IDENTIFICATION OF THE MEMBRANE ASSOCIATION OF BV/ODV E26 AND THE DOMAINS IN BV/ODV E26 RESPONSIBLE FOR NUCLEAR TRAFFICKING TO INTRANUCLEAR MICROVESICLES

A Dissertation

by

JARED K. BURKS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Biology

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Approved by:

Chair of Committee,	Max D. Summers
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	Patricia Pietrantonio
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ABSTRACT

Identification of the Membrane Association of BV/ODV E26 and the Domains in BV/ODV E26 Responsible for Nuclear Trafficking to Intranuclear Microvesicles.

(December 2005)

Jared K. Burks, B.S.; B.S., Florida Institute of Technology

Chair of Advisory Committee: Dr. Max D. Summers

The baculovirus *Autographa californica* nucleopolyhedrovirus (*Ac*NPV) has two viral forms, budded virus (BV) and occlusion derived virus (ODV). The envelopment of these two viral forms occurs at different locations: BV acquires envelopes at the plasma membrane while ODV acquires envelopes in the nucleus. The two viral forms carry out different functions in the viral life cycle. The purpose of this study is to investigate how viral envelope proteins sort/traffic to the nucleus. Of particular interest is BV/ODV E26 (E26). E26 is an envelope protein of both BV and ODV (Braunagel and Summers, 1994); therefore it must traffic to the plasma membrane and the nucleus during infection. Thus, E26 is a bi-directional trafficking protein, which interacts with membranes in both locations of the cell. As such it has been shown that there are several immunoreactive forms of E26 (Beniya, Braunagel, and Summers, 1998). The *da26* gene produces at least 2 protein products of 26 and 28 kDa with different functions respectively, which correlate with localization, solubility, membrane association, and temporal requirements. The 28 kDa form is likely a soluble protein that interacts with transcriptional activators and DNA in the nucleus in the early stages of infection. A part of the 26 kDa population is a membrane bound form interacting with an integral membrane protein in the ER and likely functions as an INM sorting factor. The 26 kDa membrane bond form is also found in the inner nuclear membrane, intra-nuclear microvesicles, ODV envelopes, and ODV in the nucleus.

ACKNOWLEDGEMENTS

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I would like to thank the many individuals that have served on my committee for their time and insightful criticism including Dr. Hagan Bayley, Dr. Efthimios Skoulakis, Dr. Bruce Riley, Dr. Andreas Holzenburg, and Dr. Patricia Pietrantonio. Thank you all.

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I would like to thank my family, both the Burks and Neely clans for their support. I would like to specifically thank my parents, Roy and Karen Burks, for their support and love. More specifically I would like to thank my parents for always telling me I can do anything and believing me when I told them I was going to do it. I would like to thank my sisters, Audra, Dana, and Megan for their support. I would like to thank my family for never again asking the ever present question, "Are you finished yet?"

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INTRODUCTION

1. Project Overview

The baculovirus *Autographa californica* Nucleopolyhedrovirus (*Ac*NPV) has two viral forms, budded virus (BV) and occlusion derived virus (ODV). The envelopment of these two viral forms occurs at different locations: BV acquires envelopes at the plasma membrane while ODV acquires envelopes in the nucleus. The two viral forms carry out different functions in the viral life cycle. The purpose of this study is to investigate how viral envelope proteins sort/traffic to the nucleus, of particular interest is BV/ODV E26 (E26). E26 is an envelope protein of both BV and ODV (Braunagel and Summers, 1994), therefore it must traffic to the plasma membrane and the nucleus during infection. Thus, E26 is a bi-directional trafficking protein, which interacts with membranes in both locations of the cell, as such it has been shown that there are several immunoreactive forms of E26 (Beniya, Braunagel, and Summers, 1998). The goals of this study are: to determine which of multiple protein products the *da26* gene produced during infection associate with membranes in the nucleus (i.e. microvesicles and ODV envelopes); to determine how E26 associates with membranes (specifically the ER, nuclear envelope, microvesicles, and ODV envelope); and to identify the molecular basis for trafficking the membrane associating form of E26 to membranes located in the nucleus for inclusion in the ODV envelope. There are many questions the last point raises; does E26 associate with the ER and if so, in a co- or post-translational manner? What are the properties of the membrane associated E26 and after ER association does E26 remain associated with membranes throughout the trafficking pathway to the ODV envelope?

1.1 Baculovirus - Viral Classification

The viral family Baculoviridae contains arthropod specific viruses capable of infecting over 600 host species from the orders Lepidoptera, Diptera, Hymenoptera, and

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This dissertation follows the style of *Virology*.

Decapoda. Due to baculovirus' specific host range they have been used for bio-control applications. Improvement of the bio-control applications and development of the baculovirus expression system have resulted in many studies characterizing the infection process (Herniou et al., 2003).

Baculoviruses are rod shaped, double-stranded DNA, lipoprotein enveloped viruses with genomes ranging in size from 90 kb to 180 kb. The Baculoviridae family is subdivided into two genera: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Herniou et al., 2003; van Regenmortel et al., 2000). This subdivision is primarily based on the morphology of the occlusion bodies (OBs) formed around the virion during infection. The genus *Granulovirus* produces ovoid OBs containing a single virion, where as *Nucleopolyhedrovirus* form polyhedral OBs containing many virions.

The research presented in this dissertation utilizes *Autographa californica* Nuclear Polyhedrosis Virus (*Ac*NPV). *Ac*NPV is a member of the genus *Nucleopolyhedrovirus*, which infects over 27 species of insects (Miller, 1997). The *Ac*NPV virion is bacilliform in shape and contains 1 to 29 nucleocapsids, each containing the complete viral genome. Transfection of *Ac*NPV DNA is sufficient for viral infection (Adams and McClintock, 1991).

1.2 Viral Infection Cycle

Baculovirus infection produces two phenotypic forms of enveloped bacilliform virions. ODV is the form responsible for natural ingestion in susceptible insects. It is enveloped in the nucleus and becomes occluded in polyhedrin, figure 1 (Miller, 1997). Microvesicles (intra-nuclear membranes) are hypothesized to bud from the inner nuclear membrane and considered precursors of the ODV envelope (Braunagel et al., 1996a; Braunagel et al., 1996b; Braunagel et al., 2004; Hong, Summers, and Braunagel, 1997; Rosas-Acosta, Braunagel, and Summers, 2001; Saksena et al., 2004). BV is the hemocoelic form, which acquires its envelope by budding through the plasma membrane of the cell, figure 1 (Knudson and Harrap, 1976).

Primary infection in vivo is initiated by ingesting polyhedra which contain the occluded virus, ODV. The occlusion is a highly ordered crystalline matrix composed of the polyhedrin protein. Under conditions of high pH (>10.5), polyhedrin protein interactions are disrupted to essentially dissolve the crystalline occlusion and release the infectious ODV to invade and infect the midgut cells, see figure 1 (Granados, 1978; Summers and Smith, 1975a; Summers and Smith, 1975b). The virions may be released from the polyhedra with a mild alkali treatment. Dissolution of the crystal allows for membrane fusion between the ODV viral envelope and the microvillar membrane of columnar midgut epithelium cell. Once inside the cell, the ODV viral nucleocapsids traffic with the aid of microtubules to the nuclear pore and then through the nuclear pore into the nucleus (Charlton and Volkman, 1993; Lanier and Volkman, 1998). The nucleocapsids uncoat, releasing the viral DNA to initiate replication (Harrap, 1970; Summers, 1969; Summers, 1971).

