Using a Commonly Down-Regulated Cytomegalovirus (CMV) Promoter for High-Level Expression of Ectopic Gene in a Human B Lymphoma Cell Line

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Background: Vectors are widely used to drive gene expression using a promoter. However, not all promoters are able to drive ectopic gene expression efficiently, including CMV promoter. Here, we report our data using CMV promoter for high-level gene expression in a B lymphoma cell line DG75.

Material/Methods: A plasmid (pcDNA3.1(+)) containing the CD21 gene driven under CMV promoter was constructed. The plasmid was stably transfected into a human B lymphoma cell line DG75 for cellular surface CD21 expression, and flow cytometry was used to monitor CD21 expression. CD21+ cells in the stable cell line were purified using anti-CD21 antibody-coupled Dynabeads for CD21-mediated antigen presentation experiment.

Results: The percentage of CD21+ cells in newly generated stable DG75 pcDNA3.1(+) CD21 cells was only 6.5% as determined by flow cytometry, which was unexpected and did not fit the requirements for further experiments. However, CD21+ cells could be purified to 100% using antiCD21 antibody-coupled beads. The percentage of CD21+ cells in purified cells can be kept at 95%, 82%, 42%, 15%, and 42% at 7 d, 14 d, 34 d, and 42 d after purification, respectively. Specific T cell response against CD21 mediated antigen presentation can be activated successfully only when surface CD21 expression remains high.

Conclusions: A commonly down-regulated CMV promoter can be used to drive ectopic gene expression at a high-level in stable cell lines. Our results should facilitate future experimental design using other down-regulated promoters containing vectors such as SV40 and PGK1.

MeSH Keywords: Burkitt Lymphoma • Cell Line • Down-Regulation • Gene Expression • Promoter Regions, Genetic

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Background

Vectors are widely used to deliver ectopic genes into cells in vitro and in vivo, and the ectopic gene expression efficiency varies depending on cell types and utilized vectors [1–3]. Many attempts had been made to improve ectopic gene expression efficiency, such as constructing vectors using different transcriptional regulatory elements [4,5], or using different vectors such as viral or nonviral vectors [6]; however, no vector has been found to be able to achieve efficient ectopic gene expression in all situations, and some commonly used promoters in the vector were found to be frequently suppressed in the transfected cells [7]. Thus, vectors with different promoters may have to be evaluated in different settings when designing scientific experiments.

Human cytomegalovirus (CMV) is a Herpes virus that can infect various human cell types such as epithelial cells, endothelial cells, fibroblasts, smooth muscle cells, connective tissue cells, macrophages, dendritic cells, and lymphocytes [8,9]. During productive infection, CMV genes are expressed from immediate early (IE) genes to early genes and then to late genes in a coordinated order, with gene expression kicking off by CMV IE promoter with assistance from its proximal and distal enhancer [10].

Thus, CMV IE promoter together with enhancer is widely used as a constitutive promoter (often abbreviated as CMV promoter) to drive gene expression in a variety of cell types [8,11,12]. However, the strength of the target gene expression driven by CMV promoter varies depending on cell types; for example, CMV promoter driven-green fluorescence protein (GFP) signal was strong in human embryonic kidney cells (293T) and human fibrosarcoma cells (HT1080), while it was weak in fibroblasts (MRC5) [1] and inactivated in mouse embryonic stem cells (D3 and J1) [2]. Xia et al. showed that the reporter gene was shut off in more than 95% of targeted cells under CMV promoter in human embryonic stem cells, and which no experiment could be performed using such a gene expression system [7].

Here, we report our data using a CMV promoter-driving ectopic gene expression system in a cell line derived from human B lymphoma cells. The ectopic gene was cloned into a pcDNA3.1(+)-CD21 plasmid under the control of a CMV promoter using the neomycin resistance gene as a selection marker. The CD21 gene (together with a signal peptide) was cut out using the EcoRI cutting site from a CD21 expression plasmid, generated from the laboratory as described previously [15]. This was done by partial digestion with EcoRI (Fermentas) (because there is also 1 EcoRI cutting site within the CD21 gene itself) at 37°C for 4 min (5 μg DNA in 5 reaction tubes were loaded with 10, 3, 1, 0.3, and 0.1 U EcoRI, respectively). The insert was cut out of the agarose gel after gel electrophoresis (intact CD21 gene is 3165 bp compared to other completely digested fragments of 4927, 1721 and 1444 bp) and purified by using phenol and butanol. The pcDNA3.1(+) vector was cut by the EcoRI endonuclease restriction enzyme. The obtained insert and the vector were ligated by T4 ligase and transformed into a competent E. coli DH5α strain. The plasmid DNA prepared by the boiling Mini-preparation from the transformed DH5α bacteria was analyzed by using BamHI and EcoRI endonuclease restriction enzymes and thus the CD21-containing clones were identified. The new CD21-containing plasmid contained a neomycin resistance gene, which was used as a selection marker following stable transfection. The plasmid was tested in 293 cells by transient transfection for CD21 expression, which was further confirmed by immunostaining.

