 family of genes. Pax5 is a well-characterized human gene involved with B cell development. The B-cell specific Pax5 also promotes the transcription of EBV latent genes from the Wp promoter. Our work clearly demonstrates that the Drosophila system is an appropriate and powerful tool in identifying the underlying genetic networks involved in human infectious disease.
INTRODUCTION

Epstein-Barr virus (EBV) is a ubiquitous herpesvirus, having infected most of the world’s adult population. Primary infection with EBV may result in infectious mononucleosis, and predisposes certain individuals to cancer (Rickinson and Kieff, 2001; Zur Hausen et al. 1970). EBV infects mainly two cell types: epithelial cells, where it undergoes lytic replication, and B cells, where it initially replicates in a lytic manner before it enters a latent or dormant state (Rickinson and Kieff, 2001). B cells normally may differentiate into plasma cells, which secrete Ig, or memory B cells (Calame et al., 2003). During EBV lytic replication in B cells, the B cells have been shown enter a plasma cell differentiation pathway (Niedobitek et al., 1997), yet EBV persists in its latent form in memory B cells (Babcock et al., 1998), indicating that the virus takes advantage of B cell differentiation pathways in order to efficiently replicate and maintain the virus.

EBV replication is dependent upon its host’s cellular environment, hence the virus encodes proteins that interact with and alter the function of cellular proteins, thus altering the intracellular environment to suit the virus. One such EBV protein is BZLF1, an immediate-early protein expressed during lytic replication. BZLF1 (Z) is a transcriptional activator that binds to and activates the promoters of early EBV genes (Chevallier-Greco et al. 1986; Cox et al. 1990; Giot et al. 1991; Holley-Guthrie et al. 1990; Kenney et al. 1989; Quinlivan et al. 1993). The DNA binding domain of Z bears homology to the AP1 site binding proteins, c-Jun and c-Fos (Farrell et al. 1989). Therefore, Z is able to bind to AP1 and AP1-like sites, which are present in the promoters
of the EBV early genes (Kieff and Rickinson 2001, Urier et al. 1989). A loss of Z DNA binding ability, such as with the Z mutant Z311 (Giot et al. 1991), leads to a loss of Z transactivation function, and prevents EBV lytic replication from occurring (Giot et al. 1991).

BZLF1 has also been shown to interact with and/or modify the function of several key cellular proteins, including p53, PML, CBP, NF-κB, and signal transduction proteins (Adamson et al. 2000, Adamson and Kenney 2001, and reviewed in Sinclair 2003). BZLF1 physically associates with the p53 protein, and this interaction has been shown to prevent p53 from activating p53-responsive promoters (Zhang et al. 1994, Mauser et al. 2002b). In addition, overexpression of p53 prevents BZLF1 transactivation of EBV early promoters (Zhang et al. 1994). BZLF1 also physically associates with the p65 subunit of NF-κB, and this association was found to inhibit EBV lytic replication (Gutsch et al. 1994). Interestingly, BZLF1 increased the levels of nuclear NF-κB, yet did not increase the levels of NF-κB binding to DNA, and in fact inhibited NF-κB transactivation of IκBα and ICAM-1 (Morrison and Kenney 2004). CREB-binding protein (CBP) also physically associates with BZLF1, and this interaction increases BZLF1 transactivation of EBV early genes as well as increases EBV lytic replication (Adamson and Kenney, 1999). Expression of BZLF1 alters the cellular localization of the promyelocytic leukemia protein (PML), from nuclear dots to a diffuse nuclear pattern, as well as alters the localization and morphology of mitochondria in the cytoplasm (Adamson and Kenney, 2001; LaJeunesse et al. 2005). Since PML is part of the cell’s response to viral invasion, the relocalization of PML by BZLF1 may enhance EBV lytic replication. BZLF1 overexpression has also been shown to affect the cell cycle. Although these effects seem
to be cell-type dependent, BZLF1 generally promotes arrest in the G\textsubscript{1} or G\textsubscript{2}/M phases of the cell cycle (as reviewed in Sinclair 2003). The cell cycle arrest may occur through a variety of mechanisms, such as a p21 increase via C/EBP\textalpha, or upregulation of p53 and p27 proteins (as reviewed in Sinclair 2003).

Since EBV is a human virus, the main means of studying the virus thus far has been through cell culture systems. A wealth of information about EBV has been gained via cell culture, yet we became interested in studying EBV protein function in the context of an intact organism, by expressing EBV proteins in tissues that exist in their natural habitat and are composed of a variety of cell types. Such a system may better reflect how EBV proteins function in their natural human host.

The \textit{Drosophila} model system has become a useful and powerful tool to study a variety of human genetic diseases (Rebay et al. 1993; Rebay et al. 2000; LaJeunesse et al. 2001; Bonini, 2001; Bonini and Fortino, 2003; Schreiter et al. 2004; Bier, 2005). The \textit{Drosophila} genome possesses a remarkable level of homology with the human genome, with as many as 70\% of genes conserved between the two species (Edgar and Lehner, 1996; Bernards and Haritharan, 2001). Furthermore, genetic techniques such as dominant second-modifier screens enable a genetic dissection of a target gene’s cellular function, signal transduction patterns associated with the target gene, and the identification of genetically-interacting genes which may encode proteins that physically interact with the target gene. However, no study on genes involved in EBV replication has been conducted using the \textit{Drosophila} model system.

