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Analysis of Papilloma Virus E1 Protein: Creation of Stable Cell Lines to Generate Sufficient Amounts of Viral E1

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Abstract

The immediate objective of our research aims to create stable cell lines to generate sufficient amounts of viral E1. The pMSG vector was chosen for its ability to amplify gene expression by way of hormonal or chemical induction, and for its ability to be selected under ampicillin medium by way of the Amp gene. pMSG was ligated with E1 insert in two fashions, blunt or modified ends. Inserts were created by way of digesting them with BamH1. The inserts were blunted and ligated with a blunt ended pMSG, or blunted, modified, digested with Sal 1 and then ligated with Sal 1 digested pMSG. Recombinants were transformed in E. coli cells, plated in ampicillin medium, and analyzed by way of electrophoresis. When analyzing the blunt ended technique, bacterial contamination was depicted by faint bands in the upper region of the gel. Products could not be analyzed from the Sal 1 ligated technique, due to complications and errors in plating. reating sufficient amounts of E1 protein by way of using blunt and modified ends to form recombinants proved difficult, yielding no desired products. Therefore, there must be further research to find a protocol to generate sufficient amounts of E1 from mammalian cell lines.

Introduction

Cervical cancer is one of the leading causes of cancer-deaths in women worldwide (1). From the 150 genomic strains of the human papillomavirus (HPV), two strains, HPV 16 and 18, are known to cause cervical cancer, yet most commonly, other strands of the papillomavirus are known to produce benign hyperproliferative lesions (warts) in epithelial cells they infect (1, 2). The papillomavirus encodes a protein called E1, which is essentially responsible for initiating the replication of viral DNA (2, 3). E1 is highly controlled by the host cell. Hopefully, understanding the mechanism by which the host cell initiates viral DNA replication, will give insight to the mechanism by which the host cell initiates its own DNA replication, as well as, shed light on ways to avert cervical cancer caused by HPV (4). Thus, the objective of this research is to map and compare phosphorylation sites on the E1 protein from HPV and the bovine papillomavirus (BPV). However, due to the difficulty to attain large amounts of E1, the immediate objective of this research is to create stable cell lines to generate sufficient amounts of viral E1 protein.

HPV and BPV Genome

The BPV and HPV genome are fairly similar and differ only slightly, therefore, we are able to utilize both forms of the papillomavirus in forming recombinants. The virus is enclosed within a protein capsid and has eight genes (5). It exists as a closed double stranded 8kbp and is usually found in cells as a supercoiled closed circular molecule or episome (1, 2, 5, 6). It has eight viral open reading frames (ORFs) and has three regions (2, 5): early region,

nonstructural viral replication proteins E1-E7; late region, two structural proteins L1 and L2 that create the capsid; the long control region (LCR), for regulation of viral replication (5). The E1 protein from both BPV and HPV is the most highly conserved of the papillomavirus genes (3).

Viral Life Cycle

It is the virus' peculiar life cycle that makes it indispensable to this research. The virus undergoes stages during its life cycle: entry, viral replication, productive viral infection, and host containment, which is depicted in Figure 1 (5). Upon infection, the virus sheds its protein coat, injects its viral material in the cytoplasm of the host cell, and then travels to the nucleus, where it resides as an episome (5). A primary target for infection is the stratum germinativum also called the basal layer (5, 6). The productive stage is marked by the translation of the late genes, which occurs in the outer layers (5).