Stable transfection

Stable transfection was performed to introduce CD21 into DG75 cells using electroporation. Briefly, 20 μg pcDNA3.1(+)–CD21 was combined with 3×10⁶ DG75 cells in RPMI 1640/10% FBS on ice, used in similar experimental settings to improve ectopic surface molecule or selectable intracellular molecule expression.

Material and Methods

Cell culture

Cell cultures were maintained in a HERA cell 150 incubator (Thermo Scientific) with constant 5% CO₂ and 37°C temperature under humidified conditions. Cell handling was done in a Herasafe KS12 Safety Cabinet (Thermo Electron Corporation) laminar flow workstation. Cell lines including DG75 [13] and HEK293 cells [11] were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) and were split 1:10 twice per week. CD4+ T cells were maintained as described below [14], and stable cell lines were maintained as described below in the stable transfection section.

CD21 cloning

To stably transflect a CD21 expression plasmid into the DG75 cell line, a plasmid expressing CD21 was generated. The CD21 gene was cloned into the pcDNA3.1(+) plasmid under the control of a CMV promoter using the neomycin resistance gene as a selection marker. The CD21 gene (together with a signal peptide) was cut out using the EcoRI cutting site from a CD21 expression plasmid, generated from the laboratory as described previously [15]. This was done by partial digestion with EcoRI (Fermentas) (because there is also 1 EcoRI cutting site within the CD21 gene itself) at 37°C for 4 min (5 μg DNA in 5 reaction tubes were loaded with 10, 3, 1, 0.3, and 0.1 U EcoRI, respectively). The insert was cut out of the agarose gel after gel electrophoresis (intact CD21 gene is 3165 bp compared to other completely digested fragments of 4927, 1721 and 1444 bp) and purified by using phenol and butanol. The pcDNA3.1(+) vector was cut by the EcoRI endonuclease restriction enzyme. The obtained insert and the vector were ligated by T4 ligase and transformed into a competent E. coli DH5α strain. The plasmid DNA prepared by the boiling Mini-preparation from the transformed DH5α bacteria was analyzed by using BamHI and EcoRI endonuclease restriction enzymes and thus the CD21-containing clones were identified. The new CD21-containing plasmid contained a neomycin resistance gene, which was used as a selection marker following stable transfection. The plasmid was tested in 293 cells by transient transfection for CD21 expression, which was further confirmed by immunostaining.

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and the mixture was electroporated (260 Voltage, 1050 μF) for 1 min using gene pulser II (Bio-Rad). At 3 days after transfection, the culture medium was exchanged for the selection medium, which was the same medium but containing 800 μg/ml G418 (Geneticin; Gibco), which was subsequently changed every 3–4 d. The concentration of G418 in the selection medium was reduced to 400 μg/ml after establishment of the stable cell lines was confirmed. The pcDNA3.1(+) empty control plasmid was transfected into DG75 cells in parallel as negative control.

**CD21+ DG75 cells selection**

To obtain a pure population of CD21+ DG75 cells and perform T cell recognition assays, CD21+ DG75 cells were selected 1 week prior to the assay. Selection was performed by incubating DG75-pcDNA3.1(+)–CD21 cells with mouse anti-human CD21 antibody (Santa Cruz, #sc-18857) at 1 μg per 1×10⁶ cells and subsequently extracting the bound cells using anti-mouse IgG Dynabeads (Invitrogen). The selected CD21+ cells were cultured for an additional 3–5 days to amplify the CD21+ population, and subsequently the Dynabead-bound cells were removed by negative selection under a Dynal single-tube MPC 1 magnetic particle concentrator (Invitrogen) and the unbound cells were washed and collected for further culture. The purity of the selected CD21+ DG75 cells was confirmed by FACS (fluorescence-activated cell sorting, BD Biosciences) analysis.

**Immunostaining**

To determine whether CD21 gene was successfully cloned into the pcDNA3.1(+) plasmid, immunostaining was performed. Briefly, cells (approx. 1–2×10⁶) were harvested and resuspended in 200 μl PBS, and 20 μl cells were dropped onto each well of an 8-well microscope glass slide (Medco). After 5–10 s, excess liquid was removed and cells on each well were air-dried. Slides were fixed in Acetone (Sigma-Aldrich) for 20 min followed by 30-min incubation at 37°C with 20 μl primary antibody (1.3 μg/ml, undiluted or antibody transfections diluted in RPMI 1640+10% FBS medium) per slide. After washing the slide 2–5 min with PBS, a secondary antibody conjugated to an immunofluorescent dye (Goat anti-Mouse IgG-Cy3, Dianova), which was diluted 1: 300 in PBS previously, was added to the slide for 30 min at 37°C. The slide was then washed 2–5 min with PBS again. A glass coverslip was mounted with 90% glycerol. The slide was observed under a Leica DMS000B fluorescent microscope and stored at 4°C.