A. Primary Infection B. Secondary Infection

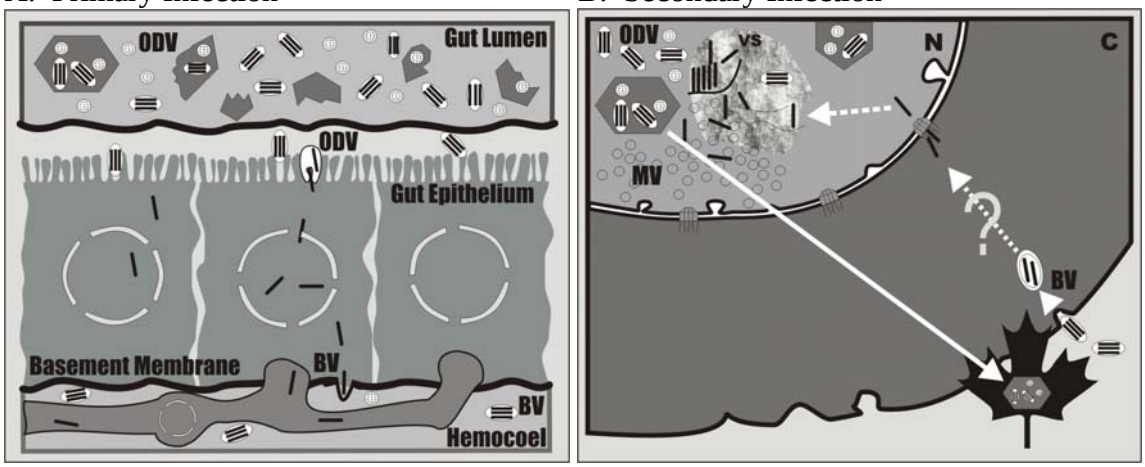

Figure 1. Baculovirus maturation cycle. Panel A represents primary infection of the insect occurring when the insect consumes occlusion derived virus (ODV). ODV is encapsulated in a polyhedrin crystal that is dissolved in the insect gut lumen. This allows the ODV to infect the gut epithelia cells, tracheal cells, and to invade the hemocoel. Once primary infection occurs, the infected cell starts to produce budded virus (BV). BV is responsible for secondary infection by receptor mediated endocytosis, panel B, which allows peripheral cells to be infected. As infection progresses the nucleus enlarges and produces the virogenic stroma (VS), considered the site of viral DNA replication. Later in the infection process the virus switches from production of BV to ODV which, once the insect is liquefied, leaves ODV for infecting a new insect.

In infected cells the nucleus enlarges allowing for the formation of the virogenic stroma (VS), considered the site of viral DNA replication. The VS is easily identified as an intensely DAPI stained region in the nucleus. During the development of the virogenic stroma, viral nucleocapsids assemble around its periphery. Nucleocapsids have two fates, early in infection they traffic to the plasma membrane for BV production and late they become enveloped in the nucleus for ODV production. During the early trafficking of the nucleocapsid to the plasma membrane the nucleocapsids traffic from the periphery of the VS to and through the inner nuclear membrane (INM) into the perinuclear space, obtaining a temporary envelope. These nucleocapsids then lose this envelope and traffic to the plasma membrane where they align anterior end-on with the plasma membrane (Knudson and Harrap, 1976). The membrane aligned with the anterior end of the nucleocapsid is modified with glycoprotein spikes composed of the viral protein gp64 and termed peplomers (Adams and McClintock, 1991). The virions bud through the plasma membrane forming the virus termed budded virus (BV) (Knudson and Harrap, 1976).

Secondary infection of the insect is caused by the spread of BV through the surrounding tissues, see figure 1. The basal lamina, a fibrous matrix of glycoprotein secreted by the epithelial cells, supports the midgut epithelial cells at their base. The basal lamina initially limits the progeny virions from directly entering the insect hemocoel by acting as a physical barrier. Virions can bypass the basal lamina by secondary infection of the tracheoblasts, which penetrate the basal lamina. These tracheoblasts deliver virus to both the tracheal epidermal cells and the hemocoel of the insect (Engelhard et al., 1994; Kirkpatrick et al., 1994). However, according to Krikpatrick et al., 1994 and Engelhard et al., 1994 the BV carried in the circulating hemolymph does not directly infect other tissues, secondary infection depends on passage through the tracheoblasts as they serve as portals across the basal lamina barriers. However, injection of BV into the insect hemocoel results in infection of the insect. BV invades other tissues through receptor-mediated endocytosis via gp64, the protein responsible for membrane fusion and a protein component of the BV envelope

(Wang, Hammer, and Granados, 1997). Once endocytosed, gp64 aids in uncoating the envelopes in a process that is not completely understood. The nucleocapsids then traffic to the nucleus to uncoat and initiate the infection process (Granados, 1978; Hess, Summers, and Falcon, 1978; Summers, 1971). Infection continues resulting in liquefaction of the host cadaver. This liberates the ODV on the surface of plants to infect future hosts (Adams and McClintock, 1991; Miller, 1997).

1.3 Virus Structure

The nucleocapsids of BV and ODV are similar in structure and are approximately 50 nm x 275 nm. There are several structural proteins of the nucleocapsid for which a comprehensive background is beyond the scope of this dissertation; however two of these proteins are relevant to this research and are vp39 and FP25K. The major protein of the capsid is vp39. When FP25K is deleted the resulting mutant virus infection produces a phenotype of "few polyhedra," a dramatic decrease in the number polyhedra produced (Beames and Summers, 1989; Braunagel and Summers, 1994; Harrison and Summers, 1995a; Harrison and Summers, 1995b; Miller, 1997). Also of note, when FP25K is deleted ODV E66 (E66 has been shown to co-translationally insert via the translocon in to the ER) does not cross the nuclear envelope via the lateral channel, but remains in the outer nuclear membrane (Braunagel et al., 1999; Rosas-Acosta, Braunagel, and Summers, 2001). E66 is the most carefully studied ODV envelope protein that traffics to the nuclear envelope, microvesicles, and the ODV envelope, more detail about this protein will follow later. FP25K has also been reported to interact with E26 in yeast two hybrid experiments (Beniya, Braunagel, and Summers, 1998).

The nucleocapsids are enveloped in a typical trilaminar envelope, composed of a lipid bilayer and an internal lumenal space. Lipid and protein composition of BV and ODV envelopes differ markedly, however they also share several proteins. This is due to the fact that ODV and BV derive envelopes from different sources, the nuclear envelope and plasma membrane, respectively. This requires viral envelope proteins to

sort and locate to the nuclear envelope, plasma membrane, or both for insertion or integration into BV and/or ODV envelopes.

1.3.1 BV Envelope

Viral proteins traffic to the plasma membrane via the same pathway as cellular proteins via vesicular trafficking, ER to golgi, golgi to the plasma membrane (Lecuit, 2004). The major glycoprotein of BV is gp64, which is also the envelope fusion protein activated by the endocytic pathway (Zhang, Han, and Blissard, 2003). *Gp64* is an early and late baculovirus gene allowing for expression throughout infection. Immunologic and biochemical studies show gp64 accumulation in the plasma membrane in spike-like structures called peplomers, the sites where nucleocapsids of BV bud through the cell surface. Because of this process peplomers are located only on the BV envelope surface at one end of the nucleocapsid, figure 2. The gp64 protein anchors to the membrane by a C-terminal acylation anchor domain where palmitic acid is ester linked to the protein and inserted into the membrane (Miller, 1997; Zhang, Han, and Blissard, 2003).