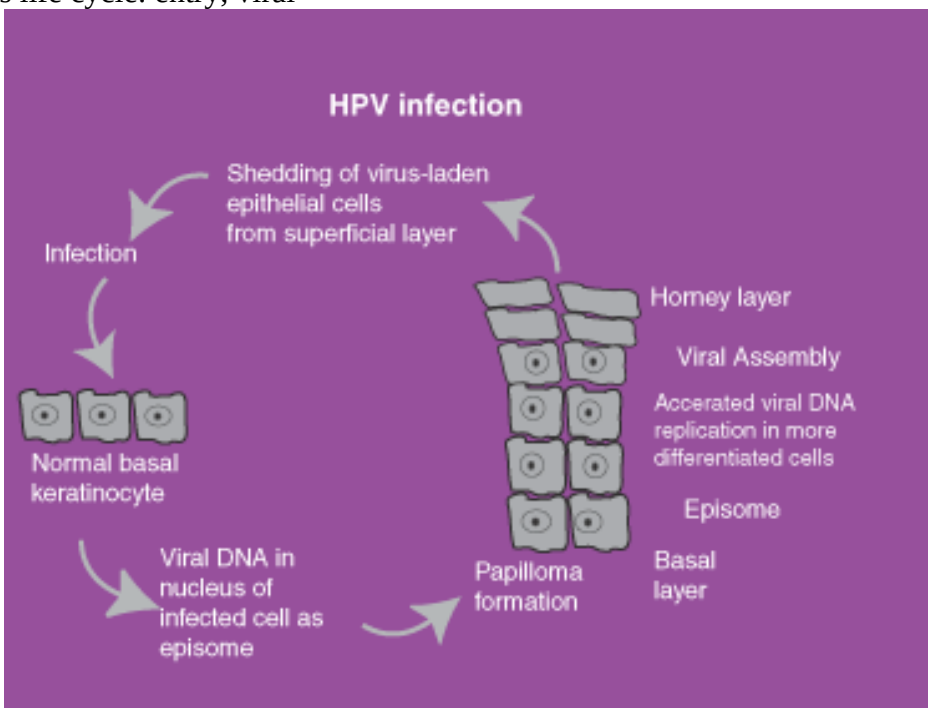


Figure 1. Infection of HPV and location of viral assembly within the layers of the epidermis (5).

As infected basal cells undergo mitosis, daughter cells begin to carry transcriptionally active episomal HPV DNA. Migration of daughter cells initiate a differentiation pathway (2, 7, 8). Within the granulosum and the corneum, terminal differentiation occurs (8). Then viral DNA is amplified, which is followed by the production of proteins produced by the L1 region that envelope each episomal DNA with a protein capsid (3, 7, 8). In summary, differentiation signals a change in the amount of viral DNA produced, synthesis of virus structural proteins, and assembly of progeny virions (2, 3, 8).

A depiction of the link between the life cycle of HPV to cellular differentiation can be seen in Figure 2 (8). The diagram comparing the location of where DNA synthesis and late gene expression take place, as well as which viral particles are produced, aid in visually representing how and where the

reproductive stage switches to the productive stage.

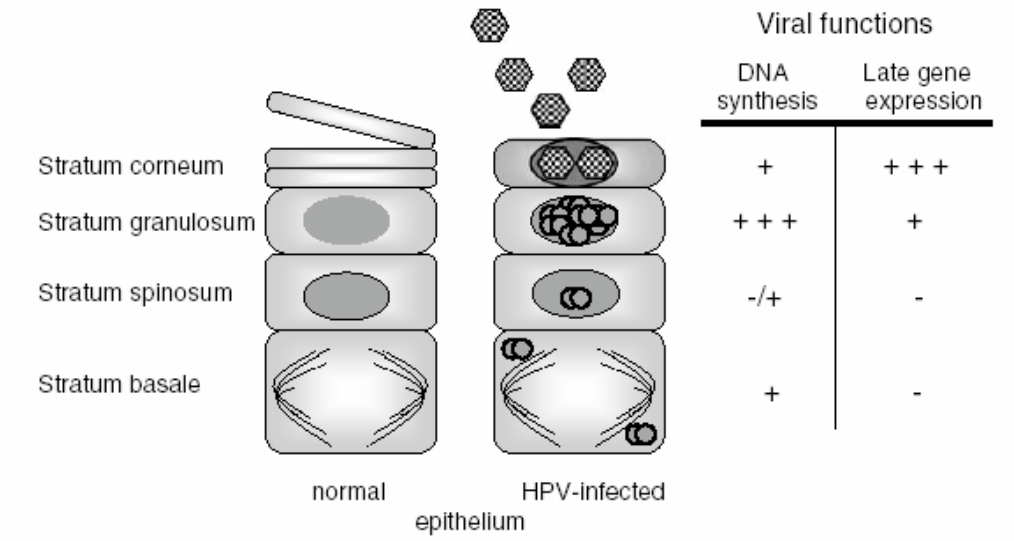


Figure 2. Schematic representation of the abnormal differentiation induced by HPV Infection. Viral replication occurs all throughout the layers, whereas late gene expression or the production of virions is found only in the stratum granulosum (8).

During the reproductive stage, roughly 50 to 200 copies of viral DNA are present and no structural proteins are produced (2, 8). However, when differentiation occurs, there is a change in the transcription program of the cell, causing the synthesis of thousands of viral DNA molecules (2, 5, 7, 8). The exact mechanism by how this occurs is still unknown. However, we can speculate that there is interplay of host, viral, and environmental factors that may induce these changes (2, 3). Ultimately, we speculate that the life cycle of the human papillomavirus is differentially induced (2, 4, 7). Thus, we focus on the E1 protein of papillomavirus protein.

