ESTABLISHMENT OF A LYMPHOBLASTOID CELL LINE USING A MUTANT MDV CONTAINING A GREEN FLUORESCENT PROTEIN EXPRESSION CASSETTE

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Summary. – We have previously described the construction and characterization of mutant Marek's disease viruses (MDVs) having mutations within the unique-short (U_S) region of the genome that have retained oncogenicity (Anderson *et al.*, 1998; Parcells *et al.*, 1995). We have also reported the characterization of lymphoblastoid cell lines (LBCLs) derived using these mutant viruses (Parcells *et al.*, 1998). These mutant MDVs were constructed using a *lacZ* expression cassette. Expression of *lacZ* was found to be constitutive during lytic infection but was found to be tightly repressed in tumors and the derived LBCLs. The construction of these viruses and the analysis of *lacZ* induction required the use of toxic substrates or antibody staining to detect *lacZ* expression. We now report the establishment of an MDV lymphoblastoid cell line, MDCC-UA04, that was derived from a tumor induced by an MDV having an insertion of a green fluorescent protein expression cassette into the US2 gene. Like previous mutant-derived LBCLs, expression of the marker cassette is constitutive in lytic infection, but repressed in tumors and in the UA04 cells. UA04 cells express $CD3^{low}$, CD4, TCR-2^{low}, MHC class II, and CD28 antigens on their surface. The percentage of UA04 cells expressing GFP is generally low (5–7%), but increases markedly within 48 hrs of 5'-iododeoxyuridine (IUdR) treatment (20–30%) in a manner similar to many MDV lytic antigens. Thus, induction of GFP expression in UA04 cells can serve as a non-invasive marker for MDV reactivation from latency.

Key words: MDV; mutants; cell lines; latency; mutagenesis

Introduction

Marek's disease (MD) of chickens is characterized primarily by the rapid appearance of T-cell lymphomas (Calnek and Witter, 1997; Venugopal and Payne, 1996). MD is caused by an acute-transforming, cell-associated alphaherpesvirus called MDV that has many biological properties of gammaherpesviruses. MDV establishes a lytic albeit productive-restrictive infection in B-cells and some T-cells and establishes latency primarily in CD4+ T-cells (Calnek, 1985). Of the T-cells latently-infected, some become transformed and give rise to lymphomas that lodge in various organs and tissues. Cell lines derived from MDV lymphomas are largely CD4+ lymphoblasts which appear to be activated (Nazerian and Sharma, 1975; Powell, 1975; Schat *et al.*, 1982, 1991).

The molecular basis of MDV transformation remains unclear, although several MDV gene products have been implicated due to their expression in MDV LBCLs, their attenuation-associated loss of expression during repeated passage in cell culture, or their lack of expression in non-oncogenic serotypes of MDV (Bradley *et al.*, 1989; Chen *et al.*, 1992; Cui *et al.*, 1991; Hong and Coussens, 1994; Hong *et al.*, 1995; Ikuta *et al.*, 1985; Jones *et al.*, 1992; Nakajima *et al.*, 1987; Peng *et al.*, 1992, 1994). In MDV tumors and LBCLs, the expression of MDV genes is tightly regulated and limited to the repeated regions of the virus genome (Bradley *et al.*, 1989; Calnek *et al.*, 1981; Hirai *et al.*, 1981; Maray *et al.*, 1988; Silver *et al.*, 1979). MDV LBCLs can be induced to express lytic antigens via treatment with nucleoside analogs (Dunn and Nazerian, 1977), and this has served as a model for MDV reactivation from latency.

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The direct analysis of the genes associated with MDV pathogenicity and oncogenicity has been hampered by the cell-associated nature of MDV and the consequent difficulty in generating mutants. To this end, we have recently reported the construction and characterization of mutant MDVs using the RB1B strain of MDV that retained oncogenicity (Anderson *et al.*, 1998; Parcells *et al.*, 1995). Moreover, we have established lymphoblastoid cell lines from tumors induced by the recombinant viruses (Parcells *et al.*, 1998). These cell lines are predominantly CD4+ T-cells, as are most MDV-derived cell lines (Nazerian and Sharma, 1975; Powell, 1975; Schat *et al.*, 1982, 1991). We have found that the *lac*Z cassette within the recombinant MDV genomes is regulated in a manner similar to other MDV lytic genes (i.e., pp38, gB, gI, etc.). The *lac*Z gene in this cassette is expressed from an SV40 early promoter and its expression is constitutive during the lytic infection of chicken embryo fibroblasts (CEFs) (Anderson *et al.*, 1998; Cantello *et al.*, 1991; Morgan *et al.*, 1996; Parcells *et al.*, 1994a,b, 1995). The regulation of the *lac*Z cassette within tumors and tumor-derived cell lines, however, appears to be highly repressed. The expression of *lac*Z could be induced with IUdR making these cells useful in the study of MDV latency and reactivation.