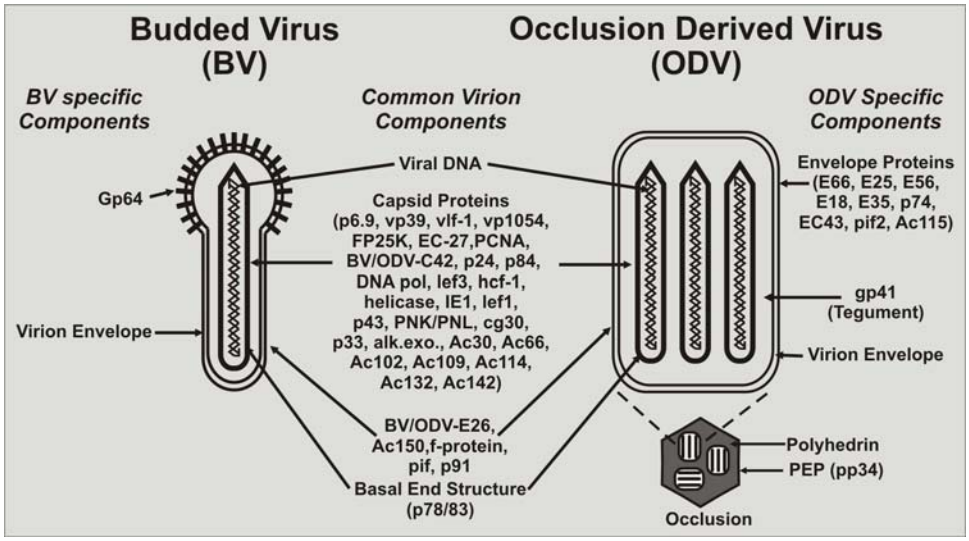

Figure 2. Virion structure and protein components localized in budded and occlusion derived virus, the two phenotypes of baculovirus. Proteins common to both viral forms are indicated in the center of the figure with specific proteins to either BV or ODV noted on the left and right respectively. ODV is also shown encapsulated in polyhedrin with the polyhedron envelope composed of pp34 noted. (Adapted from Miller, 1997)

There are several BV associated proteins (envelope and capsid proteins) including BV/ODV E26, ubiquitin, IE1, cathepsin, and actin; relevant proteins to this dissertation will be discussed below (Braunagel and Summers, 1994; Miller, 1997). BV/ODV E26 as the focus of this dissertation will be discussed in detail later. IE1, an immediate early protein of the virus, is believed to be a transcriptional activator, which is capable of binding DNA. *Spodoptera frugiperda, Sf*9, insect cells recognize the IE1 promoter resulting in production of IE1 protein. Due to this fact this promoter is used commonly for gene expression in uninfected insect cell culture (Carson, Summers, and Guarino, 1991; Kovacs et al., 1992).

1.3.2 ODV Envelope

Several proteins have been identified in the ODV envelope that are not relevant to this dissertation, a recent comprehensive summary is reported in Braunagel et al., 2003 with an abbreviated overview presented in figure 2. The ODV structural envelope proteins relevant to this dissertation include BV/ODV E26, ODV E66, and ODV E25 (Braunagel et al., 1996a; Braunagel et al., 2003) A brief summary follows.

ODV E66 is produced by a late gene and is an integral membrane protein detectable in infected *Sf* 9 cell extracts from 24 to 72 h p.i. by Western blot analysis. ODV E66 has an N-terminal 33-amino acid signal anchor which is not cleaved and functions to sort and traffic ODV E66 or reporter fusion proteins (GFP, β-galactosidase) to the intra-nuclear microvesicles and the ODV envelope. This 33-amino acid motif is referred to as an inner nuclear membrane sorting motif or INM-SM. The INM-SM consists of a single hydrophobic transmembrane domain and two associated charged amino acids, lysines which are located 6 and 11 amino acids from the end of the transmembrane domain (TMD) (Braunagel et al., 2004). This INM-SM is not cleaved and has been found to be sufficient to locate reporter proteins, GFP and β-galactosidase, to the viral-induced intranuclear microvesicles and the ODV envelope (Hong, Summers, and Braunagel, 1997). It is also capable of delivering fusion proteins to the inner nuclear membrane (INM) in the absence of infection (Braunagel et al., 2004; Hong, Summers,

and Braunagel, 1997). Covalent cross-linking studies performed using the sorting motif fused with GFP show that the lysines of the motif (+6 and +11 a.a. from the TMD) are proximal to BV/ODV E26 and/or FP25K in infected insect cell microsomal membranes (membrane fractionation technique which predominantly isolates the ER) during the integration and release of the INM-SM from the translocon (Braunagel et al., 2004). This putative interaction between E66 and E26 could create and/or stabilize a post-translational membrane association/integration between E26 and the ER, through either a protein-protein interaction or by lowering the pKa of palmitoylation allowing E26 to be lipid anchored to the membrane.

Initial evidence of viral INM-SM sorting has been found as early as integration at the translocon (Saksena et al., 2004). Distinctive photocrosslinking patterns were observed with INM transmembrane sequences indicating that INM and non-INM transmembrane sequences occupy distinctly different sites within the insect and canine translocons, which in turn suggests that INM-directed transmembrane sequences are first identified and sorted by translocon components. INM directed transmembrane sequences are proximal to both Sec61α and TRAM; either or both could be involved INM transmembrane sequence sorting. INM transmembrane sequences remain proximal to TRAM longer than Sec61α, therefore the authors propose that TRAM is involved in the hand-off of INM transmembrane sequences to the next participant in the putative INM sorting pathway. Viral INM-directed proteins, upon leaving the translocon, interact with other viral proteins that are required for sorting to the INM. As such FP25K and BV/ODV E26 were identified from crosslinking with the INM-SM. As viruses typically appropriate host mechanisms, the identification of viral proteins required for INM sorting strongly suggests the presence of similar host proteins involved in INM sorting. Therefore sorting to the INM for some viral and cellular integral membrane protein begins with protein translocation in the ER, which could be mediated by specific protein-protein interactions (Saksena et al., 2004).

In the absence of FP25K, E66 does not traffic to the INM but is found to accumulate in the ONM and ER (Braunagel et al., 1999; Rosas-Acosta, Braunagel, and Summers,

2001). Braunagel et al., 2004 propose that the INM-SM sorts proteins to the inner nuclear membrane. The authors hypothesize that E66 and the sorting motif fusions do not randomly diffuse from the endoplasmic reticulum, site of co-translational insertion, to the nuclear envelope and microvesicles during infection (Braunagel et al., 2004; Saksena et al., 2004). For the viral integral membrane proteins their current opinion is that this trafficking is mediated by specific protein-protein interactions during integration at the translocon, the ER, the outer nuclear membrane (ONM), and the inner nuclear membrane (INM). The authors suggest that E26 and/or FP25K might be directly or indirectly involved in facilitating the nuclear trafficking of E66 (Braunagel et al., 2004).