E1 Function

E1 is a helicase that triggers and induces viral DNA synthesis, and is known to be upregulated during differentiation (2, 3). E1 is regulated by way of phosphorylation and plays a role in ATPase, helicase, and viral replication (2, 3, 8, 9). Although the entire protein structure is unknown, we know that E1 protein has two domains (2, 10). The first domain binds to the origin and the other domain acts as a helicase. E1's DNA binding domain (E1 DBD) binds adjacent to E2 while it binds to its DBD. The binding of both proteins to DNA creates a sharp bend that will allow E1's helicase domain (E1HD) to interact with E2's transcriptional activation domain (E2 AD). E2 leaves the complex, and E1 is now recruited to the complex. The helicases can now move in both directions to unwind DNA, thus resulting in replication (10). Figure 3 depicts the proposed mechanism.

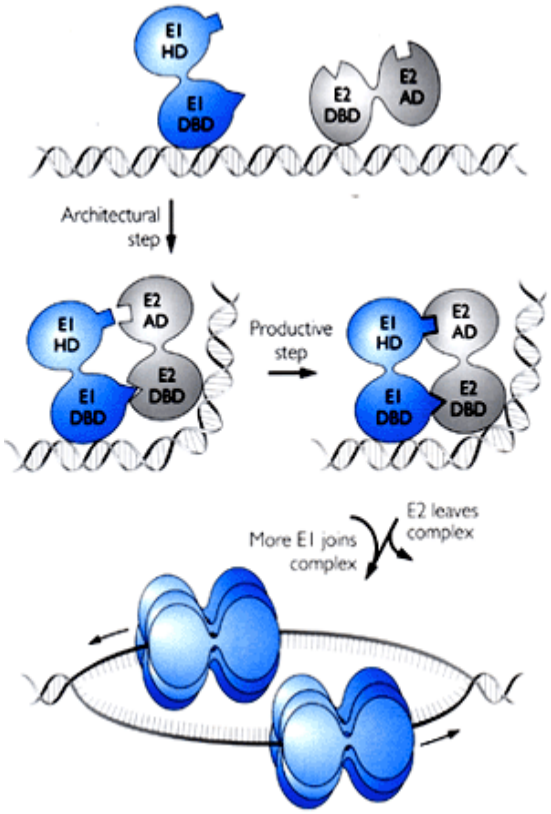


Figure 3. E1 interacts with both the DNA biding domain of the virus as well as the E2. E2 is kicked off while additional E1 proteins complex to form the E1 helicases (10).

Phosphorylation

Phosphorylation plays a role in regulating the function of E1. It is known that kinases, such as Cdks, PKC, and CK2, phosphorylate various positions in the amino-terminal third of E1 (2, 3, 8, 9). E1 is necessary for regulating the replication of viral DNA both positively and negatively (3).

Obtaining sufficient amounts of E1 to analyze is difficult. Currently, E1 is being successfully produced by way of insect cells. One drawback to using insect cells, is that insect kinases may target different sights. This again emphasizes the need to compare and map E1 protein from different sources. In mammalian cells, E1 is has high turnover rate and degrades soon after it is transcribed preventing extensive analysis of the protein function. Being that E1 is a helicase, the production of large amounts of E1 may be toxic to the cell, therefore, it is difficult to have high expression in cells. Additionally, E1 is transient in that when it is cloned, only a very small amount actually expresses the protein. Truly, there is a need to conceive a way to produce enough E1 in mammalian cells (2, 4). To overcome this problem our immediate goal is to produce stable cell lines to generate sufficient amounts of E1 protein.

Methods and Materials

PMSG, a 7.6 kb plasmid, was chosen as a vector for its ability to induce expression of the E1 gene when exposed to dexamethasone, a synthetic glucocorticoid hormone (4, 12). We can control expression in hopes to lessen the effects of cellular toxicity from large amounts of E1. Additionally, pMSG can integrate stably into several cell lines:

mouse, hamster, and human (11). pMSG also has the Amp gene, making it resistant to ampicillin and enabling us to select for recombinants in E. coli (11, 12). It also has the gpt gene which makes the cells resistant to mycophenolic acid (12). Resistance to mycophenolic acid allows for the selection of cells containing stable recombinants (12).