We now report the establishment of a LBCL using a recombinant MDV (RB1BUS2*gfpΔ*) having a green fluorescent protein (GFP) expression cassette inserted into the US2 homolog gene. Expression of GFP was tightly repressed in tumors induced by this virus, but after storage of tumor sections for >1 hr, tumor cells began expressing GFP. Likewise, tumor cell suspensions contained a low percentage of GFP-expressing cells, but after 72 hrs in culture the number of GFP-positive cells increased dramatically. We have established a cell line from one ovarian tumor induced by RB1BUS2*gfpΔ*, MDCC-UA04 (UA04). These cells are CD4+ but express very low levels of CD3 and T-cell receptor class 2 (TCR-2). In culture, approximately 5 to 7% MDCC-UA04 cells expressed GFP. After treatment with IUdR for 48 hrs, 20 to 30% of cells expressed GFP, which is consistent with the induction of MDV antigen expression associated with the reactivation from latency. Consequently, UA04 cells can be used to study MDV latency and reactivation through their expression of GFP in these cells.

Materials and Methods

Cells and viruses. MDV strain RB1B has been described previously (Schat *et al.*, 1982), as has been the construction of recombinant MDVs using this strain (Parcells *et al.*, 1995). The construction and characterization of the GFP-expressing recombinant MDV, RB1BUS2*gfpΔ*, will be described elsewhere (Parcells *et al.*, in preparation). In short, RB1BUS2*gfpΔ* contains a 1.8 kbp expression cassette from plasmid pMD312 (Fig. 1A) inserted into a *Bgl*II site within the coding sequence of the MDV US2 gene (Cantello *et al.*, 1991). Plasmid pMD312 contains the gene encoding a red-shifted GFP (S65T-GFP, Clontech, Palo Alto, CA) subcloned into plasmid pBK-CMV (Stratagene, La Jolla, CA) in frame with the *lac*Z alpha-peptide. This plasmid also contains a 400 bp deletion of the SV40 splicing region downstream of the GFP gene. This region is believed to be responsible for the attenuated phenotype of a previously described mutant MDV, RB1BUS2*gfp* (Parcells *et al.*, 1996).

Cell line MDCC-UA04 was established from an ovarian lymphoma explanted from a RB1BUS2*gfpΔ* -infected chicken. This cell line was established essentially as described for other MDV tumor cell lines (Anderson *et al.*, 1998; Parcells *et al.*, 1995), with one exception. Due to the high level of spontaneous reactivation of virus from these tumor cells (Fig. 2B), after one week in culture, dead cells were removed via Ficoll separation (Histopaque 1119, Sigma). The viable cells were then diluted across four 96-well microtiter plates and observed for proliferation. The cell line UA04 was established from one single well and therefore represents a clonal population. These cells were adapted from modified LM Hahn medium to RPMI + 20% fetal bovine serum (growth medium, Life Technologies).

Southern blot hybridization. To verify the absence of parental RB1B virus in cell line UA04, DNA from uninfected and RB1B-infected CEFs, UA04 cells, and RB1BUS2*gfpΔ* reactivated from UA04 cells onto CEFs, was digested with *Eco*RI, separated on a 0.8% agarose gel and transferred to nitrocellulose using standard methods (Sambrook *et al.*, 1989). MDV-specific and GFP-specific probes (see Fig. 1B), were labeled with biotin using a non-isotopic labeling kit (New England Biolabs). Hybridization and detection of the probes were performed according to the instructions of the manufacturer and for final visualization of bands, Kodak BioMax MR film was used for autoradiography.

Fluorescence microscopy. When the RB1BUS2*gfpΔ*-induced ovarian tumor was explanted for cell line establishment, portions were sectioned in PBS + 80% glycerol and photographed using a Nikon Diaphot microscope with epifluorescence and a 490 nm filter (Nikon). After storage at room temperature, the sections were again photographed. Likewise, tumor cells in culture were photographed at 1 and 3 days post plating.