ODV E25 is another envelope protein produced by a late gene and is an integral membrane protein with a similar N-terminal hydrophobic transmembrane domain to ODV E66. However E25 does not have the associated charged amino acids that are found in the E66 INM-SM. The N-terminal signal anchor of ODV E25 is not cleaved and traffics ODV E25 to the microvesicles and the ODV envelope (Hong, Summers, and Braunagel, 1997). E25 localization is not altered in an FP deletion virus, which makes it different from E66 and places relevance on the size differences of the proteins and the associated charged amino acids found in E66 that are not found in E25: this could account for different localizations of these two proteins in FP mutant virus infected cells (Braunagel et al., 1999; Rosas-Acosta, Braunagel, and Summers, 2001).

1.4 Gene Expression and Viral DNA Replication

Upon release of the viral DNA viral transcription is classified in four temporal phases, immediate early (IE), delayed early (DE), late (L), and very late. RNA transcripts from the IE class of genes are detectable within the first 15 to 60 minutes after inoculation (Miller, 1997). Optimal expression of delayed early and late genes requires early gene expression. Transcription of the delayed early genes requires the presence of the immediate early genes; together the IE and DE genes represent the early phase of transcription. The early phase continues until the start of viral DNA replication

which begins about 6 to 10 hours post infection (h p.i.). Optimal expression of late genes requires the expression of 18 early genes. Late gene transcription begins approximately 10 h p.i. and continues until cell death with maximal late gene transcription occurring at approximately 24 h p.i. (Herniou et al., 2003).

E26, the topic of this study, was classified as an early protein (Guarino and Summers, 1988); however the protein is an ODV envelope protein it must also be a late protein, as such it must also have a late gene promoter. There is also some preliminary evidence from Imai et al., 2004, that suggests E26 might play a roll in viral DNA replication. Because of this some preliminary experiments were performed in this project to clarify its expression pattern and identify its other promoters. To clarify the fundamental details for E26 gene expression a short summary of baculovirus gene expression and viral DNA replication will follow.

The immediate early genes are distributed throughout the circular DNA genome of baculoviruses and transcribed from both DNA strands by the host RNA polymerase II. The immediate early genes involved in viral gene transcription are ie-0, ie-1, ie-2, ORF121, and pe38 (Carson, Guarino, and Summers, 1988; Carson, Summers, and Guarino, 1991; Gong, Jin, and Guarino, 1998; Herniou et al., 2003; Kovacs et al., 1992). The structure of the early gene promoters resembles RNA polymerase II responsive cellular gene promoters. RNA polymerase II-responsive baculovirus promoters include a core region surrounding the +1 RNA start site. This core provides a basal level of transcriptional initiation that is activated by *cis*-acting elements. These basal promoters can be classified as TATA-containing (TATA), initiator-containing (INR), or a composite containing TATA/INR promoter. The most conserved INR motif is the CAGT motif, first functionally identified in the ie-1 gene, which is a typical composite promoter (Herniou et al., 2003). The first CAGT in proximity to the start of the *da26* gene is located 256 nucleotides upstream of the gene in the *Ac*NPV E2 viral genome: to be clarified later.

Viral DNA replication of *Ac*NPV in *Spodoptera frugiperda* (*Sf* 9) cells is detected by 6 to 10 h p.i. and continues until approximately 18 h p.i., after which the rate of DNA

replication declines. Infection occurs when baculovirus DNA is transfected, therefore proteins associated with the mature virions are not essential for viral DNA replication. Viral DNA synthesis is dependent on IE and DE gene expression, which led to the discovery of a viral DNA polymerase (Mikhailov et al., 1986; Miller, Jewell, and Browne, 1981; Wang et al., 1983). Recently it has been shown that the baculovirus viral DNA polymerase is required for viral DNA synthesis (Vanarsdall, Okano, and Rohrmann, 2005). *Cis*- and *trans*-acting elements associated with DNA replication have been identified in baculovirus genomes, and are believed to be involved in DNA synthesis. One gene of interest to this study is IE-1, which is required for viral DNA replication, as it is believed to be a transcriptional activator. It is of interest as an interaction between E26 and IE-1 has been proposed due to experiments performed by Imai et al., 2004 and due to yeast two hybrid experiments performed in the Summers' lab. This will be clarified in greater detail later.

Late genes are identified as those that are expressed after viral DNA replication. During the late phase, structural proteins are predominantly produced, these include envelope proteins of ODV, vp39 (Capsid), p6.9 (Basic core protein), and during the very late phase polyhedrin, and p10. Late gene transcription begins after viral DNA replication and can continue throughout infection (Huh and Weaver, 1990). A novel virus induced RNA polymerase is produced, which is responsible for the transition from early to late gene expression (Huh and Weaver, 1990). An essential motif to late baculovirus gene promoters is the TAAG element. Transcriptional mapping studies of several late genes have identified this TAAG motif as the initiation site (Theilmann et al., 1996). E26 is an ODV envelope protein therefore it must also be a late protein; as such it must also have a late gene promoter. The first TAAG element in proximity to the start of the *da26* gene is located 783 nucleotides upstream of the gene in the *Ac*NPV E2 viral genome: to be clarified later.

1.5 BV/ODV E26

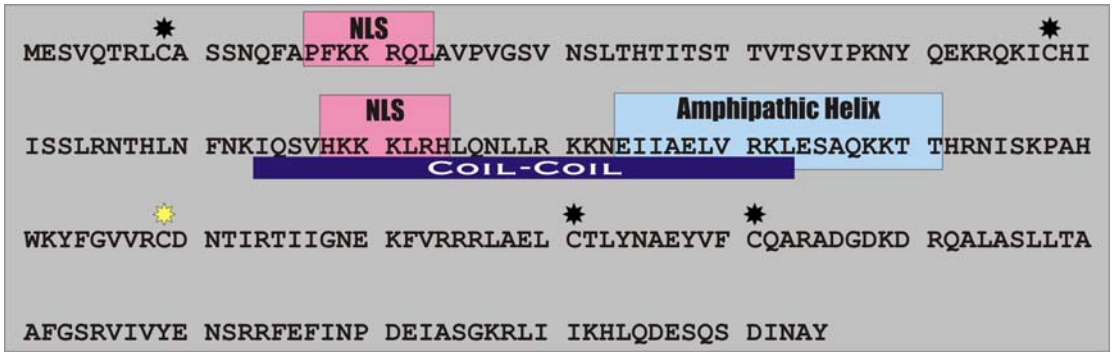

Figure 3. E26 amino acid sequence. E26 amino acid sequence with the putative domains predicted using computer bioinformatics' tools and by sequence gazing, note the two putative nuclear localization signals (NLS), the coil-coil domain, the amphipathic helix, and the 5 cysteine amino acids. Coil-coil domains are sites of protein-protein interactions. NLS function to deliver soluble and membrane associated proteins to the nucleus. Amphipathic helices and lipid anchors allow for interactions with membranes. Cysteines amino acids are where lipids are covalently attached to membrane proteins. The cysteine at amino acid 129, in yellow, has a great potential for lipidation due to the charged amino acids that surround it.

