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Changes in Herpes Simplex Virus Gene Expression Due to Commercial Serum Source and Indicator Dye in the Growth Medium

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Tissue culture growth medium derives a substantial fraction of its growth-stimulating activity from the fetal bovine serum (FBS) commonly used as a supplement. In addition to the source of serum, non-essential additives such as colorized pH indicator dyes may also affect the growth stimulating properties of complete media. We show here that both of these culture medium components can dramatically affect gene expression *in vitro*. Using a custom gene expression chip for herpes simplex virus 1, we demonstrated significant changes of expression levels in several categories of viral genes including the immediate early viral transcription factors ICP0 ($p < 0.05$), ICP4 and ICP27 (both $p < 0.001$). This dependence of virus growth on serum source and other medium components have implications for not only *in vitro* virus studies, but also viral vector design and vaccine efficacy. This is especially true when examining a large DNA pathogen that potentially contains response elements that are common in the mammalian genome.

Introduction

Fetal bovine serum has been the most commonly used tissue culture medium supplement for *in vitro* work since its inception, and regardless of the arbitrariness of the initial selection, investigators commonly cling to their choice of supplier *"because it works."* Depending on the method of collection, serum may contain substantially different levels of substances with potentially dramatic effects on cell growth and gene expression such as hormones, growth factors, lipids, and endotoxins. Although today's FBS is far more consistent than the products from decades ago, anecdotal evidence remains a significant argument in the decision-making process of choosing FBS. In addition to serum composition, phenol red, a common pH indicator for *in vitro* culture, has been indicated as an estrogen mimic. Specifically, cell proliferation increased in the breast carcinoma cell line MCF-7 and some osteoblastomas in the presence of phenol red.[1, 2] In our studies with herpes simplex virus 1 (HSV-1), a large (152 kb genome) DNA virus, we have used a bioinformatic approach to identify numerous potential sex hormone response elements in the viral genome. While a complete dissection of these response elements is beyond the scope of the present study, global effects of medium components on viral gene expression would support their presence in the HSV genome. Therefore, we set out to examine the influence(s) of FBS source and phenol red on HSV gene expression *in vitro*, and based on our results, propose that the presence of phenol red and FBS can significantly affect vaccine and viral vector development during the *in vitro* testing stage.

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Materials and Methods

Virus Preparations

Stocks of HSV-1 strain 17^+ (cell-released virus) were prepared and titered on monolayers of CV-1 (African green monkey kidney) fibroblasts. Stock virus was stored in Hanks' balanced salt solution (HBSS) supplemented with 2% FBS at −80 °C. Stock concentrations were greater than 10^9 pfu/mL and thus the dilutions required to achieve HSV infection at MOI = 2 (> 10^3-fold) made the stock-derived FBS negligible in these studies.

Cells and Culture Conditions

CV-1 cells were obtained from ATCC (CCL-70; American Type Culture Collection, Manassas, Virginia USA) and maintained in Dulbecco's Modification of Eagle's Medium (DMEM) containing phenol red (rDMEM) supplemented with 100 U penicillin, 100 µg/mL streptomycin, 2 mM glutamine, and 10% FBS. The cells were grown and passaged according to ATCC guidelines. To remove potential hormone bias from the maintained cell line, CV-1 cells were gradually weaned into hormone-free cell culture media comprised of DMEM without phenol red (wDMEM) supplemented with 100 U penicillin, 100 µg/mL streptomycin, 2 mM glutamine, and 10% dextran/charcoal-treated FBS (dFBS).

In experiments, weaned cells were treated for 24 hrs with the following prior to HSV-1 infection: 1) rDMEM and dFBS; 2) wDMEM and FBS; or 3) rDMEM and FBS or NCS. Treated cells were infected with HSV-1 strain 17^+ (MOI = 2) for 1 hr at 37 °C. Viral inoculum comprised of diluted stock virus and virus media containing DMEM (rDMEM or wDMEM, depending on treatment). Commercial suppliers of serum were (in alphabetical order and not correlated with the "A-G" used in data presentation): Atlanta Biologicals (Atlanta, Georgia USA), BioChemed Services (Winchester, Virginia USA), Caisson Laboratories (North Logan, Utah USA), Invitrogen/Gibco Life Technologies (Grand Island, New York USA), Thermo Fisher Scientific/HyClone (Waltham, Massachusetts USA) and Omega Scientific (Tarzana, California USA). Cells were harvested 20 hrs after infection with HSV-1 strain 17^+ using TRIzol LS reagent (Invitrogen) and stored at −80 °C until RNA extraction. For each treatment group, cell health was determined using PrestoBlue Cell Viability Reagent (Invitrogen) and judged to be equivalent as A570/600 nm values varied less than 10% (data not shown).