IUdR induction. To induce MDV antigen and GFP expression, UA04 cells were seeded at a density of 5 x 10^5 cells per ml in growth medium containing 25 µg/ml IUdR (Sigma) at 24 and 48 hrs prior to analysis. Untreated UA04 cells were used as the 0 hr treatment point. To serve as a non-MDV T-lymphoblastoid cell line control, RECC-CU91(Schat *et al.*, 1992) cells were treated as above.

Flow cytometric analysis. For surface antigen characterization, cell lines UD14 (RB1B-transformed, Parcells *et al.*, 1998), CU91 (Schat *et al.*, 1992) and UA04 were stained using unlabeled monoclonal antibodies to chicken CD3, CD4, CD8, CD8ß, T-cell receptor classes 1, 2 and 3 (TCR-1, TCR-2, TCR3, respectively), CD28, and surface IgM/D (Southern Biotechnology Associates) according to the manufacturer's instructions. Monoclonal antibodies to chicken MHC class II (P2M11) and pan-B-cell antigen, Bu, were gifts of Dr. Hyun S. Lillehoj, USDA-ARS, Beltsville, MD. For detecting bound monoclonal antibodies, phycoerythrin (PE)-conjugated goat-anti-mouse immunoglobulin G (whole mo-

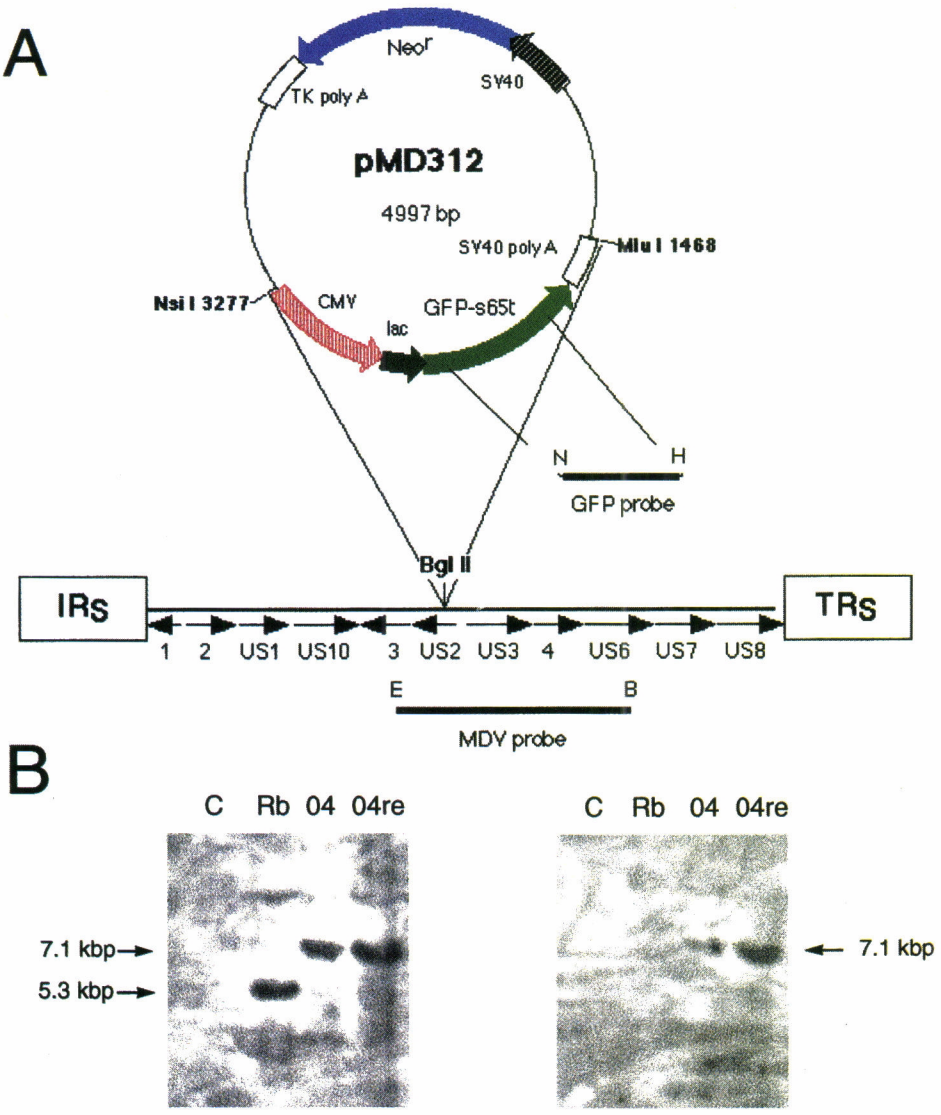

Fig. 1

Structure of the mutant MDV, RB1BUS2*gfpΔ*, in cell line UA04

(A) depicts the structure of the U_S region of MDV (Fukuchi *et al.*, 1984) and the open reading frames encoded (Brunovskis and Velicer, 1992). The site of the GFP expression cassette insertion is shown. The 1.8 kbp *Nsi*I-*Mlu*I fragment from plasmid pMD312 was inserted at the *Bgl*II site within the U_S2 coding region. Also shown are the probes used in (B). The MDV-specific probe was a 4.3 kbp *Eco*RI-*Bam*HI fragment from plasmid pMD100 (Cantello *et al.*, 1991). The GFP-specific probe was a 700 bp *Nhe*I-*Hin*dIII fragment from the GFP gene (Clontech). (B) depicts the results of Southern blot hybridization of *Eco*RI-digested DNA from CEFs (C), RB1B strain MDV-infected CEFs (Rb), UA04 cells (04), and RB1BUS2*gfpΔ* reactivated from UA04 cells (04re). The blot on the left was hybridized with the MDV-specific probe shown in (A) spanning the site of GFP-cassette insertion. The blot on the right was hybridized with the GFP-specific probe shown in (A). Since there are no *Eco*RI sites in the GFP-cassette, insertion of this cassette results in a 1.8 kbp increase in size of the 5.3 kbp MDV *Eco*RI fragment.

lecule) was used (Sigma). Cell data was acquired using a Becton Dickinson FACSort. For each sample, 10,000 ungated cells were acquired and analyzed using CellQuest® software (Becton Dickinson). Unstained and secondary reagent-treated samples were used as negative controls for staining. Live cells were gated according to their light-scattering profile (forward vs side scatter).

For flow cytometric analysis of IUdR-treated cells, cultures were collected via centrifugation after treatment (see above), washed twice with 10 ml of PBS + 1% BSA and resuspended in 0.5 ml this buffer. As above, 10,000 cells were acquired for analysis. For GFP (FL-1) fluorescence detection, detectors settings were used from previous analyses of fluorescein isothiocyanate (FITC)-la-