The purpose of this study is to clarify factors important for E26 membrane association and to investigate the mechanisms of its trafficking to the ODV envelope. Comparisons will be made with ODV E66, in order to determine if E26 follows the same pathway as E66. Examining the BV/ODV E26 (E26) primary amino acid sequence reveals several domains and amino acids of interest in the context of this study. Specifically the E26 a.a. sequence contains two putative nuclear localization signals (NLS), a putative amphipathic helix, a coil-coil domain, and 5 cysteine amino acids, see figure 3. This search also reveals that E26 does not contain any predictable transmembrane sequence or signal anchor. However, the domains identified could account for the nuclear localization and membrane association. Coil-coil domains are common sites of protein-protein interactions, three of which have been preliminarily identified including IE-1, FP25K, and ODV E66. NLSs function to deliver soluble and membrane associated proteins to the nucleus. Comparing E26 to ODV E66, E66 does not contain any predictable NLS, as such it sorts and traffics through the membranes of the ER, ONM, lateral channels of the nuclear pore complex (NPC) and into the INM of the nucleus through the "diffusion:retention" pathway (Ellenberg et al., 1997; Smith and

Blobel, 1993; Soullam and Worman, 1993; Soullam and Worman, 1995). Amphipathic helices and lipid anchors allow interactions with membranes. A lipid anchor, specifically S-acylation, covalently links a lipid inserted into the membrane bi-layer to cysteine amino acids, E26 contains 5 cysteine amino acids. As mentioned earlier gp64 is a baculovirus protein that utilizes this type of membrane association, serving as a model for E26 membrane association. The cysteine at amino acid 129, in yellow, has a great potential for lipidation due to the charged amino acids that surround it. A more comprehensive background and examples for each of these possibilities will be presented in relevant sections to follow.

1.5.1 BV/ODV E26 Literature

Da26, the gene for E26, was identified by Guarino and Summers in 1988 in the *Pst*I-G fragment of the E2 strain of the *Ac*NPV viral genome. When the gene was translated into its amino acid sequence it predicted a highly basic 226 amino acid protein with a molecular weight of 25,891 Da. Nuclease S1 mapping of *da26* RNAs from infected and transfected cells indicated that *da26* is transcribed as a delayed-early gene. An early baculovirus promoter (CATTA) was identified 34 nucleotides upstream of the ATG start of the *da26* gene (Guarino and Summers, 1988). Guarino and Summers also found that late gene expression was increased 3- to 10-fold by expression of *da26* and *da41* by measuring CAT activity expressed by a late promoter in a cotransfection assays with IE1. This increase in expression is not due to an increase in the steady state levels of mRNA, suggesting that the effect observed was post-transcriptional (Guarino and Summers, 1988).

The *da26* gene was found altered with insertions and deletions after several serial passage of the virus in cell culture, molecular characteristics that resemble those of transposable elements (O'Reilly et al., 1990). *Da26* transcripts were detected as early as 1 hour post infection (h p.i.) in *Trichoplusia ni* cells. This led O'Reilly et al., 1990, to construct a recombinant virus in which *da26* was truncated after amino acid 118 by insertion of the *lacZ* gene. They found this mutant virus to be viable in both

Trichoplusia ni and *Spodoptera frugiperdia* cells with no detectable differences from wild type infections (O'Reilly et al., 1990). Such a construct at this time was considered equivalent to a complete gene mutation or a gene knockout. Therefore, O'Reilly et al. concluded that *da26* was not essential for viral replication in either cell culture or in the insect. In this study several of my experiments were based on O'Reilly's data that E26 was not essential. As to be shown, this led to several major complications as data will be presented that contradicts these conclusions and explains why the O'Reilly et al. results were not correct.

The nucleotide sequence of the genome region (EcoRI-A) flanking *da26* is presented in Braunagel et al., 1992. In Braunagel et al., (1992) the authors predicted the amino acid sequences for 15 putative ORFs including *da26*. Amino acid homology searches using the *da26* found a high degree of homology (20% identity and 40% similarity) to the outer capsid protein, VP8, of rotavirus (Braunagel et al., 1992). Sequence similarity with VP8 indicated possible hemagglutination activity (Fiore, Greenberg, and Mackow, 1991; Lizano, Lopez, and Arias, 1991) and maybe a role in host range determination (Zapikian and Chanock, 1990).

Beniya et al., 1998, determined E26 to be an envelope protein of both BV and ODV. Thus Beniya et al., 1998, named the 26-kDa species of the protein to designate that form incorporated into viral progeny, incorporated into viral envelopes, and apparent molecular weight. The 7554 antiserum to E26 was used in a Western blot temporal course analysis, which detected steady state synthesis of E26 from 4 h p.i. throughout infection, with maximal protein levels at 16 h p.i. By 36 h p.i. several additional immunoreactive proteins were identified, which migrated at approximately 18, 26, 28, and 34 kDa. Both the 26 and 18 kDa proteins were identified in Western blots of purified BV and ODV viral envelopes indicating they are structural envelope proteins of both viral forms. By IEM E26 protein is detected in ODV envelopes at 24 and 36 h p.i. Also by IEM, E26 protein is detected in cytoplasmic vesicles and electron dense regions contained in the nucleus at 6 h p.i. (Beniya, Braunagel, and Summers, 1998).

Immuno-precipitations (IPPs) find that E26 and FP25K are in the same precipitated complex of proteins from infected cell extracts and yeast two-hybrid assays find that they are capable of interacting. IPPs find that E26 and cellular actin are also found in the same precipitated complex suggesting a possible association bewteen these two proteins (Beniya, Braunagel, and Summers, 1998). The authors' concluded, "these data suggest that FP25K and cellular actin may participate in the regulation, or movement through the cell, of baculovirus proteins and/or nucleocapsids."

Homologues to the *Ac*NPV E26 protein have been identified in seven of the sequenced baculoviruses with highly conserved homology as seen in figure 4: indentity and similarity are noted in the figure. Specifically the most conserved regions of the E26 gene are amino acids 53 through 105 and 123 through 209, as denoted by stars (indentical a.a.), colons (highly conserved a.a), and periods (conserved a.a.) below the line up of the genes. These regions contain the second NLS, the coil-coil domain, the amphipathic helix, and the 3 c-terminal cysteines. The domains in these regions which are the most conserved are the character of the second NLS and the c-terminal 3 cysteines with proximal charged or polar amino acids. Homology to E26 with *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV), ORF8, was examined by Imai et al., 2004. The authors found that *orf8* was expressed as an early gene. Immunoblot analysis failed to detect ORF8 protein associated with BV and/or ODV when using antisera developed against a bacterial expressed form of the *orf8* gene (Imai et al., 2004). This observation is in direct conflict with data from the Summers' lab (Benyia et al., 1998). Experiments have been performed and will be presented, which demonstrate E26 only associates with membranes in infected cells. These membrane association conditions are absent in bacteria, thus epitopes to the membrane associated form are absent in the bacterial expressed protein presented as antigen to rats by the Imai group. In Imai et al., confocal microscopy revealed that ORF8 protein accumulated in the nucleus with relatively constant levels of expression from 4 to 24 h p.i. and was found to co-localize with IE1 to specific nuclear foci. The authors attempted to recreate the null mutant experiment presented in O'Reilly et al. with the insertion of a reporter gene. Isolation of the null

Figure 4. E26 homologues. E26 amino acid sequences from 7 different baculoviruses compared with the AcNPV form using DBclustal: rapid and reliable global multiple alignments of protein sequences detected by database searches (Thompson et al., 2000). Stars (**⋆**) indicate identical a.a., colons (**:**) indicate highly conserved a.a., and periods (**.**) indicate conserved a.a. The relevant domains of the AcNPV E26 are denoted; cysteines in green, NLSs in yellow, coil-coil domains in red, and the amphipathic helix in pink. The percent identities, similarities, and gap are in relation to the E26 from AcNPV. The baculovirus strains listed in the figure above are as follows from top to bottom: *Autographa californica* NPV, *Rachiplusia ou* NPV, *Bombyx mori* NPV, *Epiphyas postvittana* NPV, *Orgyia pusedotsugata* NPV, *Antheraea pernyi* NPV, *Choristoneura fumiferana* defective NPV, and *Choristoneura fumiferana* NPV.

mutant was not possible suggesting that *orf8* is essential. Biochemical analyses performed indicated that ORF8 protein bound to nucleic acids suggesting a DNA binding activity (Imai et al., 2004). The authors concluded that *Bm*NPV E26 may be involved in viral DNA replication and/or transcription.