RNA Extraction and HSV-1 Microarray

Total RNA was isolated from cells using TRIzol LS reagent. Following RNA extraction, RNA clean-up was performed using RNAeasy mini columns (QIAGEN, Valencia, California USA) according to manufacturer protocols and treated with RNase-free DNAse (QIAGEN). RNA concentration and purity were detected using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, Delaware USA) with absorbance readings at 260 nM and 280 nM. 75-mer probes for custom HSV-1 microarrays[3] were purchased from Integrated DNA Technologies (Coralville, Iowa USA) and microarrays were printed in the Microbiology and Molecular Cell Biology core facility at EVMS. Array 900 (Genisphere, Hatfield, Pennsylvania USA) was used to synthesize cDNA from 1 µg total RNA and label it with Cyanine (Cy) 5 and Cy 3 following manufacturer protocols. In the experiments evaluating the effects of different cell culture media components on HSV-1 gene expression, Cy 5 was used to label samples from cells treated with wDMEM and dFBS while Cy 3 was used to label samples from cells treated with: 1) rDMEM and FBS; 2) rDMEM and dFBS; and 3) wDMEM and FBS. In experiments surveying FBS, Cy 5 was used to label samples from cells treated with rDMEM and dFBS while Cy 3 was used to label samples from cells treated with rDMEM and FBS (various sources). The microarrays were scanned using ScanArray Express microarray scanner (PerkinElmer/Packard Biosciences, Boston, Massachusetts USA) and analyzed using the built-in ScanArray Express software and Vector Xpression 3.0 (Invitrogen).

Quantitative Reverse-Transcription PCR Analysis

Total RNA previously isolated for HSV-1 microarrays was used in quantitative real time polymerase chain reaction (qRT-PCR). qRT-PCR was performed using Moloney Murine Leukemia Virus (M-MLV) (Invitrogen) reverse transcriptase (RT) following manufacturers protocols, and stored at −80 °C unless used immediately. Primers were designed using Oligo7 (Molecular Biology Insights, Cascade, Colorado USA) and qRT-PCR was performed for three HSV-1 regulatory genes (5′−>3′):

ICP0: GCGGACAGCACGGACACGGAACTG & CGCTGATTGCCCGTCCAGATAAAGTCCA,

ICP4: GGCGGCGGCGACGACGACGATAA & GAGTACAGCACCACCACGCGCACGTCCT,

ICP27: GCGTCTGGCGGACATTAAGGACATTGCATC & GCGGTGCGTGTCTAGGATTTCGATCAGGC.

qRT-PCR C_t values were normalized to β-actin (5′−>3′): GTCCGCTAGAAGCAT & ATCCGCAAAGACCTGT. (Control primers were a gift from Dr. Duffy, Dept. of Physiological Sciences, EVMS.) qRT-PCR conditions were 12.5 µL of 2× iQ SYBR Green Supermix (Bio-Rad Life

Science, Hercules, California USA), 10.5 L dH_2O, 200 nM primers, and 20 ng/µL cDNA for each 25 L reaction. Following a 5 min incubation at 95 °C to activate the iTaq polymerase, cycling parameters were constant except annealing temperatures that varied for each primer pair: 45 cycles of 95 °C for 10 sec and annealing for 30 sec, followed by a melting curve from 65–95 °C in 0.5 °C increments for 5 sec. Annealing temperatures were either 66 °C (ICP0), 67 °C (ICP4), 63 °C (ICP27), or 55 °C (β-actin). All samples were run in triplicate on a Bio-Rad iCycler. No primer dimers were detected via melt curve analysis.