When a virus invades a host cell, it ectopically expresses an array of new foreign genes that alter and commandeer the host’s cellular machinery to propagate its life cycle.
In this paper, we demonstrate the first use of the *Drosophila* model system to investigate the host cell/virus relationship found in early Epstein-Barr viral infection. Transgenic *GMR::Z* flies were generated that express a mutant dose-sensitive eye phenotype, making them amenable to dominant second site modifier genetic screens. Phenotypic characterization of *GMR::Z* flies showed that ectopic expression of Z affected the differentiation of cone cells and pigment cells within the developing *Drosophila* retina. Using these *GMR::Z* flies, we have performed a preliminary candidate gene screen and identified *shaven* as a strong enhancer of the *GMR::Z* phenotype. Interestingly, *shaven* encodes the Drosophila homolog to the human Pax2/5/8 genes (Fu and Noll, 1997; Fu et al, 1998). *shaven* and the human Pax2/5/8 genes share sequence homology including 88% identity and 91% similarity between their paired domains.

Human Pax5 is a B-cell specific transcription factor that plays a key role in B cell development. Pax5 is necessary for cells to commit to a B cell lineage, and activates several target genes including CD19 and CD79a. Pax5 inhibits plasma cell differentiation, however, and must itself be inhibited for plasma cell differentiation to occur (Calame et al., 2003). Moreover, Pax5 plays an important role in the establishment of EBV latency in EBV-infected B cells, by activating the latent promoter Wp (Tierney et al. 2000a; Tierney et al. 2000b). In this paper, we show that Z physically and functionally interacts with human Pax5, suggesting that Z may promote lytic replication in B cells by inhibiting Pax5 function.
MATERIALS AND METHODS

**Cell lines:** HeLa cells (ATCC) are cervical carcinoma cells and were maintained in DMEM with 10% fetal bovine serum as well as bacterial and fungal inhibitors (50 U/ml penicillin, 50 U/ml streptomycin, 0.25 ug/ml amphotericin B). Jijoye cells (Shannon Kenney) are EBV-positive Burkitt’s lymphoma cells and were maintained in RPMI 1640 with 10% fetal bovine serum as well as bacterial and fungal inhibitors.

**Plasmids:** The control vector is SVpIE, the Z expression vector contains the Z gene in the SVpIE vector, the Pax5 expression vector contains the human Pax5 cDNA in the EVRF2 vector (James Hagman), and the Pax5 reporter contains the Pax5 binding site from the CD19 promoter (5’ AGA ATG GGG CCT GAG GCG TGA CCA CCG C 3’), in triplicate, in the E1b-CAT vector. Transfections were carried out by the Calcium Phosphate method (Sambrook et al. 1989).

**Fly culture:** Flies were maintained at 20°C in plastic vials on a medium of cornmeal, yeast, molasses, and agar, with methyl 4-hydroxybenzoate added as a mold inhibitor. w1118 was used as the wild-type line.

**P element-mediated transformation:** cDNAs for BZLF1 and Z311 were cloned into the pGMR vector. Z311 has a mutation at amino acid 185 of BZLF1, in the DNA binding domain, of an alanine to a lysine. Germline transformations were performed using the standard P element protocol (Rebay et al. 1993). For GMR::Z, several independent lines were isolated, including GMR::Z.12, all of which had an identical phenotype. For GMR::Z311, several independent lines were isolated, including
GMR::Z311.1/TM3 and GMR::Z311.8/CyO, all with a phenotype very similar to GMR::Z.

**Weak GMR::Z:** To create a dose-sensitive GMR::Z fly, we mobilized the GMR::Z P element from one established GMR::Z line by crossing GMR::Z (Z12) males to Δ2-3 females. The resulting male progeny were crossed to w^{1118} females and their resulting progeny were screened for a more wild-type eye color. One line, referred to as weak GMR::Z, was isolated and has a milder phenotype.

**Fly crosses:** weak GMR::Z males were crossed to svspa-pol (M. Noll) females. weak GMR::Z females were crossed to GMR::Rbf (I. Hariharan; Du et al. 1996), GMR::dap2A (I. Hariharan; de Nooij et al. 1996), GMR::p35 (2-1) (G. Rubin; Hay et al. 1994), or CyclE^{AR95} (I. Hariharan; Du et al. 1996) males. The resulting double-heterozygous progeny from each cross were examined for a modification of GMR::Z eye phenotype, as compared to weak GMR::Z heterozygotes or candidate gene heterozygotes.

**DNA purification:** Plasmid DNA was purified through QIAGEN columns as described by the manufacturer.

**CAT assays:** Cell extracts were prepared 48 hr posttransfection and incubated at 37°C with [14C]chloramphenicol in the presence of acetyl coenzyme A as previously described (Gorman et al. 1982). The percent acetylation of chloramphenicol was quantitated by thin layer chromatography followed by PhosphorImager screening.

**EMSAs:** EMSAs were performed as previously described (Garner and Revzin, 1981). The synthetic double-stranded oligonucleotide used in the binding reactions was 5’-end labeled with ^32P by using the klenow reaction. The Pax5 site consists of the oligonucleotides 5’ AGA ATG GGG CCT GAG GCG TGA CCA CCG C 3’.
protein preparation, Jijoye cells were infected with adenovirus that expressed either the LacZ gene or the Z gene, at a multiplicity of infection of 50. The cells were harvested 48 hr postinfection and nuclear extracts were prepared. The binding reactions were conducted in a buffer consisting of 10 mM HEPES pH 7.9, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1% BSA, and 10% glycerol. Two micrograms of protein were added to each reaction and incubated at room temperature for 10 min before the addition of labeled probe (20,000 cpm). 100 ng of unlabeled Pax5 site was used as a competitor. After addition of the probe, the reactions were incubated 30 min at room temperature and loaded onto a 4% polyacrylamide gel and run in 0.25X Tris-borate-EDTA buffer at room temperature.