1.5.2 The Model for BV/ODV E26

A model for E26 that localizes to the nucleus during infection has two possibilities. Early during infection the protein has one role as a soluble protein and late in infection the role changes to that of a membrane associated protein. That is not to say that the two roles are exclusive of each other, they do appear to overlap in the time course of infection. Basically the *da26* gene produces at least two protein products pertaining to the study presented in this dissertation, these two products have different functions. The 28 kDa form is a soluble protein that interacts with transcriptional activators and DNA in the nucleus during the early stages of infection (Imai et al., 2004). Where as, a population of the 26 kDa form is a membrane associated form that interacts with a viral integral membrane protein (ODV E66) in the ER and is found in the inner nuclear membrane, intra-nuclear microvesicles, ODV envelopes, and ODV in the nucleus (Beniya, Braunagel, and Summers, 1998; Braunagel et al., 2004). The focus of my research is predominately on the 26 kDa membrane associated form of E26.

2. Study Relevance and Specific Aims

E26 being an envelope protein of both BV and ODV (Braunagel and Summers, 1994) traffics to the plasma membrane and the viral envelope in the nucleus during infection. Thus, E26 is a bi-directional trafficking protein (Beniya, Braunagel, and Summers, 1998). The goal of the study is to identify the molecular basis for trafficking E26 to membranes located in the nucleus.

Specific Aims:

1. Determine if there are multiple forms of E26 created or modified during infection, if so which of these forms becomes an integrated in to the ODV envelope.

2. Determine if E26 associates with ER membranes, traffics to the INM via membrane associations, and the properties of those membrane associations in correlation to the E66 association and trafficking pathway.
3. Identify regions within E26 required for the ER association and if possible trafficking of E26 to intra-nuclear membranes.
4. Investigate if protein interactions play a role in the nuclear trafficking of E26.

Understanding the process and mechanisms by which a virus sorts and targets protein to the INM can reveal insights into the molecular basis of nuclear envelopathy disease. Although E26 has been classified as a membrane protein, unpublished preliminary data suggesting it is a peripheral membrane protein will be presented in this document. These studies will attempt to answer the following questions: Are there multiple forms of E26 created or modified during infection, if so which form becomes an envelope protein of ODV? How does the E26 envelope protein interact with membranes in the cell? How does the virus sort this form of E26 to the ODV envelope, does it follow the pathway described for ODV-E66, does it aid in trafficking ODV-E66, or do the two traffic as a complex together? Since E26 crosslinks in infected microsomes with E66, that form, at least, seems to follow some aspect of the E66 trafficking pathway. Other questions could be answered, including is there a unique trafficking pathway for membrane associated proteins, or do they traffic as soluble or integral membrane proteins? Significant progress in understanding the molecular mechanisms controlling trafficking of a membrane associated protein can be made using E26 as a model and comparing it to other viral and cellular proteins including ODV E66 and LBR. Studying these trafficking patterns will potentially extend our knowledge of cellular and viral controls of cellular membrane protein trafficking during infection, with specific comparisons of nuclear trafficking in infected and uninfected cells.

For the study of pathways and mechanisms of INM proteins this research is pertinent and timely, as few inner nuclear membrane associated proteins have been studied, however this is a rapidly growing field. Mutations or alteration of INM proteins and the lamins with which they interact has been found to lead to several diseases in humans and

other organisms (Broers, Hutchison, and Ramaekers, 2004; Burke and Stewart, 2002; Gilchrist et al., 2004; Mounkes et al., 2003; Ostlund and Worman, 2003; Ostlund and Worman, 2004; Somech et al., 2005; Worman, 2005). Diseases include cardiac and skeletal myopathies, partial lipodystrophy, peripheral neuropathy, and premature aging. These diseases are collectively known as "nuclear envelopathies" (Somech et al., 2005; Worman, 2005). To date, mutations in genes encoding the nuclear envelope proteins emerin, MAN1, lamin A/C, and lamin B receptor (LBR) were found to cause nuclear envelopathies. The diseases caused by mutations in the *LMNA* gene have been subdivided and are called "laminopathies."

A mutation or lack of expression of emerin in humans causes X-linked Emery-Dreifuss muscular dystrophy. Loss of function mutations of MAN1 result in osteopoikilosis, Buschke-Ollendorff syndrome and melorheostosis, an increased bone density disorder (Somech et al., 2005; Worman, 2005). Two additional INM protein diseases are Pelger-Huet anomaly and Greenberg skeletal displasia, which are caused by mutations in the lamin B receptor (LBR) gene. Pelger-Huet anomaly results in developmental delay, epilepsy, skeletal abnormalities and changes to both nuclear morphology and chromatin organization in granulocytes (Mounkes et al., 2003; Somech et al., 2005).

The laminopathies account for the bulk of the diseases identified and are due to multiple mutations in *LMNA* gene (lamin A and C). These mutations are responsible for at least nine different diseases which affect the skeletal and cardiac muscle. These diseases are autosomal dominant Emery-Dreifuss muscular dystrophy, Limb girdle muscular dystrophy type 1B, dilated cardiomyopathy, Dunnigan's familial partial lipodystrophy, mandibuloacral dysplasia, Hutchinson-Gilford progeria syndrome, atypical Werner's syndrome, restrictive dermopathy, and Charcot-Marie-Tooth neuropathy type 2 B1(Broers, Hutchison, and Ramaekers, 2004; Burke et al., 2005; Gilchrist et al., 2004; Mounkes et al., 2003; Ostlund et al., 1999; Ostlund and Worman, 2003; Ostlund and Worman, 2004; Somech et al., 2005; Worman, 2005).

*3. Structure and Function of the Nucleus**

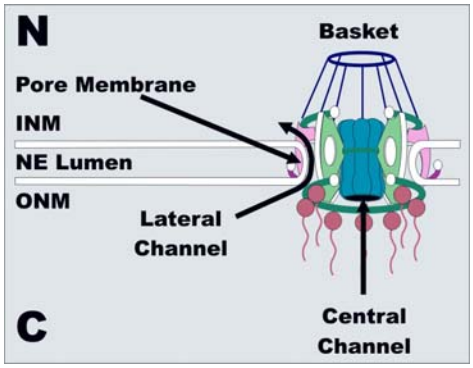

Figure 5. Nuclear envelope and pore structure. Note the central channel and lateral channel, the two passages through the NPC. The nuclear pore basket is also denoted. The nuclear envelope is composed of the inner and outer nuclear membrane, enclosing the nuclear lumen. Where they meet in the lateral channel forms the pore membrane, a biochemically distinct membrane. (Adapted from Blobel and Woznaik, 2000)

Since the nuclear trafficking of E26 is the primary focus of this dissertation a short summary of nuclear architecture and function will follow in order to identify pathways and processes where E26 is likely to traffic during its sorting into the INM, intranuclear microvesicles, and the ODV envelope.