Statistical Analysis

Differences in viral gene expression in Figures 1 and 3 (shown later) were determined via GraphPad Prism (GraphPad Software, San Diego, California USA) using one-way ANOVA and Bonferroni's Multiple Comparison Test between all pairs of columns with a significance level of $p = 0.05$; * = $p < 0.5$; ** = $p < 0.01$; and *** $p = < 0.001$. Algorithm definitions for gene expression clustering in Figure 2 (shown later) used the K-mean divisive method to build a binary tree that iteratively clustered gene expression data sets non-hierachically. Additionally, Euclidian distance analysis separated data sets into groups with similar expression profiles based on the magnitude. The clades in Figure 2 were selected on the analyzed data set by Euclidian distance following the analysis.

Results

Components of cell culture media have often been neglected in experimental designs due to either convention or convenience. For instance, FBS may be a source of hormones and growth factors that inherently depends on a given commercial source.[4-6] In addition, phenol red has been shown to be an estrogen mimic that affects cell proliferation and interferes with cell cycle progression.[1, 2] We propose that cell culture media components may bias experimental results, and in this study, we tested the potential for FBS and/or phenol red to alter HSV gene expression *in vitro*. To examine the effects of medium components on gene expression, we first weaned CV-1 cells to grow on phenol red-free medium supplemented with dextran and charcoal-treated FBS. This treatment of FBS depletes most hormones and other growth factors, allowing determination of the influence of medium supplementation, both phenol red and serum source.

Phenol Red and FBS Alone Alter HSV Gene Expression

Total RNA extracted from CV-1 cells 20 hrs post-HSV infection was used in custom microarrays to assess changes in viral gene expression. Figure 1 (following page) shows distinct changes in several categories of HSV genes due to exposure to phenol red and/or non-depleted FBS. For instance, HSV ICP27 (immediate-early [IE] regulatory protein that functions to inhibit splicing) and U23 (thymidine kinase) were down-regulated when stimulated with either phenol red or FBS, compared to it being up-regulated when stimulated only with phenol red or FBS ($p < 0.05$) (Figure 1A). The actual mechanism behind such a complex regulatory system is beyond the scope of this study, but similarly, the various splice forms of HSV latency genes were also differentially regulated ($p < 0.01$–0.05) (Figure 1B). This suggests that the added effects of FBS-derived hormones and growth factors, and