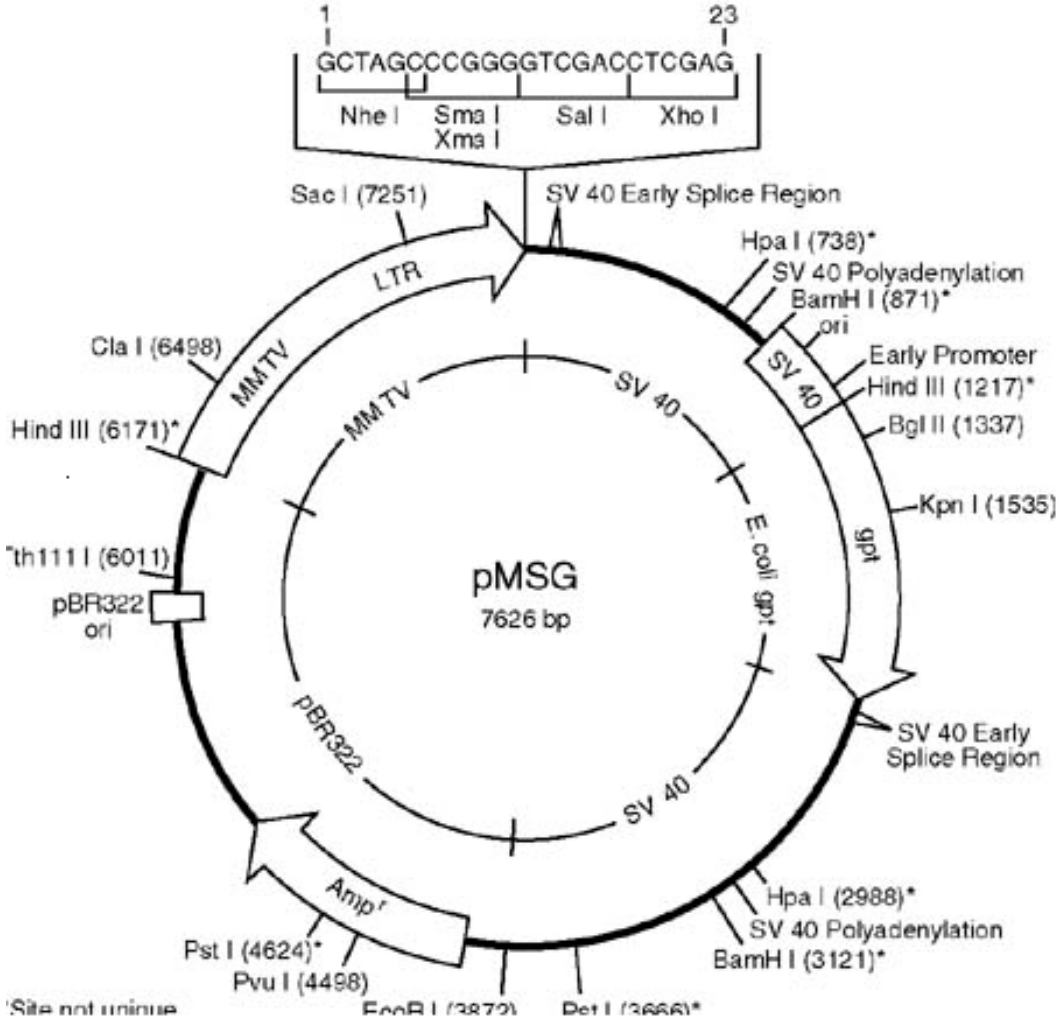


Figure 4. pMSG vector containing a Sma1 and Sal 1 restriction sites. Inserts are induced by the MMTV promoter and selection is aided by the Amp gene (obtained from Dr. M. Lentz).

We used two methods to create a recombinant. First, recombinants were made by ligating blunted ends, and second, the creation of recombinants by ligating modified ends. A depiction of both methods can be seen in Figure 5. Successful completion of each step will be monitored using gel-electrophoresis.

