Analysis of the Effects of Phosphorylation on Bovine Herpesvirus-1 Transcription

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During productive infection Bovine Herpesvirus-1 (BHV-1) initiates transcription in a characteristic temporal pattern. Viral genes are classified as Immediate Early, Early, and Late, and their transcripts can be quantified by reverse-transcriptase polymerase chain reaction (RT-PCR). We are interested in studying the effects of phosphorylation on viral transcription during productive infection. We designed primers to amplify three Immediate Early genes, two Early genes, two Late genes, and a cellular gene using the Primer3 Plus software. Optimization of primers for PCR was conducted by taking into account specific annealing temperature, $MgCl_2$ concentration, DNA polymerase concentration, content of glycerol, and the total amount of DNA added. We have found optimum conditions for the amplification of all the selected genes. We infected cells in the presence or absence of phosphatase inhibitors at 2, 4, 8, and 16 hours post infection. Due to previous related experiments conducted in our lab, we expect to find that an over-phosphorylated environment will cause the virus to produce a decreased amount of mRNA transcripts therefore reducing the productivity of infection.

Bovine Herpesvirus-1 (BHV-1) belongs to the Alphaherpesviridaesubfamily within the Herpesviridae family of viruses. BHV-1 has an isocohedral capsid, short replication cycle, and double-stranded linear DNA molecule. BHV-1 primarily infects cattle weakening the immune system and making the animal more susceptible to secondary bacterial infections that lead to respiratory complications and pneumonia. This illness is termed bovine respiratory disease (BRDC) complex or shipping fever. Shipping fever costs the cattle industry billions of dollars per year (Muylkens 2007). After initial infection of the oral, nasal or ocular route, the virus establishes latency in the nervous system for the life of the infected animal. Viral reactivation can be induced by diverse environmental stresses, shedding virus that spreads to other cattle in the herd. This spread of infection can cost the owner of the herd cattle and money, which is one of the reasons why research on BHV-1 is important to the agriculture industry. Understanding more about the virus allows for scientists to research ways to prevent the spread or find a possible cure.

During productive infection, BHV-1 initiates transcription in a characteristic temporal pattern as shown in Figure 1. Viral genes are classified as Immediate Early, Early, and Late. The synthesis and translation of mRNA transcripts of these genes is tightly regulated. Phosphorylation is a post-translational modification of proteins and is one of the main forms of regulation of enzyme and protein function. Preliminary studies in our lab suggested that viral replication is slightly impaired in an over-phosphorylated environment. We are interested in studying the effects of phosphorylation on viral transcription at early times after infection. Through quantification of viral transcripts, we will be able to determine if an over-phosphorylated environment affects mRNA transcription during productive infection. The

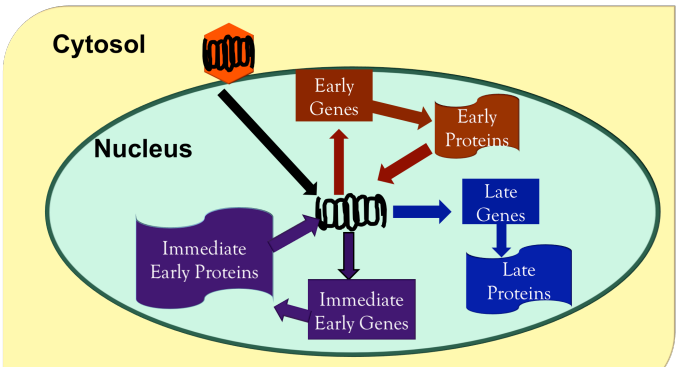

Figure 1: BHV-1 Transcription Cascade

abundance of mRNA transcripts can be studied through reverse transcriptase polymerase chain reaction (RT-PCR). We hypothesize that the over phosphorylated environment will reduce viral transcription and thus viral replication. This work focuses on the optimization of the RT-PCR technique and early finding for the effects of phosphorylation.