3.1 Nuclear Envelope (NE)

Nuclei are typically depicted as spherical in shape; however the shape of the nuclear envelope can diverge greatly from this model. Grooves and invaginations have been found throughout the nuclear envelope of many eukaryotes (Fricker et al., 1997). The envelope is made of two concentric membranes, the inner (INM) and outer nuclear membrane (ONM), see figure 5. The ONM is contiguous with the endoplasmic reticulum (ER) (Harris, 1978). The INM and ONM are continuous through the nuclear pore membrane, however they maintain distinct protein compositions (Goldberg and Allen, 1995; Harris, 1978). The nuclear envelope is not just a boundary of the nucleus,

*Figure 5 with adaptation reprinted with permission from "Proteomics for the pore" by Blobel, G. and Wozniak, R. W., 2000, *Nature*, 403, 835-836. © 2000 by Nature Publishing Group. www.nature.com.

but also an interface between the nuclear and cytoplasmic compartments via the nuclear pore complex (NPC).

3.1.1 Outer Nuclear Membrane

The ONM resembles the RER as it is a contiguous membrane system sharing many characteristics including attached functional ribosomes (Harris, 1978). This continuous membrane allows integral membrane proteins that are inserted in the ER a path through the ER to the ONM and into the INM via the lateral membrane channels of the NPC (Gant, Goldberg, and Allen, 1998).

3.1.2 Nuclear Envelope Lumen

The nuclear envelope membranes enclose an aqueous domain called the nuclear envelope lumen or perinuclear space. The function of the NE lumen potentially overlaps with the RER lumen, but the NE lumen may have some unique functions yet to be identified. The NE lumen provides an environment for the lumenal domains of integral membrane proteins of the ONM, INM, and pore membrane. These integral membrane proteins may anchor the NPC and lamina, or, may provide a functional connection or communication between the lumen and the nucleus, NPC, or cytoplasm (Goldberg and Allen, 1995).

*3.1.3 Inner Nuclear Membrane**

Baculovirus infection creates an amplified pulse of viral integral membrane proteins (IMP) trafficking through the continuous membranes of the ER, ONM, and INM during transit to the viral envelope of ODV (Braunagel et al., 2004). Recall that the ODV envelope is derived of intra-nuclear membranes, microvesicles, which are precursors to the ODV envelopes and are hypothesized to bud from the inner nuclear membrane of

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*Figure 6 with adaptation reprinted with permission, from the *Annual Reviews of Cell and Developmental Biology*, Volume 21 © 2005 by Annual Reviews, www.annualreviews.org

infected nuclei (Braunagel et al., 1996a; Braunagel et al., 1996b; Braunagel et al., 2004; Hong, Summers, and Braunagel, 1997; Rosas-Acosta, Braunagel, and Summers, 2001; Saksena et al., 2004). It is presumed that these viral proteins are trafficking through the same pathways as cellular IMPs. An understanding of the mechanisms required to traffic cellular proteins to the INM should provide insights into viral protein trafficking to the nucleus and vice versa.

Although the INM is connected to the ER via the NPC and the ONM, it does not resemble the RER or the ONM in protein composition, structure, or function. Recent studies have found that some of the proteins of the INM interact with proteins of the ONM and connect the peripheral nucleoskeleton to the cytoskeleton. Examples are *C. elegans'* UNC-83 and ZYG-12, which connect to the microtubule cytoskeleton and centrosomes respectively. UNC-83 and ZYG-12 interact with UNC-84 and SUN1 (or matefin) respectively, which are proposed to locate to the INM (Hetzer, Walther, and Mattaj, 2005). These interactions and connections are responsible for nuclear positioning, nuclear movement, and possibly other as of yet, unidentified functions.

The nesprin family of proteins (Syne, Myne, and NUANCE) demonstrates that NE proteins also fulfill a specialized roll in establishing and maintaining the three-dimensional architecture of certain differentiated cells. These proteins are large, integral proteins that are proposed to locate to the INM. They contain many spectrin repeats, a C-terminal transmembrane domain, and an actin binding motif. Some nesprins bind both lamin A and emerin in vitro (Apel et al., 2000; Zhang et al., 2005; Zhang et al., 2001).

Nuclear lamins were the first proteins identified of the INM and are a type of V intermediate filament proteins (Rzepecki and Fisher, 2002; Worman and Courvalin, 2000). Lamins have an amino-terminal head and a carboxyl-terminal tail domain separated by an alpha-helical rod domain, these are highly conserved domains of the intermediate filament protein family (Shumaker, Kuczmarski, and Goldman, 2003). The lamins form the nuclear lamina on the inner surface of the INM interacting with several INM proteins, see figure 6. These proteins can be divided into two classes, A-type and

B-type, based on expression patterns, properties and localization. A-type lamins are typically expressed in differentiating cells and are generally more soluble than B-type lamins during M-phase. B-type lamins are expressed in almost all cells and are usually associated with the membrane vesicles during mitosis. There are three types of lamins in vertebrates, A, B, and C. Lamins A and C are alternative RNA splice variants of the *LMNA* gene. Lamin B, B1 and B2, are expressed from the *LMNB* genes, *LMNB1* and *LMNB2* respectively (Taddei et al., 2004). These genes produce more than ten different lamin proteins due to both alternative splicing and post-translational modifications in different types of cells (Rzepecki and Fisher, 2002). There are two lamin genes in *D. melanogaster*; one codes for lamin Dm_0, a homologue of vertebrate B-type lamins, and the other codes for lamin C, which is similar to vertebrate A-type lamin (Rzepecki and Fisher, 2002). Most lamins are farnesylated at their carboxyl-termini, which facilitates their membrane association via lipid anchor. The exception to farnesylation is vertebrate lamin C, which is never been detected in a prenylated state (Gruenbaum et al., 2000; Worman and Courvalin, 2000).

Otefin and Bocksbeutel are *Drosophila* proteins, which contain similar LEM (Lamin, Emerin, and Man1 like) domains (53% identical amino acids) (Wagner, Schmitt, and Krohne, 2004). Otefin is one of the few INM peripheral proteins identified (Wagner, Schmitt, and Krohne, 2004; Worman and Courvalin, 2000). Otefin is a 45 kDa protein with a large hydrophilic domain, a single C-terminal hydrophobic sequence of 17 amino acids, and a high content of serine and threonine (Goldberg et al., 1998). The C-terminal 17 amino acid hydrophobic sequence is essential for targeting otefin to the nuclear periphery with amino acids between 173 and 372 targeting it to the nuclear envelope, while amino acids between 35 and 172 stabilize the interaction with the nuclear envelope (Ashery-Padan et al., 1997a; Ashery-Padan et al., 1997b).

Various integral membrane proteins of the INM are also components of the nuclear lamina by binding to the lamins, peripheral INM proteins, chromatin, or other structures of the INM, see figure 6. The Bocksbeutel gene, CG9424, produces two isoforms via alternative splicing. The α-isoform is 399 amino acids and contains a c-terminal

transmembrane domain. The β-isoform is 351 amino acids, which is missing the c-terminal transmembrane domain. When expressed in transfected cells as GFP fusions the β-isoform localizes to the nucleoplasm and the α-isoform is targeted to the nuclear envelope. The α-isoform is the predominantly expressed in cells, larvae, and flies. Bocksbeutel is localized in the nuclear envelope and in the cytoplasm by indirect immunofluorescence using Bocksbeutel antiserum (Wagner, Schmitt, and Krohne, 2004).