**Immunoprecipitation:** HeLa cells were transfected with expression vectors for Z and Pax5, washed twice with cold 1xPBS, and resuspended in Buffer 8 (25 mM HEPES pH 7.5, 100 mM NaCl, 5 mM MgCl2, 100 mM EDTA, 200 ug/ml BSA, 0.01% Tween-20, protease inhibitors). After brief sonication and centrifugation, the lysate was diluted to 500 ul with Buffer 8. 2 ug of anti-Pax5 antibody (Santa Cruz) was added and the mixture incubated for 2 hr at 4°C. Protein G beads (from Zymed) were added and incubated for 1 hr at 4°C. The beads were washed 5 times with Buffer 8, resuspended in SDS-PAGE loading dye, and loaded onto an SDS-PAGE gel.

**Western blot analysis:** Immunoblot analysis was performed as previously described (Adamson and Kenney, 1999). The anti-Z antibody (Argene) and anti-Pax5 (Santa Cruz) were used at a dilution of 1:500. The secondary antibodies [goat-anti-mouse-HRP or donkey anti-goat-HRP (Jackson Immunoresearch)] were used at a dilution of 1:20,000. SuperSignal (Pierce) was used for detection.
**Immunostaining of imaginal discs:** Eye-antenna imaginal discs were immunostained as described (Wolff, 2000). The anti-Z antibody (Argene) was used at a 1:50 dilution, the anti-Cut antibody (Developmental Studies Hybridoma Bank) was used at a 1:50 dilution, the anti-Sv antibody (Markus Noll) was used at a 1:20 dilution and the anti-Elav antibody (Developmental Studies Hybridoma Bank) used at a 1:500 dilution. Each primary antibody was incubated with several (~10) discs overnight. The secondary antibodies [donkey-anti-mouse-CY3 and donkey-anti-rabbit-FITC (Jackson Immunoresearch)] were used at a 1:2000 dilution, and were incubated with the discs for 2 hr. Discs were mounted in anti-fade media (Molecular Probes).

**Acridine Orange staining of imaginal discs:** Eye-antenna discs were dissected in S2 cell media and incubated in S2 cell media containing 0.0016 mM acridine orange (Sigma) for 5 min. The discs were rinsed with S2 cell media and mounted live for confocal microscopy.

**BrdU labeling:** Eye-antenna discs were dissected in S2 cell media and incubated in 1 mg/ml BrdU solution in S2 cell media for 30 min. The discs were washed 3X with 1xPBS, fixed in 4% paraformaldehyde/PBS for 30 min, washed 3X with 1xPBS, and their DNA denatured in 2N HCl/0.1% Triton X/PBS for 30 min. The discs were washed 3X with PBS, blocked in 5% Goat serum/1% BSA/0.1% Triton X/ PBS for 30 min., and incubated overnight at 4°C in anti-BrdU antibody (1:1000 dilution; Phoenix Systems, Inc.). The remainder of the protocol was carried out as for immunostaining of imaginal discs (Wolff, 2000).

**Immunocytochemistry:** HeLa cells were immunostained as previously described (Adamson and Kenney, 2001), except that the incubation mix consisted of 1xTS (20 mM
Tris base, 137 mM NaCl; pH 7.6) and 5% donkey serum, and the wash solution consisted of 1xTS only. The anti-Pax5 antibody (Santa Cruz) was used at a dilution of 1:100 and the anti-Z antibody (Argene) was used at a dilution of 1:50. Secondary antibodies [donkey anti-mouse-FITC and donkey-anti-goat-CY3 (Jackson Immunoresearch) were used at a 1:100 dilution. Hoechst stain was used to visualize nuclei.
RESULTS

BZLF1 expression produces a severe phenotype in Drosophila: To generate BZLF1 transgenic flies, we cloned the coding region of Z into the Drosophila P-element eye-specific expression transformation vector pGMR (Glass-mediated response) (Hay et al. 1994), which expressed Z specifically in all cells posterior to the morphogenetic furrow in the developing (larval) eye (Figure 3A). GMR::Z/+ adult flies expressed a very striking eye phenotype in which all structures except the interommatidial bristles were absent (Figure 1C & D). The bristles were disorganized and scattered throughout the thin cuticle that remained. There was also a loss of pigment cells, since the GMR::Z eyes had no pigmentation and were white in color. In addition, the GMR::Z eye was slightly smaller than the wild-type control. We observed no difference in the phenotype between homozygous and heterozygous GMR::Z flies, demonstrating that GMR::Z lines are not dose sensitive (with the exception of weak GMR-Z, see below). Sectioning of the adult eye revealed that no recognizable eye structures were detectable in GMR::Z eyes (Figure 2B), implying an absence of all normal eye cell types in the adult eye. This result indicated that Z is biologically active in Drosophila cells, and suggested that experiments performed in the fly will be at least partially relevant to human cells.