**Flow cytometry assay**

To assess the expression of CD21 on the cell surface, a flow cytometry assay was performed. Cells were handled in a 1.5-ml Eppendorf tube. All the procedures were done on ice. Briefly, 1×10⁶ cells/samples were washed in PBS. For the detection of CD21, cells were incubated with primary (unconjugated) anti-CD21 antibody (1.3 μg/ml) for 20 min, washed twice with PBS, then incubated with PE-labeled secondary antibody (anti-mouse IgG-PE, Jackson Immunoresearch #115-116-068, diluted 1: 100 in PBS) and corresponding predicted bands were 5428 bp for pcDNA and 6596 bp+2001 bp for pcDNA-CD21, which were shown in the gel.

**Results**

**Generation of CD21 expression plasmid pcDNA3.1(+)–CD21 and its functional testing**

To generate the CD21 expression plasmid, the DNA fragment of CD21 from a plasmid expressing CD21, which was generated previously [15], was cloned into a pcDNA3.1(+) plasmid.
Figure 2. Expression of CD21 on DG75 cells using generated pcDNA3.1(+)-CD21. (A) The generated CD21 expression plasmid (DG75-pcDNA3.1(+)-CD21) was tested by transient expression of 293 cells for 3 d, which was confirmed by immunostaining. LCLs and DG75 cells were used as positive and negative controls for CD21 expression, respectively. Untransfected 293 cells were also compared in parallel as a negative control. Both immunofluorescent and phase-contrast pictures were taken, and scale bars represent 100 μm. (B) Stable transfection of DG75 cells with pcDNA3.1(+)-CD21 plasmid was performed by electroporation. FACS analysis of untransfected cells (DG75) and cells transfected with pcDNA3.1(+)-CD21 plasmid (DG75-pcDNA3.1(+)-CD21) or empty vector (DG75-pcDNA3.1(+)), the expression of CD21 was tested after establishment of the stable cell line, and the selected CD21+ DG75 was confirmed by FACS analysis. The grey shading represents unstained samples alone, the black line represents samples stained with secondary fluorescent antibody alone, and the light dark line represents samples stained with anti-CD21 antibody coupled with secondary fluorescent antibody. pcDNA3.1(+) is shortened to pcDNA for convenience.
under a CMV promoter with a neomycin gene under a SV40 (Simian virus 40) promoter (Figure 1A). The CD21 gene-containing plasmid was analyzed and selected by using BamHI (Figure 1B). The obtained pcDNA3.1(+)−CD21 plasmid was then tested in 293 cells for CD21 expression by transient transfection, which showed a strong positive signal by immunostaining at day 3 after transfection (Figure 2A).

Stable transfection of pcDNA3.1(+)−CD21 into DG75 cell line

To stably express CD21 in DG75 cells, pcDNA3.1(+)−CD21 plasmid was transfected into DG75 by using electroporation, and a pcDNA3.1(+)−CD21-containing stable DG75 cell line was generated under antibiotic G418 selection. CD21 expression of the newly generated DG75-pcDNA3.1(+)−CD21 cell line was analyzed using anti-CD21 antibody by flow cytometry. Surprisingly, the percentage of CD21+ population in the selected pcDNA3.1(+)−CD21-containing DG75 cells was only 6.5% after a stable cell line was generated (Figure 2B).

Down-regulation of CD21 expression driven by CMV promoter in DG75 cells

Because the proportion of CD21-positive cells in the stable transfected DG75 cells only reached 6.5% after establishment
of the stable cell line, questions arise regarding the dynamics of CD21 expression in these cells. To further evaluate CD21 expression, CD21+ cells were purified using mouse anti-human CD21 antibody-coupled with anti-mouse IgG-conjugated Dynabeads (Figure 2B). The Dynabead-bound cells were removed with a magnet (negative selection) after 3–5 d of growing. FACS analysis was done to assess CD21 expression on these selected cells. The results clearly showed that CD21 expression on the selected CD21+ DG75 cells decreases over time (Figure 3). In fact, the half-life of CD21 expressed on these selected cells is only around 30 d.