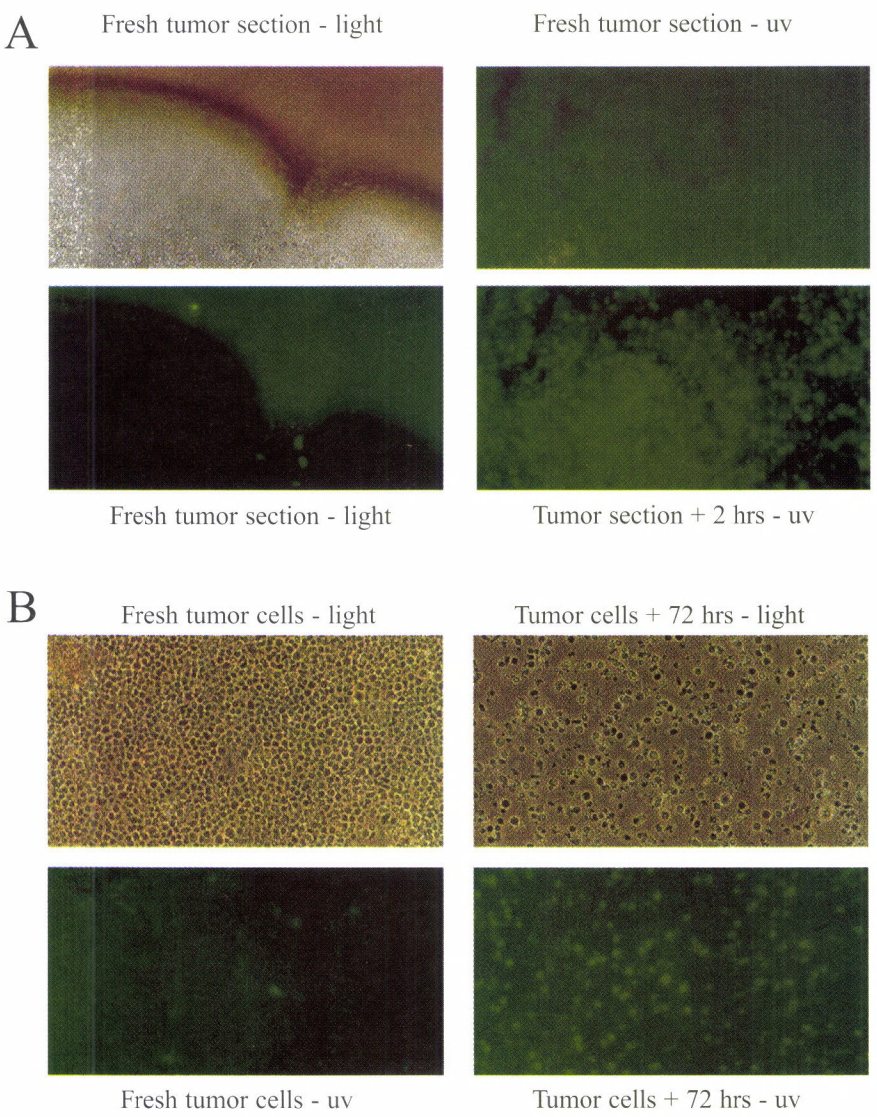

Fig. 2
Light and fluorescence micrographs of an RB1BUS2*gfpΔ*-induced ovarian tumor (A) and tumor cells in culture (B)
Total magnification is approximately 40x.

beled cells (Parcells *et al.*, 1998). CU91 cells were used as a non-fluorescent, non-MDV-transformed T-lymphoblastoid control.

Results and Discussion

We report the establishment of an MDV-transformed LBCL using a GFP-expressing MDV. This cell line, UA04, and the virus that is reactivated from this cell line, RB1BUS2*gfpΔ*, contain only the mutant MDV genome as demonstrated by Southern blot analysis (Fig. 1B). Since there are no *Eco*RI sites within the expression cassette (Fig. 1A), a probe spanning the site of insertion hybridizes to a 5.3 kbp fragment of the parental virus, RB1B, DNA and 7.1 kbp fragments in UA04 and reactivated RB1BUS2*gfpΔ* DNAs (Fig. 1B). These results indicate that the UA04 cells were transformed by the mutant virus and contained no parental virus.

The UA04 cell line was established from an ovarian lymphoma induced by RB1BUS2*gfpD*. This lymphoma was not spontaneously fluorescent but contained relatively few fluorescent cells (Fig. 2A). This observation was consistent with our previous findings that tumors induced by *lac*Z-

A

cell line	virus	tumor spont. react.	immunophenotype
MDCC-UD14	RB1B	200 PFU/10^4 cells	CD3+, CD4+, TCR-2+, TCR-3+ MHC-II+, CD28+
MDCC-UA04	RB1BUS2gfpΔ	20 PFU/10^4 cells	$CD3^{low}$, CD4+, $TCR\text{-}2^{low}$ MHC-II+, CD28+
RECC-CU91	REV-T	ND	$CD3^{low}$, $TCR\text{-}3^{low}$, MHC-II+, CD28+

B

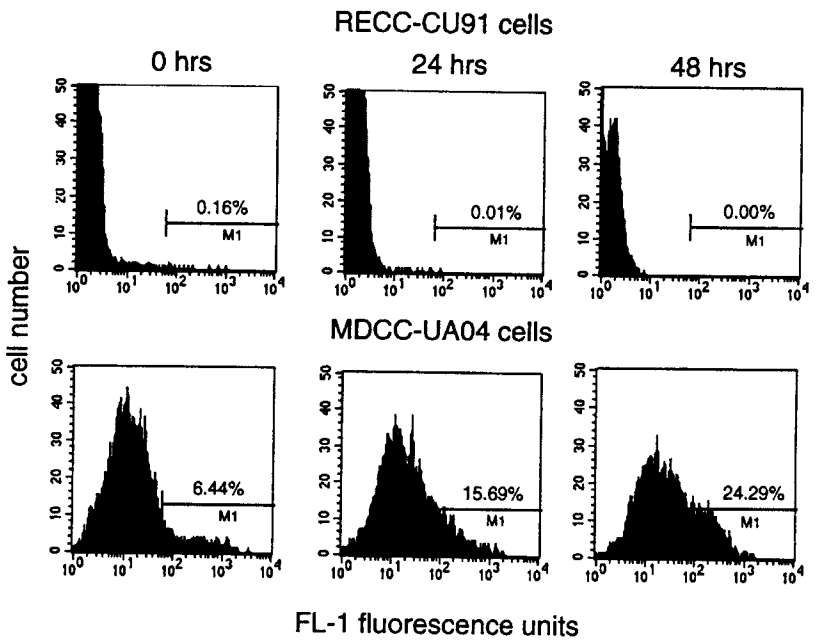

Fig. 3

Characterization of the surface antigens and spontaneous and induced GFP expression in UA04 cells