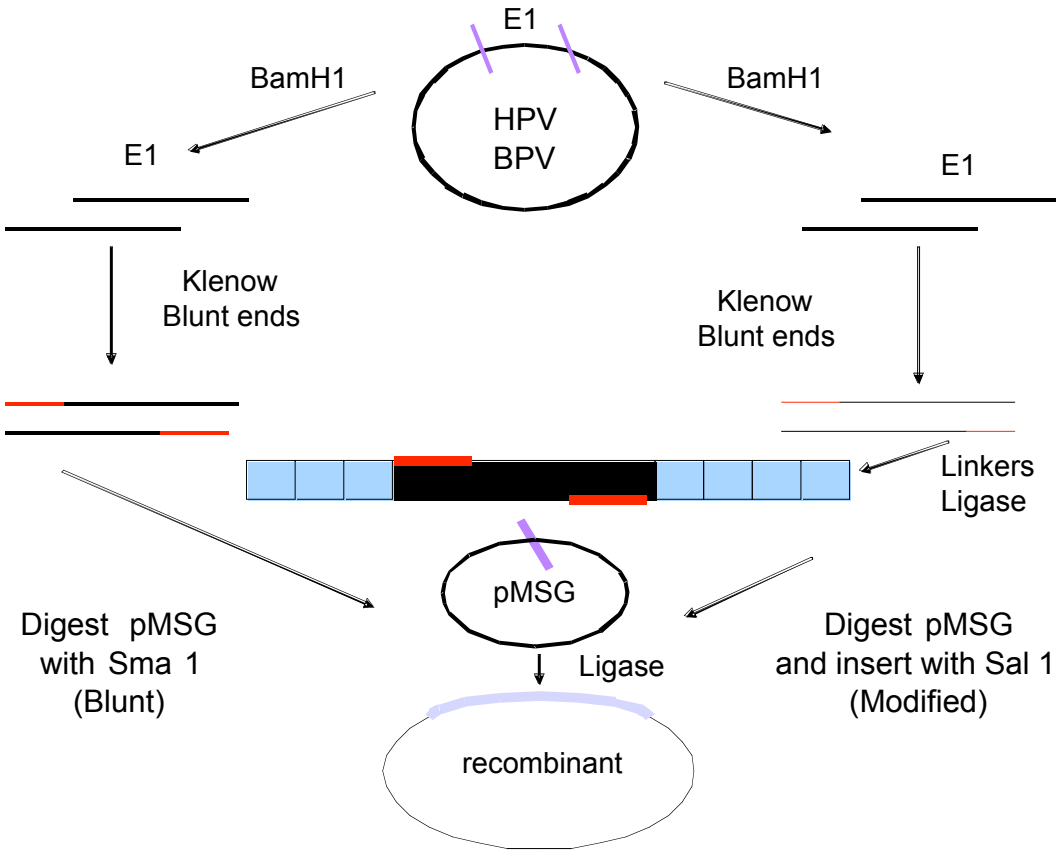


Figure 5. Two approaches to create a recombinant. Both inserts are digested with BamH1 and staggered ends from Bam H1 are filled with dNTPS with the aid of a pol 1 Klenow. For the first technique pMSG is digested with Sma 1 and ligased with the blunt ended insert. The second technique, blunt ended inserts are modified with the addition of linkers, then digested with Sal 1. This modified insert will then be ligased with Sal 1 digested pMSG.

Overall, inserts will be digested with Bam H1 to form staggered ends, which will be filled to form blunt ends by using Klenow, portions of a Pol 1 enzyme derived from E. coli. The first portion of the research aims to join blunt ends created from the Klenow to the blunt ends from a Sma 1 digest on pMSG. The second portion of the research adds portions of DNA called linkers to the insert, which will then be digested by Sal 1 and ligated to pMSG which has also been digested with Sal 1.

SMA 1

Two sources of E1 were used: HPV E1 and BPV E1. BPV E1 and HPV E1 were digested with BAMH1, while pMSG was digested with Sma1 as seen in Table 1. pMSG was treated with phosphatase to prevent recircularization. Purified water was used throughout the entire project to prevent contaminants. Electropheresis gels were used to determine completion of digests, separation, and isolation. The correct bands were identified, removed, and isolated from gels.

	pMSG (µL)	HE1 (µL)	BE1(µL)
H20	21	21	21
DNA	5	5	5
10X Buffer	3	3	3
Bam H1	---------	1	1
Sma 1	2	---------	----------
Total	30	30	30

Table 1. Amount of Solution Used for Digest.

E1 was isolated from the gel by gene clean. This process removed DNA from the gel by using glass milk (silica) and several rounds of centrifugation in order to isolate the viral DNA. Again, analyzing the results from a gel electrophoresis aids to determine the concentration of our DNA. The ends of HE1 and BE1 are blunted by filling in the staggered ends. Pol I enzyme Klenow was used along with the addition of dinucleotide triphosphates (dNTPs) and added in the volume amounts seen in Table 2. This process was followed by another gene clean.

Table 2: Volumes Used to Blunt Ends

	HE1 (µL)	BE1(µL)
DNA	24	24
dNTPs	2	2
10X buffer	3	3
Klenow	1	1

Table 2. Volumes Used to Blunt Ends.