High-level surface CD21 on DG75 cells restores CD21-mediated antigen presentation

Because there is no CD21 expressed on DG75 cells [13], and antibody-mediated antigen delivery to antigen presenting cells could activate CD4+ T cells [16], we tried to test whether restoring surface CD21 would restore cognate CD4+ T cell response by B cell lymphoma DG75 cells. Our results clearly showed that T cell response could be activated by CD21+ DG75 cells targeted by anti-CD21 antibody-mediated antigen delivery in the first week after CD21+ selection (Figure 4). Interestingly,
The selected cells can be enriched for further experiments. CMV promoter-containing vector (in our case, recovery of CD21 lymphoma cell line based on antibody-based selection from a down-regulated cellular surface molecule expressed on a B cell) quite common [7,17–19]. We report here a method to recover down-regulation of ectopic gene expression in targeted cells is important method with which to investigate functions of genes. Unfortunately, down-regulation of ectopic gene expression in targeted cells is quite common [7,17–19]. We report here a method to recover a down-regulated cellular surface molecule expressed on a B lymphoma cell line based on antibody-based selection from a CMV promoter-containing vector (in our case, recovery of CD21 expression driven by CMV promoter in DG75 cells); therefore, the selected cells can be enriched for further experiments.

The CD21 gene generated in the lab previously [15] was cloned into a pcDNA3.1(+) plasmid using respective cutting sites, which was compatible with eukaryotic cells to express CD21 in DG75 cells. The plasmid was proved to strongly express CD21 by transient transfection (Figure 2A), confirming correct cloning of CD21 gene in the plasmid when low CD21+ cells occurred after establishment of a stable cell line (Figure 2B). The percentage of CD21+ cells in stable DG75-pcDNA3.1(+) (CD21 cells generated based on standard protocol was very low; therefore, we focused on CMV promoter activity of the plasmid after considering the problem, as there were reports on suppression of CMV promoter activity [1,7]. As CD21 detection was done by FACS analysis on the pools of transfected cells, as it is proposed that loss of CD21 expression is caused by CMV promoter inactivation in the minority of cells initially and outgrowth of such cells afterwards.

Since low CD21 expression on stable DG75-pcDNA3.1(+) (CD21 cells does not meet the requirement of following the CD21-mediated antigen presentation experiment, which requires high CD21 expression, so that CD21+ purification was attempted to restore CD21+ cell purity based on antibody-based selection which included an anti-CD21 antibody for binding to cellular surface CD21 molecule and a secondary antibody for purification. Purification was done successfully (Figure 2B). However, high CD21 expression on these purified CD21+ DG75 cells does not keep long and its expression level was decreased continuously (Figure 3). The stable cell line was generated under antibiotic G418 selection; therefore, loss of CD21 expression is not expected to be simply due to loss of the plasmid from DG75 cells; it may be caused by methylation, deacetylation, or inhibitors of DNA methylation and histone deacetylation, thus leading to CD21 down-regulation [7,18–20], but this needs to be further investigated. Intriguingly, in this experiment we could restore CD21 expression by using anti-CD21 antibody-based positive selection without additional inhibitor treatment of the cells, which is useful and efficient enough for further experiments.

The advantage of this method is applicable for long-timeline experiments such as stable transfection experiments that normally last for several weeks or months, as promoter activity is often down-regulated, such as CMV promoter during such a long period [1]. However, for short experiments such as transient transfection experiments that last for only several days, purification is not necessary, as expression of ectopic gene usually does not change much for a promoter activity such as CMV. For example, in this study, the “half-life” of CD21 driven by CMV promoter is estimated to be around 30 d; therefore, there is still enough time left to do purification after establishment of the stable cell line (it takes around 30 d); on the other hand, down-regulation of CD21 will be faint for transient transfection that normally takes 3–5d, so CD21+ purification will not be useful. Thus, we recommend the ectopic gene purification method for long-term experiments. This method could be potentially used in cells that need a sustained high level of

**Figure 5.** Schematic view of the method workflow. The original cells without target gene A are transfected with vector containing target gene A and gene B for positive selection. In many cases, target gene A under promoter 1 (P1) is expressed insufficiently. Following target gene A specific antibody-based purification, sufficient target gene A expression can be achieved for further experiments and this protocol could be repeated if necessary.
ectopic surface molecules or selectable intracellular molecules, and the normal expression level is lower than that required. For example, cell surface molecule or GFP driven by CMV promoter lost during culture in embryonic stem cell lines could be restored to high-level expression by this method (GFP-positive cells could be enriched by FACS sorting).

Conclusions

We described a novel method to recover down-regulated target gene expression driven by a CMV promoter using antibody-based selection, so that ectopic molecule-positive cells could be enriched for further experiments. This study provides an example of recovery of a down-regulated molecule using antibody-based selection and should facilitate future experimental design when using CMV promoter-containing vectors.

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Conflicts of interests

None.

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