Z is a transcription factor that binds to AP1 and AP1-like binding sites in DNA (Rickinson and Kieff, 2001; Urier et al. 1989). To determine whether the GMR::Z phenotype was due to rampant and promiscuous transcription via Z-binding sites (or other similar sites within the Drosophila genome), we expressed the DNA-binding defective Z mutant Z311 in the Drosophila eye via the pGMR system. Z311 contains a
mutation in the DNA binding domain of Z that abolishes DNA binding (Giot et al., 1991). The loss of DNA binding ability inhibits Z-mediated transactivation and consequently inhibits EBV lytic replication (Giot et al., 1991). \( \text{GMR::Z311/} + \) flies expressed an identical phenotype as \( \text{GMR::Z/} + \) flies (Figure 1E & F), such that there was a loss of ommatidial structures and pigmentation, and only bristles and cuticle remained. This suggests that the Z-induced phenotypes are not due to unregulated transcriptional activation by Z, but rather to explicit protein-protein interactions between the Z protein and endogenous host proteins.

Since all of our \( \text{GMR::Z} \) lines displayed an invariant and genetically unchangeable eye phenotype (not dose-sensitive), one line with a moderate and dose sensitive phenotype (referred to as \( \text{weak GMR::Z} \)) was created by mobilizing the P element in one of our “strong” \( \text{GMR::Z} \) lines. \( \text{Weak GMR::Z} \) eye imaginal discs expressed less quantity of Z protein than \( \text{GMR::Z} \) (Figure 3B). While the \( \text{GMR::Z} \) lines produced abundant Z protein, the \( \text{weak GMR::Z} \) line produced a small amount of protein (compare Figure 3A & B). Western analysis confirmed that \( \text{weak GMR::Z} \) eye discs expressed greatly reduced levels of Z protein (data not shown).

The \( \text{weak GMR::Z} \) phenotype is dose-dependent; flies heterozygous for this insertion display a moderate eye phenotype (Figure 1G & H), while flies homozygous for \( \text{weak GMR::Z} \) appear similar to the original \( \text{GMR::Z} \) flies (Figure 1I & J). Sectioning of the \( \text{weak GMR::Z/} + \) adult eyes showed that, although some eye tissue was present, there were no recognizable ommatidial clusters (Figure 2C).
**Z inhibits cell proliferation and increases cell death.** In human cells, Z has been shown to inhibit the cell cycle (Cayrol and Flemington, 1996a; Cayrol and Flemington, 1996b; Mauser et al. 2002a; Sinclair 2003). To determine whether GMR::Z inhibited cell cycle we examined cell proliferation via BrdU incorporation (Figure 4). GMR::Z / GMR::Z eye discs showed a significantly lower number of cells that had incorporated BrdU when compared to wild-type eye discs (Figure 4A & B and Table 1). Therefore less cells entered the cell cycle in GMR::Z discs. This result is consistent with previously published data from human tissue culture cells showing that Z expression can arrest the cell cycle (Cayrol and Flemington, 1996a; Cayrol and Flemington, 1996b; Mauser et al. 2002a). Moreover, this reduction in cell division may account for the slightly smaller size of the GMR::Z discs.

We also examined whether the ectopic expression of Z affected cell viability by using acridine orange, a vital stain that is taken up by cells undergoing apoptosis (Figure 4) (Wolff, 2000). We found that, while the level of cell death was comparable between wild-type and weak GMR::Z / + tissues (Figure 4C & D), there was in increase in cell death in the GMR::Z/GMR::Z eyes (Figure 4E and Table 1), suggesting that, at least in part, the smaller size of the imaginal eye discs may also be due to a loss of cells via apoptosis. The levels of cell death and inhibition of cell cycle are clearly dependent upon the level of Z protein present, since the low levels of Z in weak GMR::Z flies did not induce these effects (Figure 4D and data not shown). However, the level of Z produced during lytic replication from an EBV genome is relatively high and is mimicked by the level of Z protein produced from transfected plasmids (unpublished data). Thus we are confident that the level of Z protein produced in GMR::Z flies is comparable to the level
of Z protein produced by EBV. The above results, taken together, suggest that Z’s arrest of the cell cycle and induction of apoptosis can account for the small imaginal eye discs observed in GMR::Z flies. Moreover, the cell cycle arrest by Z supports our line of reasoning that Z’s function within fly cells parallels what has been shown in human cells.

**Z does not affect photoreceptor cells in the developing larval eye.** Since we observed a loss of specific eye cell types in GMR::Z adult eyes, we investigated the fate of cell types in developing larval eye discs, after the Z protein had been expressed, yet before the pupal stage. The eye contains photoreceptor cells, cone cells, bristle cells, and pigment cells. To examine the effect of Z upon developing photoreceptor cells, we employed the anti-Elav antibody, which detects the photoreceptor-specific Elav protein (Robinow and White, 1988). We immunostained third instar larval eye discs with the anti-Elav antibody and found that there was no loss of photoreceptor cell clusters at this early stage in GMR::Z discs (Figure 5A-C). To confirm this finding, we generated a fly line that contained the Z gene under the control of the upstream activating sequence (UAS) promoter element. We crossed these UAS::Z flies to sev::GAL4 flies, creating flies that produce GAL4 only in sevenless-containing (photoreceptor) cells. In these flies, the GAL4 binds to UAS and activates expression of Z only in the photoreceptor cells. No mutant phenotype was produced (data not shown). Therefore, even though Z is expressed in photoreceptor cells and appears to promote cell death in the imaginal discs (as in Figure 4), Z does not appear to negatively affect the photoreceptor cell precursors.
Z prevents Cut expression by interfering with Shaven function. Since we found that Z does not affect developing photoreceptor cells in GMR::Z discs, we turned our attention to the cone cells and bristle cells. As shown in Figure 1, bristles are present in GMR::Z discs, albeit in an unorganized manner. Therefore Z does not appear to prevent bristle cell differentiation. To determine whether there was a disruption of cone cell development in GMR::Z eye discs, we examined the expression of cut, a gene which is expressed in cone and bristle cells of the eye (Blochlinger et al. 1993). We immunostained third instar larval eye discs with the anti-Cut antibody and found that, although the presumptive bristle cells of GMR::Z discs express Cut, the cone cells did not express Cut (Figure 6D). Thus the cut gene did not appear to be expressed in cone cells when Z was expressed.