Table 3 shows the volumes used to prepare the ligation. A negative control is found in tube 1 because it does not contain either BE1 or HE1. Therefore, no E1 insert will be found and cannot form a recombinant. Tube 2 is our positive control because kinase is present. Kinase adds phosphates to the ends making them able to form recombinants. Tubes 3, 4, 5 contained BE1 increasing in 3ul increments (3, 6, 9). Tubes 6, 7, 8 contain HE1 and also increase in amounts of HE1 (3, 6, 9). Total volume of each tube equals 30 uL. Each tube was vortexed and then slightly shaken to make sure all solution was at the bottom of the tube and placed to incubate at 37C for greater than 1 hour. .5 uL of ligase was then added again and incubated at 16C over night in a freezer. Ligation was again followed by performing a gene clean.

	1 (µL)	2 (µL)	3 (µL)	4 (µL)	5 (µL)	6 (µL)	7 (µL)	8 (µL)
H20	23.5	22.5	20.5	17.5	14.5	20.5	17.5	14.5
pMSG	3	3	3	3	3	3	3	3
BE1	------	------	3	6	9	-----	-----	----
HE1	-----	-----	-----	---	----	3	6	9
10X Buffer	3	3	3	3	3	3	3	3
Ligase	.5	.5	.5	.5	.5	.5	.5	.5
Kinase	-----	1	-------	-----	------	-----	-----	-----
Total Volume	30	30	30	30	30	30	30	30

Table 3. Ligation Volumes.

Ligation was followed by transformation and plating on LB ampicillin plates. Next, E. coli cells were created that will uptake the circular recombinants. The solution was then plated on LB ampicillin plates at 37C upside down overnight. 6 colonies from BE1 plates and 6 from HE1 plates were chosen. They were transferred to tubes containing 4ml of LB amp broth. DNA was isolated via detergent lysis and centrifugation and was ran through a gel in order to determine if it contained the desired product.

SAL 1

Procedures for the modified end approach closely mimicked the Sma 1 approach as seen in Table 4. For further information see the Sma 1 digest. To blunt the inserts, 2.5 uL of 10X buffer, 1uL of 2mM dNTPs, and 1uL of Klenow was incubated at 30C for thirty minutes, followed by a gene clean.

	pMSG (µL)	HE1 (µL)	BE1(µL)
H20	21	21	21
DNA	5	5	5
10X Buffer	3	3	3
Bam H1	---------	1	1
Sal I	2	---------	----------
Total	30	30	30

Table 4. Sal 1 Volumes for Digest.

Blunted ends are followed by the linker-ligase treatment. DNA samples of HE1 and BE1 were dried, and then H20 was added to make the total volume of DNA and water equal to 14μL. Added to this mixture was 2μL of 10X buffer, 2μL of linker, and 1μL of Sal 1 ligase which is left at room temp for 1-3 hours. 1μL of ligase is again added to the solutions, which is placed in a water bath. The water bath is placed with the lid on inside a freezer to slowly decrease the temp of the bath over night.

Sal 1 is used to digest the modified inserts. After removing the DNA samples from the freezer, they are heated to 65-70C for 15 minutes. A solution containing, 68uL of H20, 20uL of DNA, 10uL of 10X buffer, and 2uL of Sal 1 is created to perform a digest on the modified inserts. After incubation at 37C for more than two hours, 1uL of Sal 1 is again added and incubated for at least 2 more hours, which is followed by performing a gene clean.

The ligation of Sal I inserts with the Sal 1 digested pMSG can be seen in Table 5. Everything remains constant except for the volume of HE1 used. There is a negative control seen in Tube 1 containing no HE1. Ligation was followed by a gene clean. Ligation was followed by transformation and plating, which is similar to the Sma 1 process.

	1 (μL)	2 (μL)	3 (μL)	4 (μL)
H20	20	16	12	8
pMSG	6	6	6	6
HE1	---------	4	8	12
10X Buffer	3	3	3	3
Ligase	1	1	1	1

Table 5.

Results

The following are Polaroid's of the gels. Figure 6 shows successful digestion of pMSG by Sma 1. The boxed bands are removed and isolated. Figure 7, shows successful isolation and aids in determining the concentration of HE1 and BE1 present. Figure 8 confirms the presence and successful blunting with Klenow.