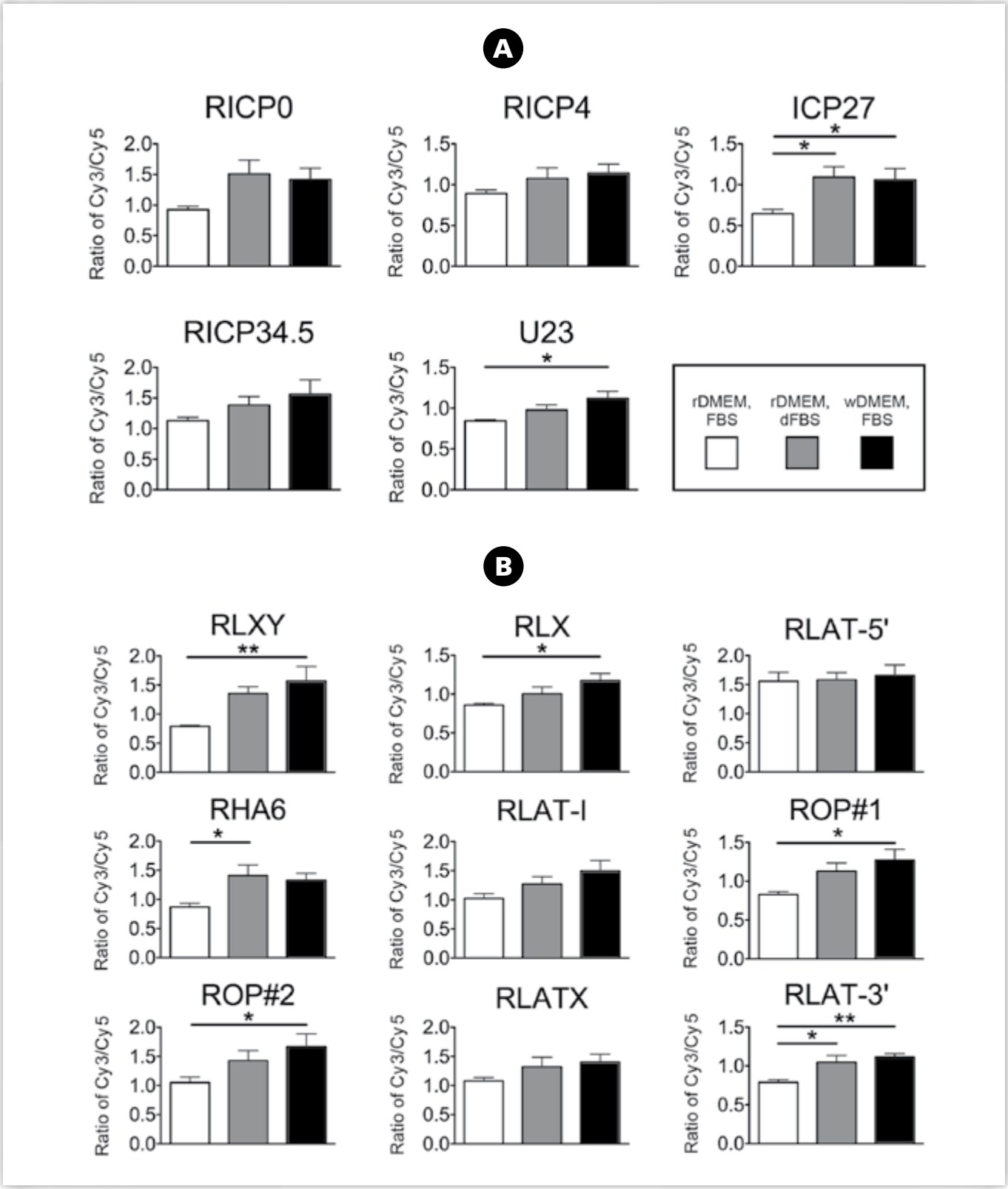
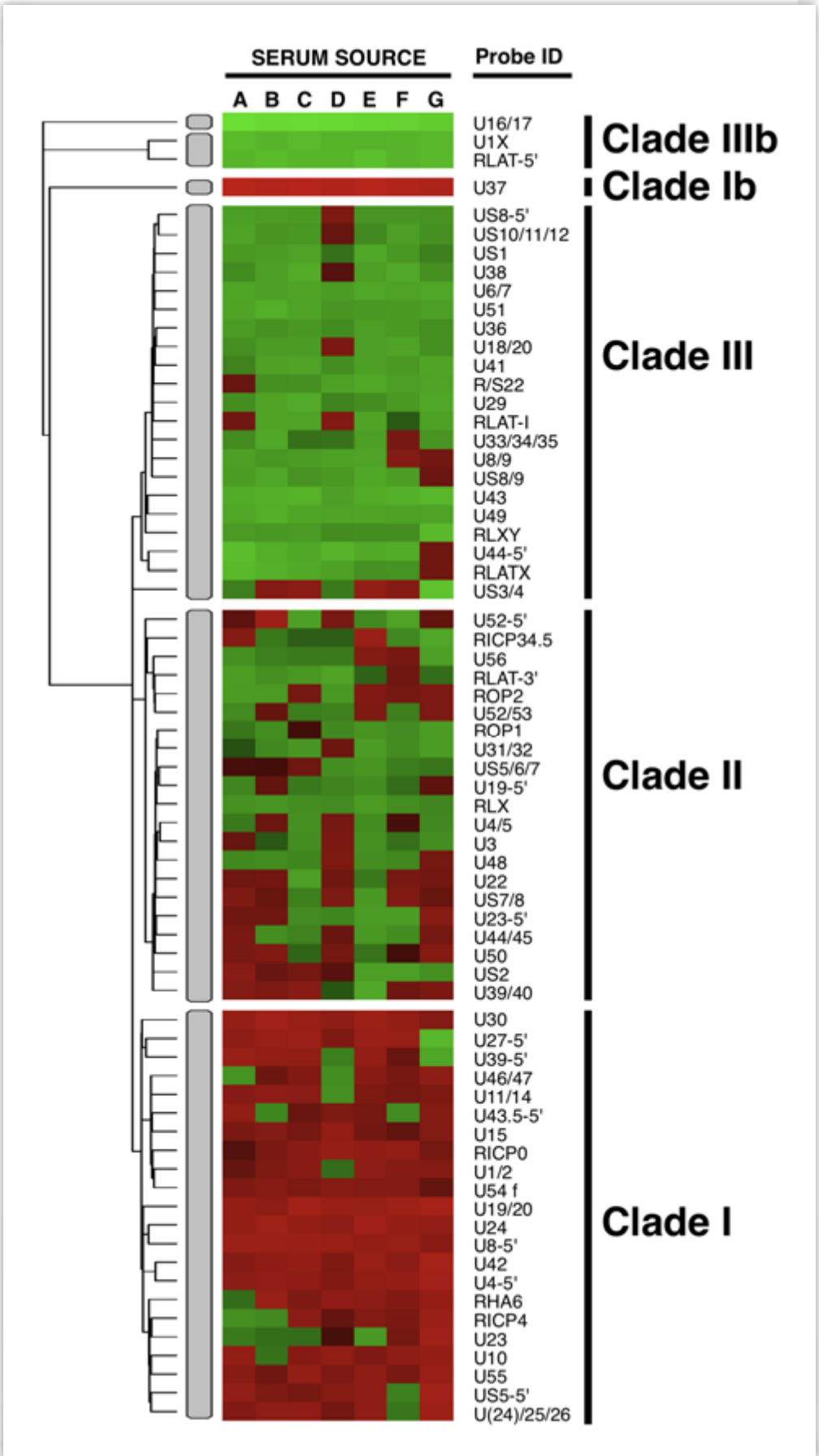