To elucidate the loss of cut expression in GMR::Z eye discs, we examined an additional cone cell marker, shaven (also known as sparkling). shaven (sv) is required for specification and differentiation of cone cells, primary pigment cells, and bristle cells in Drosophila and has been shown to transactivate Cut expression by activating the cut promoter (Fu and Noll, 1997; Fu et al, 1998). We immunostained third instar larval discs with anti-Shaven antibody and found that, in contrast to the Cut results (where no Cut was present), the GMR::Z cone cells expressed Shaven (Figure 6B). This result suggested to us that Z interfered with Shaven’s transactivation function and therefore prevented cut gene expression, contributing to a loss of cone cell differentiation. The loss of the cone cells at this stage may lead to the subsequent disruption of the entire eye (Siddall et al, 2003).
shaven is a dose-sensitive modifier of GMR::Z. Using our phenotypic characterization of the GMR::Z flies, we decided to perform a candidate gene screen to look for genetic interactors. The centerpiece of such genetic screens is the generation of a dose-dependent mutant phenotype, typically upon the ectopic expression of either the wild-type or mutant target gene in a nonessential tissue such as the eye or wing (Rebay et al. 2000; LaJeunesse et al. 2001). A resulting mutant phenotype represents a defect in a mechanism associated with the target gene. For instance, expression of a dominant negative form of the Drosophila Neurofibromatosis homologue Merlin results in an over-proliferation phenotype characteristic of loss-of-function for a tumor suppressor gene (LaJeunesse et al. 1998). Genes identified as enhancers of the sensitized phenotype can be thought of as genes that encode proteins that function as antagonists or negative regulators of the molecular mechanisms of which the target gene is part; while suppressors of the phenotype can be thought of as genes that encode proteins that function as protagonists or positive regulators of the molecular mechanisms that the target gene is part of. Such genetic screens have been used to identify genes associated with the molecular mechanisms underlying a number of inherited human genetic diseases, including Neurofibromatosis II, neurodegenerative diseases, and obesity (LaJeunesse et al. 2001; Bonini, 2001; Bonini and Fortino, 2003; Schreiter at al. 2004; Bier, 2005).

Using our dose-sensitive weak GMR::Z line (since our strong GMR::Z lines were not dose-sensitive), we performed a candidate gene screen of genes known to affect proliferation, apoptosis, and differentiation in the developing eye. We crossed weak GMR::Z to fly lines that overexpressed the Drosophila E2F/Dp, the Drosophila Rb (Rbf),
the Drosophila p21 (dacapo), or the anti-apoptosis baculovirus p35 gene in the eye, as described in the Methods section. We also crossed weak GMR::Z to a cyclin E null allele (CyclE\textsuperscript{AR95}). All of these crosses yielded flies whose eyes maintained the same phenotype as weak GMR::Z eyes (data not shown). Therefore, the overexpression of a cell cycle promoter (E2F), cell cycle inhibitors (Rb, p21, and the null cyclin E allele), and even an anti-apoptotic gene (p35) were unable to overcome the Z-induced phenotype.

Since we found that Z may be preventing the differentiation of cone cells, we further examined the relationship between Z and sv and investigated whether there was a genetic interaction between weak GMR::Z and sv. To do this, we crossed weak GMR::Z to sv\textsuperscript{spa-pol} (a sv null allele that yields no functional Sv protein in the eye) (Fu and Noll, 1997), and examined the heterozygous sv and GMR::Z progeny (Figure 7). sv is recessive and flies heterozygous for sv mutations express a normal eye phenotype (Figure 7A & B). However, flies heterozygous for sv\textsuperscript{spa-pol} and weak GMR::Z displayed a strong GMR::Z phenotype, where there was a loss of facets (Figure 7E & F). Therefore there is a genetic interaction between Z and sv, such that the presence of Z sensitizes the cells to a reduction in Sv levels. These results support our hypothesis that Z functions, at least in part, to disrupt the development of the Drosophila eye by interfering with sv function. This Z/Sv interaction likely contributes to the loss of differentiated cone cells.

**Z inhibits human Pax5-mediated transactivation.** Since the goal of our study was to identify proteins that interact with Z in Drosophila and translate these findings to the normal hosts for EBV, humans, we next turned our attention to sv human homologs. The sv gene encodes a paired-box gene that is the homolog of the human Pax2/Pax5/Pax8
proteins (Fu and Noll, 1997; Fu et al, 1998). Interestingly, Pax5 is a transcription factor that is expressed in B cells and central nervous system cells, while Pax2 is expressed in kidney and central nervous system cells, and Pax8 is expressed in kidney, thyroid, and central nervous system cells (Chi and Epstein, 2002). Pax5 is required for the development of B cells (Urbanek et al. 1994; Baker and Reddy, 1995; Enver, 1999; Mikkola et al. 2002), and has been shown to activate the EBV latent Wp promoter (Tierney et al. 2000a; Tierney et al. 2000b). We focused on this homolog in view of the fact that Pax5 and EBV share the same environment (B cells) in the human host’s immune system. No connection had been previously established between Z and Pax5.