Materials and Methods

RNA Preparation: RNA was isolated from tissue culture using Trizol reagent followed by phenol-chloroform extraction. RNA pellets were washed with ethanol, dried and resuspended in DEPC water. Spectrophotometry (260nm) was used to determine RNA concentrations.

DNase I Treatment and Reverse Transcriptase: 2 micrograms of RNA were treated with 2 U of DNase I (RNase-Free; Optizyme) for 20 minutes at 37°C in the presence of 40 U of RNase inhibitor (RNasin; Promega). 50mM of EDTA was then added and the sample incubated at 65°C for 10 minutes. 2 micrograms (µg) of DNase treated RNA plus 1microliter (µL) of Random Hexamers were incubated at 65°C for 5 minutes, then immediately placed on ice for 1 minute. Eleven microliters of ice-cold RT mix (10RT buffer, 25mM $MgCl_2$, 0.1 M DTT, RNaseOUT (40 U/µgL), SuperScript III RT (200 U/µL), and 10 mM dNTP mix [Invitrogen by Life Technologies]) was added to the 2 µg of DNase I treated RNA to give a 20 µL solution. The reaction mixture was left at 25°C for 10 minutes followed by incubation at 42°C for 50 minutes. Termination of the reaction was performed by incubating at 85°C for 5 minutes then placing directly on ice. One microliter of RNase H was added to each sample then incubated at 37°C for 20 minutes. The cDNA samples were purified using a QIAquick PCR purification kit[Qiagen]. The cDNA concentration was measured spectrophotometrically (at 260nm) and diluted to 25ng/ µL. A summary of the process of going from RNA to cDNA can be found in Figure 2.

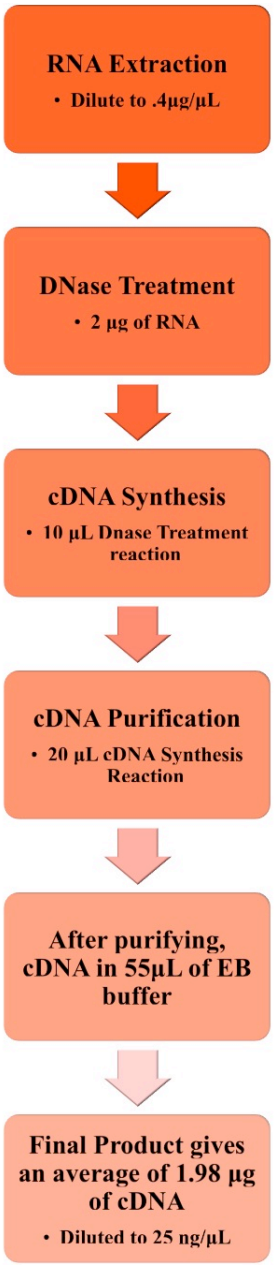

Figure 2: Summary of RNA Extraction and Treatment Process

PCRs: 25-50ng of cDNA was used for each PCR reaction. 25µL reactions were carried out inGoTaq Flexi Buffer, 10mM PCR nucleotide mix, 10µm of each primer, and GoTaq DNA polymerase (Promega). Ten percent glycerol was added to each reaction as indicated in Table 1. $MgCl_2$ concentration varied for each primer and can be found in Table 1 along with DNA Polymerase concentrations. The number of amplification cycles for each primer set is unique and ranges from 25 to 40 cycles. The denaturing phase was for 2 minutes at 95°C, annealing ranged from 45-65°C for 45 seconds, and extensionfor 45 seconds at 72°C. A final extending phase at 72°C for 1 minute was used to ensure complete extension of all products. Amplification products were analyzed via electrophoresis in 1.5% agarose gel with 2.5% ethidium bromide for 1 hour at 100 volts.

Results

Primers were designed to amplify three Immediate Early genes, bovine Infected Cell Protein 4 (bICP4), bICP-22, and bICP-0, two Early genes, Thymidine Kinase (TK) and Ribonucleotide Reductase (RR), two Late genes, Viral Protein 16 (VP 16) and Glycoprotein C (GC). In addition, the cellular protein, bovine Growth Hormone (bGH) was used as a control. We used the Primer3 Plus software for the design of oligonucleotides. A summary of each primer can be found in Table 1.