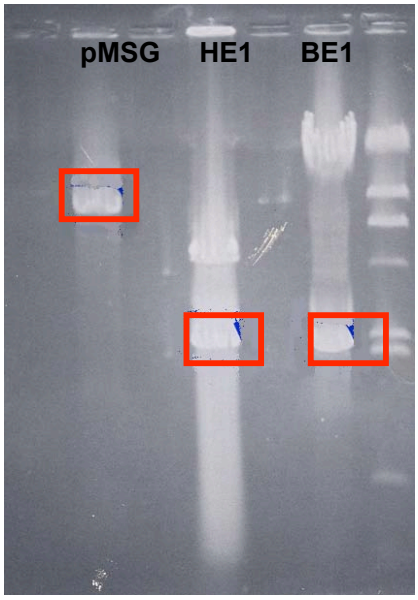


Figure 6. Digest and isolation of pMSG by Sal 1 enzyme. Digest of HPV and BPV by Bam H1 enzyme. Red boxes Represent vector and insert to be isolated.

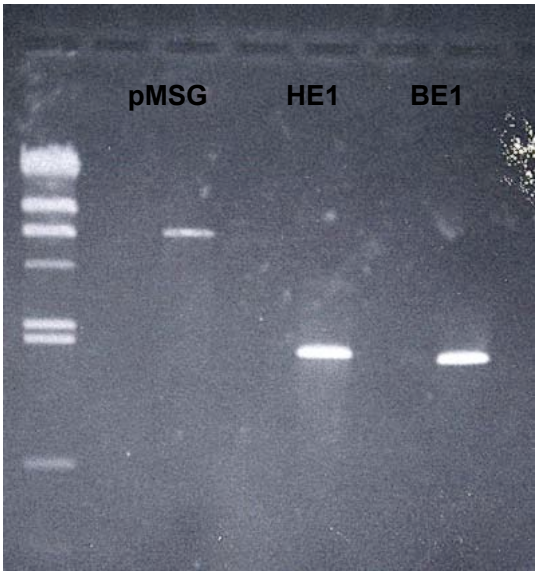


Figure 7. Analysis of DNA concentration of pMSG, HE, and BE1 after isolating the bands of DNA from the previous gel.

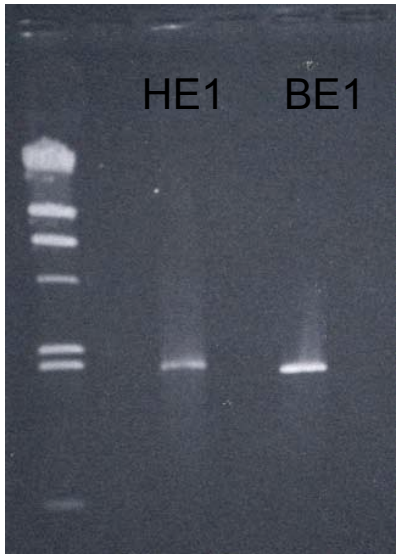


Figure 8. The two lower bands labeled HE1 and BE are the inserts and display the successful filling in of the staggered ends with Klenow.

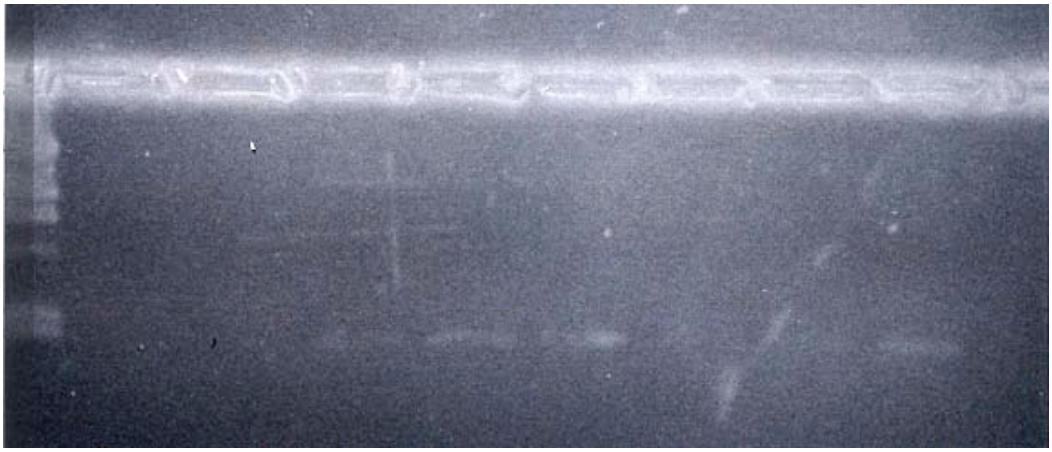
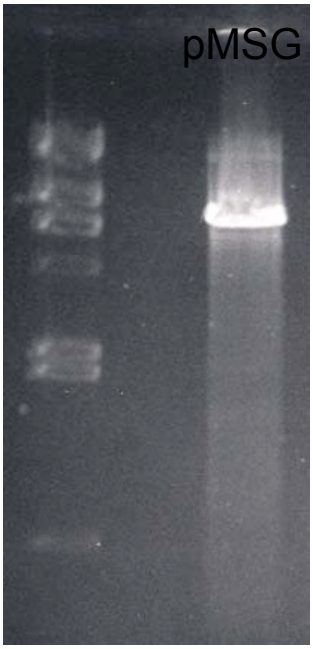