To determine whether Z altered Pax5 function in human cells, we examined Z’s ability to disrupt human Pax5 transcriptional function. HeLa cells were transfected with Pax5 and Z expression plasmids and a reporter plasmid containing a Pax5 binding site upstream of a minimal promoter element and the chloramphenicol transferase (CAT) gene (Figure 8A). In repeated experiments, addition of Z with Pax5 compromised the ability of Pax5 to drive CAT expression from the reporter construct, suggesting that the interaction that we observed between Z and Sv in Drosophila cone cells is also conserved between Z and Pax5 in human cells. Given these results, we sought to investigate the mechanism that Z may use to neutralize Pax5 function. To test whether Z altered the ability of Pax5 to bind DNA, we performed electromobility shift assays using several different Pax5 binding sites. We found that Z had no effect upon Pax5 binding to DNA, and an example of this is shown in Figure 8B. The levels of Pax5 binding in the presence of Z was identical to, if not more than, the levels of Pax5 binding in the presence of the
control protein LacZ (compare in Figure 8B, lanes 3 & 4). These results suggest that Z may abrogate Pax5 transcriptional activity by a mechanism not altering DNA binding.

**Z physically associates with and stabilizes Pax5 protein.** We next examined the ability of Z to physically associate with Pax5. We transfected HeLa cells (which do not contain endogenous Pax5 protein) with expression plasmids for Z and Pax5, coimmunoprecipitated the proteins with an anti-Pax5 antibody, and performed immunoblot analyses with anti-Z and anti-Pax5 antibodies (Figure 9). We found that the anti-Pax5 antibody immunoprecipitated the Pax5 protein (Figure 9, lanes 1 and 3), as well as co-immunoprecipitated Z protein (Figure 9, lane 3). Z did not co-immunoprecipitate with the anti-Pax5 antibody in the absence of Pax5 protein (lane 2). Thus there is a physical interaction between Z and Pax5. In support of a Z/Pax5 physical interaction, we have previously found that Z is able to relocalize Pax5 protein within the nuclei of human cells, such that when Z binds to mitotic chromosomes it tethers Pax5 to these chromosomes when Pax5 does not normally bind to mitotic chromosomes (Adamson, 2005).

In addition, we discovered that expression of Z seemed to stabilize Pax5 protein levels. HeLa cells that co-expressed both Pax5 and Z proteins had much higher levels of Pax5 protein present than cells that expressed the Pax5 protein alone (Figure 10, compare B & D). In a similar scenario, it has been previously observed that Z stabilizes the p53 protein, while preventing p53 from transactivating its target promoters (Mauser et al. 2002b). These results, taken together, suggest that Z might interfere with Pax5 function
by binding to Pax5 and preventing a critical interaction necessary for expression of Pax5 target genes.
DISCUSSION

In this paper we demonstrate that the Drosophila model system can be a powerful discovery tool for new genes that are involved in viral pathogenesis. We have expressed the Epstein-Barr virus immediate-early protein Z in the Drosophila eye and found that Z induced a mutant eye phenotype. To establish the Drosophila system as a model for early EBV infection it was necessary to determine whether the phenotype was biologically relevant and whether the phenotype was amenable to genetic manipulation. The expression of a mutant eye phenotype demonstrated that the EBV Z protein had biological activity within the context of a foreign cell type; however whether this was relevant remained to be tested.

Z is a transcription factor that binds to and activates the promoters of early EBV genes (Chevallier-Greco et al. 1986; Cox et al. 1990; Giot et al. 1991; Holley-Guthrie et al. 1990; Kenney et al. 1989; Quinlivan et al. 1993) via AP1 and AP1-like binding sites (Rickinson and Kieff, 2001; Urier et al. 1989). Given that Z functions as a transcriptional activator, one rationale for the GMR::Z eye phenotype might have been simply rampant misexpression of endogenous Drosophila genes leading to aberrant development of the eye. To test this possibility we examined the phenotypes expressed by a DNA binding null mutant of Z referred to as Z311; Z311 cannot transactivate promoters. Ectopic expression of Z311 in the Drosophila eye produced the same phenotype as wild-type Z, and demonstrated that the phenotypes we initially observed were not due to unregulated transcription but likely due to other protein-protein interactions.
In human cells, Z physically interacts with a number of different cellular factors and through these interactions affects the cell cycle and several different signal transduction pathways (Adamson and Kenney, 1999; Adamson et al. 2000; Adamson and Kenney, 2001; Mauser et al. 2002b). In Drosophila cells, we examined the effect of Z on the cell cycle, specifically examining its effects upon cellular proliferation. In human cells, expression of Z has been shown to arrest the cell cycle (Mauser et al. 2002b), and likewise, the Drosophila eye imaginal disc cells that express Z have a reduced number of cells entering S phase. This result, along with the interesting phenotype, suggested to us that Z may be functioning in fly cells in a homologous manner to what has been demonstrated in human cells. Therefore, we believe that Drosophila is an appropriate system in which to hunt for proteins that physically and functionally interact with the Epstein-Barr virus Z protein.