Table 1: Primer Summary; IE indicates Immediate Early; E indicates Early; L indicates Late

Primer Name	Primer Abbreviation	Kinetic Stage	Primer Sequence	Amplification Fragment in base pairs (bp)
Bovine Infected Cell Protein-4	bICP-4	IE	+: GCG CGT GGA GGT GCT CTC CTC -: CCC TCC CTC CCT TCC CGC G	150 bp
Bovine Infected Cell Protein-22	bICP-22	IE	+: GCG CTG GTC CTC CGG CTC C -: CTC GCT GGC GGC GCT TGG	110 bp
Bovine Infected Cell Protein -0	bICP-0	IE	+: TTC TCT GGG CTC GGG GCT GC -: AGA GGT CGA CAA ACA CCC GCG GT	192 bp
Thymidine Kinase	TK	E	+: TCA AAG CTC AGA GAC ACG CC -: GAA CCT GGC CTG GTA GTA CG	501 bp
Ribonucleotide Reductase	RR	E	+: TTT TAC GAG ACC GAG TGC CC -: GAC GAA AAG GTT GTG GGT GC	513 bp
Viral Protein 16	VP 16	L	+: CTA ATG CTG TTG CGT CTG GC -: CAG GGA GTC ATC GAA CGC TT	630 bp
Glycoprotein C	GC	L	+: TGA TCG CAG CTA TTT TCG CC -: TTC TGG GCT ACG AAC AGC AG	598 bp
Bovine Growth Hormone	bGH	Control	+: TTT CGC CCT GCT CTG CCT GCC -: CCC TTC CTG CCT CCC CAC CCC	183 bp

Each PCR reaction must be optimized to obtain an amplification product of the expected size (in base pairs) and no additional non-specific bands. In order to find the optimum annealing temperature for each primer, we performed several PCR reactions, using a gradient of temperatures to anneal the primers in each reaction. The range of temperatures tested was from 45°C to 65°C. An example of bICP-0 amplification products across the different temperatures can be seen in Figure 3.

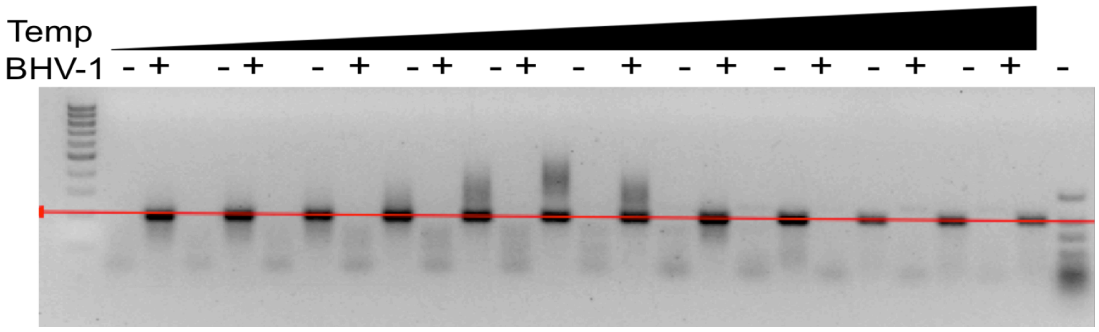

Figure 3: Temperature Gradient Analysis using bICP0 primers. Temperatures range from 45°C to 65°C.The red line indicates the correct size amplification product. The best annealing temperature gives the most precise band along with no non-specific amplification products (extra bands). □

The concentration of other PCR reagents was also adjusted for each primer pair. $MgCl_2$ determines the specificity of primer binding and the concentration range tested was from 37.5 mM to 62.5 mM. The amount of DNA polymerase was also adjusted and we tested a range of .625U to 1.5U per reaction. Due to the 72% GC content of the BHV-1 genome, 10% glycerol was added to increase the effectiveness of the strand separation during the denaturing phase. Finally, the amount of cDNA added was either 50ng or 75ng. For all primers, the correct PCR amplification products can be seen in Figure 4 and correct PCR reaction concentrations can be found in Table 2.