Figure 9. Analysis of DNA from Colonies Grown in Ampicillin to determine desired product. Lower bands represent RNA and faint upper bands represent bacterial chromosomal contamination.



Firgure 10. Successful Sal 1 digest of pMSG.

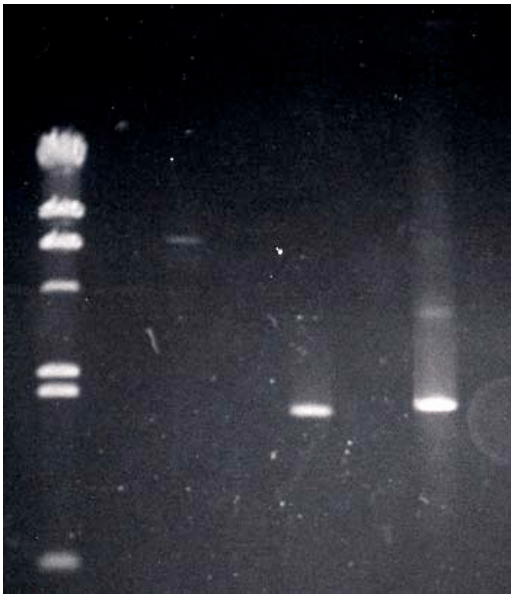


Figure 11. Analysis of DNA concentration of pMSG, HE1, and BE1 after isolating the bands from the previous gel.

Figure 10, depicts successful digest of pMSG by Sal 1. Figure 11 depicts the successful isolation of bands from digests in Figure 10. Unfortunately, no Polaroid's were taken after the transformation, because no growth was seen on the plates.

Discussion

Although scientists have yet to understand the exact mechanism of how cellular DNA replication is induced, much insight can be gained by understanding how cellular mechanisms affect viral E1 protein to initiate viral replication. Difficulties still abound in generating sufficient amounts of viral E1 protein from mammalian cells to analyze, which can be seen in our research. Forming recombinants to introduce into mammalian cells posed a difficult task, due to the lack of success in creating recombinants. It is difficult to determine which method is more efficient in producing recombinants to generate stable mammalian cell lines. Although growth was seen from the blunt ended treatment, the desired product was not found in Figure 9. Alternatively, because no growth was found from the modified treatment, we are not able to analyze the desired product as seen in Table 6. As seen in Table 6, it is expected that the staggered ends of Sal 1 should be more able to produce recombinants because the ends created by Sal 1 exposes hydrogen that can readily bond with single and complimentary stranded Sal 1 digested pMSG (13). The inability to produce recombinants could stem from human error or old enzymes and DNA. Further research should aim at discovering where the errors occur to best gain recombinants. Because neither approach resulted in stable recombinants, further research must be done to create stable recombinants to incorporate into mammalian lines. If stable recombinants were created it would result in a vector that could be placed into a mammalian cell lines to produce E1. Large amounts of E1 would then be readily available for extensive analysis. Scientists would then be able to map and compare phosphorylation sites from both sources of HPV and BPV to determine similarities and differences.

	Sma 1	Sal 1
E1 ends	Blunt 5'ccc ggg3' 3'ggg ccc5'	Modified 5'G TCGAC-3' 3'-CAGCT G-5'
Growth	YES	NO
Desired Product	NO	No gel --------

Table 6. Summary of results showing which enzyme was used, the ends that results from each enzyme, the type of insert used to make a recombinant, and if the recombinant resulted in growth in an ampicillin medium, and whether the DNA recovered from the colonies resulted in the desired product.

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Acknowledgments

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Dedication

For Eva Furmage: “Though you are seven years old, you have shown me more courage and life than all others. Thank you.”