FIGURE 1. **Phenol red and FBS alter HSV-1 gene expression.** CV-1 cells grown in wDMEM and dFBS were treated for 24 hrs with various cell culture media backgrounds: 1) rDMEM and FBS; 2) rDMEM and dFBS; and 3) wDMEM and FBS. Following treatment, cells were infected with HSV-1 (MOI = 2) and harvested for RNA at 20 hrs post-infection. HSV-1 regulatory (A) and latency-related (B) gene expression data was obtained from custom HSV-1 microarrays, and statistical analyses were done as described in Materials and Methods. Probe names for each gene are as listed online[3]. Expression levels were normalized to wDMEM and dFBS (=1.0). All reactions were performed in triplicate, and C_t values were normalized to β-actin. Data are representative of two experiments.



phenol red (the estrogen mimic) in cell culture media may bias experimental interpretation or simply viral vector growth.

HSV Gene Expression is Altered When Stimulated with FBS from Different Suppliers

Our initial results showed similar patterns of HSV regulatory and latent gene expression when stimulated with phenol red and FBS. HSV gene expression was generally down-regulated in the presence of phenol red and up-regulated when stimulated with FBS in the absence of phenol red. To determine if FBS source could differentially alter HSV gene expression, we used our HSV microarrays on total RNA isolated from HSV-infected CV-1 cells treated with cell culture medium composed of rDMEM and FBS from various suppliers.

Indeed, Figure 2 is a heat map showing that HSV gene expression is altered when stimulated with cell culture media supplemented with FBS from different suppliers. We used a K-mean divisive algorithm and Euclidian

FIGURE 2. **HSV gene expression clustering due to serum source.** Gene expression data for each serum source supplementation was analyzed for K-mean and Euclidian distance according to the description in Materials and Methods. Probe IDs are as listed online.[3] Data are representative of two experiments.

distance to cluster genes into three expression-based clades. Clades I and Ib show HSV genes that were mainly up-regulated when stimulated with cell culture media supplemented with dFBS. Clade II shows HSV genes that were variably regulated when stimulated with FBS from different sources. Additionally, clades III and IIIb show HSV genes that are mainly up-regulated when stimulated with FBS from different suppliers. The HSV regulatory genes ICP0, ICP4, ICP27, and U23 were clustered into clade I, whereas ICP22 (another immediate early gene with the ability to regulate these genes) is in clade III. Similarly, UL46/47, modulators of UL48 (a virion-associated transcriptional activator that enhances IE transcription) were also in clade I, whereas the target of their regulation, UL48, fell into clade II. Interestingly, there was no distinct clustering of groups involved with HSV kinetics (IE, E, and Late), the genes required for replication in cell culture, or the structural integrity of the virion itself into any clade.