(A) is characteristics of cell lines MDCC-UD14 (RB1B-transformed, Parcells *et al.*, 1998), MDCC-UA04 and RECC-CU91 (Schat *et al.*, 1992). All cell lines were stained for CD3, CD4, CD8, CD8ß, TCR-1, TCR-2, TCR-3, MHC-II, CD28, IgM/D, and Bu expression. The immunophenotype is listed as those antigens which were present (all others were absent). The spontaneous reactivation frequency is given as the number of PFU obtained after cocultivation in triplicate with secondary CEFs and was not determined (ND) for CU91 cells. (B) depicts histogram analysis of the percentage of RECC-CU91 (top) or MDCC-UA04 (bottom) cells having green fluorescence (FL-1) after treatment with IUdR (25 µg/ml) for 0, 24 and 48 hrs. The x-axis is a log scale of green fluorescence channels. The y-axis is a linear scale of cell number. The percentages shown on each histogram represent the percentage of gated cells having green fluorescence in the range denoted M1.

containing mutant MDVs showed little to no *lacZ* expression (Parcells *et al.*, 1995, 1998). Our results with RB1BUS2*gfpΔ* further support the hypothesis that autologous promoters within the context of the MDV genome are regulated in a manner similar to MDV lytic genes in MDV tumors and cell lines.

Moreover, after letting the sections of ovarian lymphoma stand for over an hour, fluorescent cells began appearing in increasing number, suggesting that the RB1BUS2*gfpΔ* genome was beginning to reactivate (Fig. 2A). The rest of the ovarian lymphoma was ground up for the establishment of a cell line. Most of these cells were also non-fluorescent, but a majority became fluorescent within three days of being placed in culture (Fig. 2B). Concomitant with their fluorescence, these cells died in culture, suggesting that the virus was indeed reactivating and the cells were undergoing lytic infection. In order to obtain a cell line, the dead cells were removed from culture via Ficoll gradient centrifugation and

the viable cells were diluted and plated across four 96-well microtiter plates. From one of these wells we were able to establish the UA04 cell line. The difficulty in establishing this cell line may be due to the activity of the CMV promoter in the context of the unique short (U_S) region which encodes genes associated primarily with lytic infection (Brunovskis and Velicer, 1992; Brunovskis *et al.*, 1992). An enhancement of the lytic infection through upregulation of MDV U_S region genes has also been implicated in the phenotype of the RM-1 virus (Jones *et al.*, 1996) which contains an LTR insertion near the junction of the IR_S and U_S regions.

Like most MDV cell lines described to date, the UA04 cell line is CD4+ and expresses MHC class II suggesting that the cells are activated T-helper cells. A puzzling phenomenon concerning MDV T-cell lines is the disproportionate expression of T-cell receptor and CD3 to the level of CD4 expression (Parcells *et al.*, 1998, and this report). This observation may be due to the interaction of MDV gene products with the factors regulating CD4 and MHC class II expression, or may be due to the immaturity of the cells transformed by MDV (Göbel, 1996). We believe the former assertion, as we have begun to study changes in surface receptor expression during virus reactivation which support this notion (data not shown).

As with an SV40-driven *lac*Z cassette in the MDV genome, the GFP gene within the MDV genome was regulated like a lytic gene and could be induced by IUdR treatment (Fig. 3B). The kinetics of the induced expression of GFP is very similar to that of pp38, another gene product induced during virus reactivation that is associated with lytic infection (Baigent *et al.*, 1996; Chen and Velicer, 1992; Cui *et al.*, 1991; Ikuta *et al.*, 1985; Nakajima *et al.*, 1987; Parcells *et al.*, 1998). The UA04 cell line now provides a novel, rapid and non-invasive means to conduct studies of MDV latency and reactivation.

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