The phenotypes expressed by all lines of GMR-Z (with the exception of weak GMR-Z) demonstrated no dose-sensitivity. In these flies we observed a smoothened eye with disorganized bristles and a loss of pigment cells. However, we were able to identify a weakly-expressing GMR::Z line, which we refer to as weak GMR::Z, that expressed reduced levels of Z protein. Heterozygous weak GMR::Z eyes had a slightly smoothened eye phenotype with a reduction of some (but not all) pigment cells. However, homozygous weak GMR::Z eyes expressed a phenotype that resembled the “strong” GMR::Z eyes (smoothened with no pigment). This suggested to us that weak GMR::Z would be a useful tool for identifying dominant second-site modifiers. With weak GMR::Z we began a candidate-gene genetic screen, to identify known genes or pathways that could modify the weak GMR::Z phenotype. We looked for interactions between Z
and E2F/Dp, Rbf, p21, cyclin E, p35, and shaven. The cell cycle promoters and inhibitors had no effect upon the weak GMR::Z phenotype. The anti-apoptotic baculovirus p35 protein also did not alter the weak GMR::Z phenotype. The only candidate that did modify the weak GMR::Z phenotype was the shaven mutant.

Characterization of the GMR::Z eye phenotype revealed that expression of Z prevents the formation of lenses and pigmentation, presumably due to a loss of pigment and cone cells in the developing eye. This is strikingly similar to what has been described for loss of sv; expression of Z induces pigment cell and cone cell defects, as well as an increased number of apoptotic cells, all similar to sv loss of function (Siddall et al., 2003). Loss of Sv activity in eye imaginal disc cone cells leads to a disruption of signaling to the surrounding cells, which induces apoptosis of these surrounding cells (Siddall et al., 2003). Furthermore, in a manner similar to sv loss-of-function, we observed a reduction of downstream activation of cut, suggesting that Z functions in the Drosophila eye, at least in part, by disrupting the normal transcriptional function of the Sv protein.

Given that the goal of this work was to establish the Drosophila model system as a tool for identifying genes involved in EBV virus/host cell interactions, after identifying the strong genetic interaction between Z and sv in the Drosophila eye, we wished to determine whether this genetic interaction was biologically significant in human cells. Since a dose-dependent genetic interaction may reveal an underlying functional or physical interaction between the protein products of the two genes involved (Rebay 2000; LaJeunesse et al., 2001), we wished to determine whether Z disrupted the transcriptional activity of the human sv homologue, Pax5, and whether it did so through a physical
interaction. Using co-immunoprecipitation, we showed that Z physically associated with Pax5. Moreover, we demonstrated that Z inhibited Pax5-mediated transcriptional activation in reporter assays, even though Z seemed to stabilize Pax5 protein levels. This is similar to what has been shown with the interaction between Z and p53, where, in Z-expressing cells, there is an increase in p53 protein levels (as well as an increase in p53 binding to p53 sites in DNA), yet there was an inhibition of p53 transactivation ability such that p53 targets p21 and MDM2 were not induced (Mauser et al. 2002b). In regard to Pax5, Z may bind to and block the transactivation region of Pax5, so that even though Pax5 can still bind to DNA, it cannot recruit transcriptional machinery to a promoter.

The EBV Z protein has been shown to disrupt the function of several human genes involved in transcription, including CBP, p53, and NF-κB, through direct protein-protein interactions (Adamson and Kenney, 1999; Gutsch et al., 1994; Mauser et al., 2002b). Our results suggest that Z-binding to Pax5 may disrupt the ability of Pax5 to function as a transactivator, affecting both B-cell specific genes and the EBV latent Wp promoter. It has been shown that EBV lytic replication occurs in B cells with a plasma cell morphology (Niedobitek et al., 1997), and in this state Pax5 must be inactive. The Blimp-1 protein has been shown to repress Pax5 expression during normal plasma cell differentiation (Calame et al, 2003). Z may also contribute to Pax5 inhibition during EBV lytic replication by binding to Pax5 and blocking its transactivation domain. In addition, Z may promote lytic replication in newly-infected B cells, in lieu of latency establishment, by inhibition of Pax5 transactivation of the Wp promoter. Wp is the first latent promoter activated when EBV switches from lytic to latent replication, and Wp activates transcription of the latent EBNA2 and EBNA-LP genes (Tierney et al. 2000b).
The inhibition of Pax5 by Z may prevent Wp activation and delay the onset of viral latency. The promotion of lytic replication would yield more viral particles and consequently more EBV-infected cells.

It is important to note that despite the fact that Epstein-Barr virus is a well-studied viral system, we have been able to identify a novel interaction between Z and Pax5 by using an innovative method. The effectiveness of this pilot project gives credence to the promise that larger scale screens will be able to identify other genes involved in EBV’s early commandeering of cellular machinery, and will provide a richer and fuller picture of how a virus uses cellular machinery to propagate its own life cycle.
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**FIGURE LEGENDS**

**Figure 1.** *GMR::Z* and *weak GMR::Z* adult eye phenotypes. Magnification is presented at both 500X and 2000X. A, B. Wild-type eye. C, D. *GMR::Z/ +* eye. Note loss of ommatidia. E, F. *GMR:: Z311/+* eye. Phenotype is similar to *GMR::Z*. G, H. *Weak GMR::Z/ +* eye. Phenotype is intermediate, between wild-type and *GMR::Z*. I, J. *Weak GMR::Z/ weak GMR::Z* homozygous eye. Phenotype is similar to *GMR::Z*.

**Figure 2.** Adult *GMR::Z* eyes have no recognizable eye cells. Adult heads were stained with Giemsa and sectioned. A. Wild-type eye. Arrow points to an ommatidium. B. *GMR::Z/ +* eye. C. *Weak GMR::Z/ +* eye.

**Figure 3.** *Weak GMR::Z* flies express less Z protein than *GMR::Z* flies. Late third instar eye imaginal discs were immunostained for Z. A. *GMR::Z/ +* eye disc. B. *Weak GMR::Z/ +* eye disc. Disc immunostainings were performed concurrently and photographed with the same exposure times. Results were consistently reproducible. Arrows refer to the morphogenetic furrow.