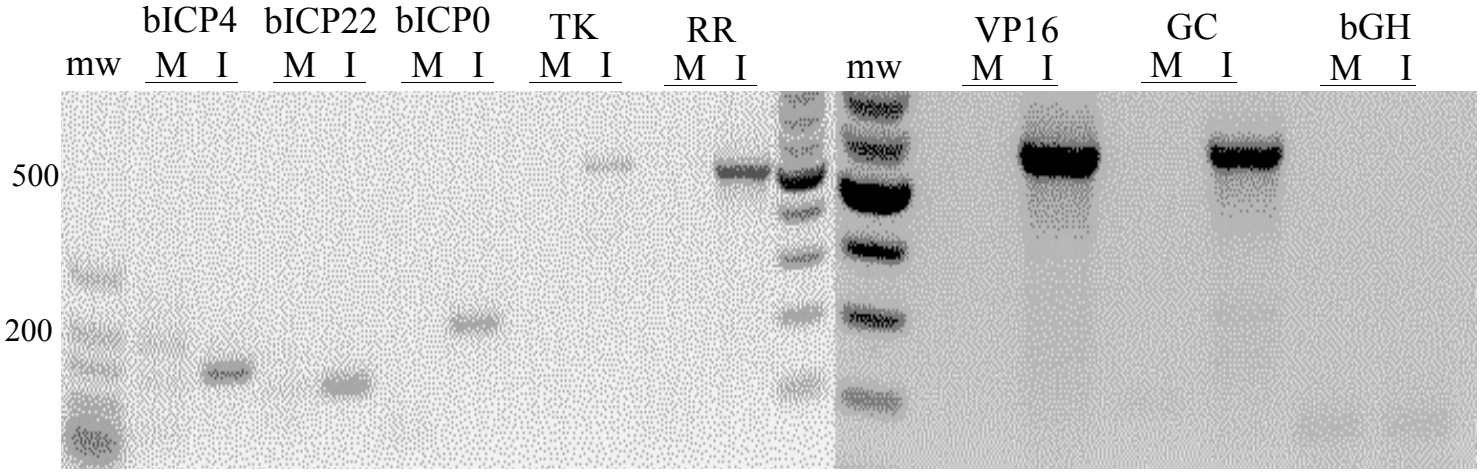

Figure 4: PCR Amplification Products for all the analyzed genes. PCR reactions were analyzed via DNA electrophoresis in in a 1.5% agarose gel run for 1 hour at 100V. M indicates mock-infected; I indicates infected; mw denotes t he molecular weight markers, and the numbers to the left indicate number of base pairs.

Table 2: PCR Reaction Mixture Summary; Gly indicates glycerol

Primer Name	[$MgCl_2$] (mM)	Gly (%)	GoTaq (U)	cDNA Amount (ng)	Optimum Annealing Temperature	PCR Cycles
bICP-4	2	10	1	50	46.6°C	40
bICP-22	1	10	1	50	65.1°C	40
bICP-0	1.5	10	0.5	50	61.1°C	40
TK	1.5	10	1	50	63.1°C	30
RR	2	-	0.625	50	58.7°C	25
VP 16	1.5	-	0.625	50	61.1°C	25
GC	1.5	10	0.625	50	53.4°C	25
bGH	2.5	10	1.5	75	65.1°C	40

Conclusion

This work focuses on the optimization of 8 PCR reactions. Now that the optimization phase of the project is complete, we will begin analyzing RNA transcripts from mock and infected cells treated with phosphatase inhibitor and collected at 2, 4, 8, and 16 hours post infection. Our preliminary findings suggest that an over-phosphorylated environment may reduce the amount of viral transcripts present in the cell during productive infection.

References

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2. **Schang, L. M., and C. Jones**. 1997. Analysis of bovine herpesvirus 1 transcripts during a primary infection of trigeminal ganglia of cattle. J. Virol.**71**:6786–6795.