HSV Regulatory Gene Expression is Altered by FBS from Different Commercial Suppliers

Since most HSV regulatory genes clustered together within clade I, qRT-PCR was performed on the same total RNA isolated for microarray analysis. We confirmed that expression levels are altered for ICP0 ($p < 0.05$), and ICP4 and ICP27 (both $p < 0.05–0.001$) when stimulated with rDMEM supplemented with FBS from different suppliers (Figure 3). Although ICP0 is dispensable *in vitro* at higher MOI (we used 2, whereas ICP0 is essential at or below 0.2[7]), the altered regulation of ICP4 and ICP27, due to medium composition, suggests that the source of FBS can alter experimental results and thus may potentially explain incongruent *in vitro* analysis. Additionally, since ICP0 has been shown to counteract the effects of Type I interferons *in vivo*[8-11], there are inherent implications for HSV-based vector uses both in terms of latency establishment and immune rejection by the host.

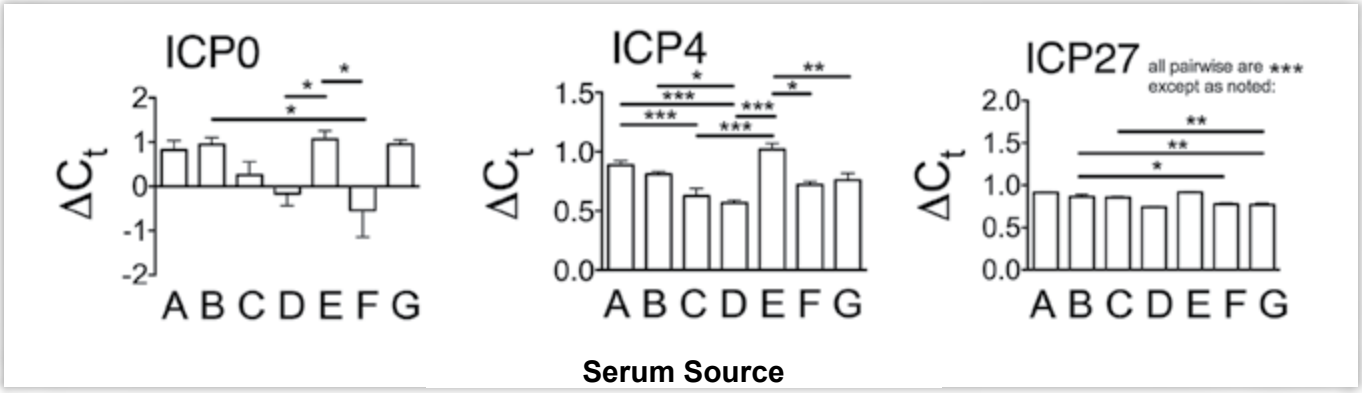


FIGURE 3. **FBS from different commercial sources ("A-G" described in Materials and Methods) alter HSV-1 regulatory gene expression.** 20 hrs post-infection, total RNA was isolated from CV-1 cells treated with cell culture media supplemented with FBS from various sources and infected with HSV-1 strain 17+. All reactions were performed in triplicate and C_t values were normalized to β-actin. Data are representative of two experiments.

Discussion

In years past, numerous studies have investigated the effects of growth medium components to include serum source and pH indicator dye.[2, 6, 12-14] More recently however, such investigation has received less attention despite the development of tools eminently suited to address these questions. One recent exception demonstrated that endocannabinoid content in sera can have dramatic effects on cells in culture[4], and hormones have been shown to alter reactivation *in vivo* or latency-associated transcripts (LAT) and/or ICP0 regulation *in vitro.*[15-18] Our primary concern in this study was the effect of culture medium components on HSV gene expression *in vitro*, although the implications of our findings extend to the use of viral vectors in a clinical setting. Using a well-defined custom array for HSV-1[19, 20], we have shown significant changes in all categories of viral genes in response to the presence of phenol red and the fetal bovine serum supply source we used. Since HSV is an obligate cellular pathogen, we cannot necessarily separate direct effects of these medium components on the virus itself from indirect effects due to changes in cellular transcription patterns. However, we conclude that medium composition can have profound effects on HSV gene expression *in vitro*, and by extension, such changes are likely to occur *in vivo* as a result of the physiological state of the infected (or in the case of a viral vector, transduced) host cell. Conversely, in the design of DNA, or live attenuated viral vaccines, the identification of the response elements responsible for the differential viral gene expression seen in this study has the potential to improve on existing vaccine efficacy with modest DNA sequence alterations.

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