**Figure 4.** Homozygous *GMR::Z* eye discs undergo less proliferation and more apoptosis than wild-type. Late third instar eye imaginal discs were stained with BrdU (A-B) or acridine orange (C-E). A. Wild-type eye disc. B. *GMR::Z/ GMR::Z* eye disc. C. Wild-type eye disc. D. *Weak GMR::Z/ +* eye disc. E. *GMR::Z/ GMR::Z* eye disc. Arrows refer to the morphogenetic furrow of each disc.

**Figure 5.** *GMR::Z* eye discs maintain their photoreceptors. Late third instar eye imaginal discs were immunostained with an anti-Elav antibody. Disc stainings were performed concurrently and photographed with the same exposure times. A. Wild-type
Figure 6. Cut protein levels, but not Sv protein levels, are reduced in GMR::Z eye discs. A, C, wild-type eye discs. B, D, GMR::Z / GMR::Z eye discs. Late third instar eye imaginal discs were immunostained for Sv (A, B), and Cut (C, D). Note the loss of Cut staining in the GMR::Z disc (D), as compared to the wild-type disc (C). In D, the arrow refers to the peripodial cells, which are part of the membrane that surrounds the disc. Some of these cells show staining with the anti-Cut antibody, but the staining is not in the disc itself.

Figure 7. Genetic interaction between weak GMR::Z and sv. A, B. sv/+ eye. C, D. weak GMR::Z/+ eye. E, F. weak GMR::Z/+ ; sv/+ eye. The phenotype shown in F is much more severe than that of B and D.

Figure 8. Z decreases Pax5-mediated transactivation. A. HeLa cells were transfected with expression plasmids as indicated, along with Pax5-CAT reporter plasmid. CAT assays were performed in four separate experiments and are presented as percent acetylation. B. Electromobility shift assay. Jijoye cells were uninfected, infected with an adenovirus containing the control gene LacZ, or infected with an adenovirus containing the Z gene. Extracts of each were incubated with the CD19 gene Pax5 binding site. comp. = cold CD19 site competitor added. * indicates partially degraded Pax5 protein that still binds to the CD19 site.

Figure 9. Z physically associates with Pax5. HeLa cells were transfected with expression plasmids for Z alone (lanes 2, 4, 6), Pax5 alone (lanes 1, 5) or Z plus Pax5 (lanes 3, 7). Anti-Pax5 antibody was used to co-immunoprecipitate the Pax5/Z complex
(lanes 1-3). An anti-Z antibody (lower panels) and an anti-Pax5 antibody (upper panels) were used for immunoblotting. The anti-Z antibody immunoblots revealed the co-immunoprecipitated Pax5/Z complex (lane 3), as well as the levels of Z protein present in the cell extracts used (lanes 6, 7). The anti-Pax5 antibody immunoblots revealed the levels of Pax5 protein immunoprecipitated with the anti-Pax5 antibody (lanes 1-3), as well as the levels of Pax5 proteins present in the cell extracts used (lanes 5, 7).

**Figure 10.** Z stabilizes Pax5 protein levels. HeLa cells were transfected with expression plasmids for Pax5 alone (A, B), Z plus Pax5 (C-F), or Z alone (G, H). Cells were immunostained with both anti-Z and anti-Pax5 antibodies, as well as the DNA stain Hoechst. Arrows in (B) indicate cells that express Pax5, albeit at low levels. Note that when Pax5 is co-expressed with Z, Pax5 is present in high levels (D). All images were taken at the same time with identical exposure times.
Table I: Cell counts for Acridine Orange and BrdU labeling in wild-type and GMR::Z eye imaginal discs.

<table>
<thead>
<tr>
<th>Genotype/Experiment</th>
<th>$n$</th>
<th>Number of cells labeled</th>
<th>Percent Deviation from wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>$w^{1118}$ (wild-type), BrdU</td>
<td>10</td>
<td>159±14</td>
<td></td>
</tr>
<tr>
<td>GMR::Z / GMR::Z, BrdU</td>
<td>10</td>
<td>112±12</td>
<td>-30%</td>
</tr>
<tr>
<td>$w^{1118}$ (wild-type), Acridine Orange</td>
<td>12</td>
<td>100±16</td>
<td></td>
</tr>
<tr>
<td>GMR::Z / GMR::Z, Acridine Orange</td>
<td>11</td>
<td>221±42</td>
<td>+221%</td>
</tr>
</tbody>
</table>
A wild-type GMR::Z weak GMR::Z

A

B

C

wild-type

GMR::Z

weak GMR::Z
sv/+  weak GMR::Z/+  weak GMR::Z/+; sv/+
A

Percent acetylation

vector  Pax5  Z  Pax5+Z

0 0.5 1 1.5 2 2.5 3 3.5 4

B


Pax5

Free probe

1 2 3 4 5 6 7
Pax5:  +  -  +  -  +  -  -  +  
Z:     -  +  +  +  -  +  +  +  
anti-Pax5 Ab.: +  +  +  -  -  -  -  -  
protein G beads: +  +  +  +  -  -  -  -  

IgG  ➔
Pax5  ➔

Z ➔

1  2  3  4  5  6  7
Transfection

Pax5 alone

A DNA

B αPax5

C DNA

D αPax5

Pax5 + Z

E DNA

F αZ

Pax5 + Z

G DNA

H αZ

Z alone