The first integral membrane protein of the INM to be identified was lamin B receptor (LBR), which contains eight transmembrane domains and binds to B-type lamins (Ellenberg and Lippincott-Schwartz, 1999; Rolls et al., 1999; Worman and Courvalin,

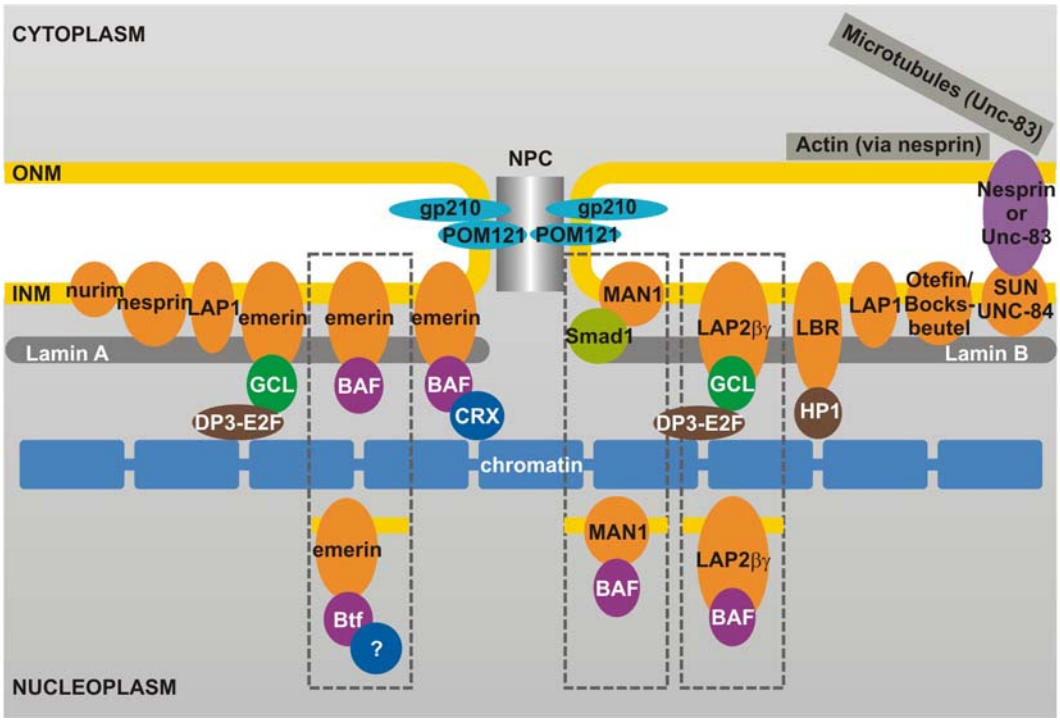


Figure 6. Illustration of the nuclear envelope. INM proteins are grouped into Lamin A and B binding proteins with their interacting transcription and chromatin regulators noted. Chromatin is in red, INM proteins orange, lamins grey. Protein abbreviations are as follows: barrier to autointegration factor (BAF), germ-cell less (GCL), Dependent protein 3-E2 transcription factor (DP3-E2F), cone-rod homeobox (CRX), Btf is a death-promoting transcriptional repressor, Mothers against decapentaplegic homolog 1 (Smad1), Lamina-associated polypeptide (Lap), lamin B receptor (LBR), uncoordinated movement (UNC), SUN is a domain consisting of a coiled-coil region, an anti-erbB-2 IgG domain, and two hydrophobic regions contained in UNC-84, pore membrane protein (POM), glycoprotein 120 (gp210)(Adapted from Hetzer, Walther, and Mattaj, 2005).

2000). The nuclear trafficking of LBR is the model for the diffusion:retention of integral membrane proteins, with an inserted transmembrane domain in the ER and trafficked through the continuous membrane system of the ER, ONM, and INM via the lateral channels of the NPC. As such it is directly comparable to ODV E66. For information on other integral membrane proteins identified in the INM see the follow articles and reviews as the comprehensive details therein are beyond the scope of this dissertation (Dechat et al., 1998; Dechat, Vlcek, and Foisner, 2000; Ellenberg and Lippincott-Schwartz, 1999; Ellenberg et al., 1997; Foisner, 2001; Gruenbaum et al., 2000; Holmer and Worman, 2001; Lin et al., 2000; Ostlund et al., 1999; Ostlund and Worman, 2003; Rolls et al., 1999; Worman, 2005; Worman and Courvalin, 2000).

Contrasting these INM proteins is Nurim. It is a multispanning INM protein with a small hydrophilic nucleoplasmic domain. This proteins seems to exclusively reside in the INM, however, its function is yet to be identified (Rolls et al., 1999). Other proteins are being currently identified in several of the proteomics experiments. Two recently reported proteins are a 100 kDa Unc-84A like protein (*C. elegans*) and a 45 kDa protein named LUMA. Functional analysis of these newly identified proteins has not been completed (Dreger et al., 2001).

*Nuclear Pore Complex (NPC)**

Regulated soluble protein transport between the nucleus and cytoplasm occurs through the nuclear pore complex (NPC). The nuclear pore complex is a large proteinaceous structure composed of roughly 40-50 distinct proteins in vertebrates totaling 120 MDa in mass (Suntharalingam and Wente, 2003). The yeast NPC has been isolated to homogeneity, and is approximately 50 Mda in mass composed of about 30 proteins (Rout and Aitchison, 2001; Rout et al., 2000). The 30 proteins were identified and structural analysis located their positions in the yeast NPC (Allen et al., 2001;

*Figure 7 with adaptation reprinted with permission from “Proteomics for the pore” by Blobel, G. and Wozniak, R. W., 2000, *Nature*, 403, 835-836. © 2000 by Nature Publishing Group. www.nature.com.

Denning et al., 2001).

The pore is composed of three stacked octagonally symmetric rings, with the center ring containing eight spoke-like structures supporting the central transporter or central channel, see figure 7. The lateral channel is created by the spokes of the central ring and the pore membrane. There are eight lateral channels around the central channel and each is ~10 nm in diameter (Hetzer, Walther, and Mattaj, 2005; Rout et al., 2003; Suntharalingam and Wente, 2003; Talcott and Moore, 1999). The pore membrane is where the ONM and INM meet and are continuous with each other in the NPC. This pore membrane is biochemically distinct from the INM or ONM (Hinshaw, Carragher, and Milligan, 1992). The lumenal space of the ER and nuclear envelope meet in this pore membrane region. Moving away from the NPC eight short filaments extend from the cytoplasmic ring and on the nuclear ring, the symmetry continues with the nuclear basket (Allen et al., 2001; Bayliss, Corbett, and Stewart, 2000; Blobel and Wozniak, 2000; Rout and Aitchison, 2001; Rout et al., 2000).

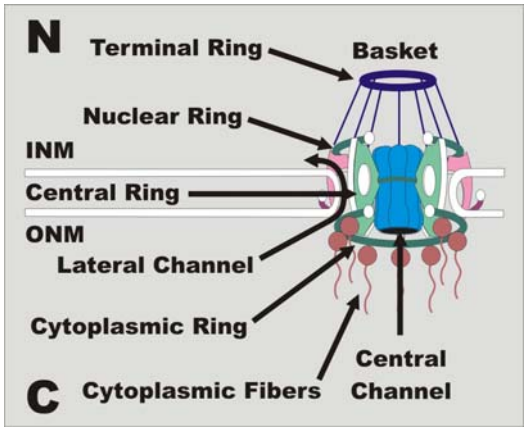


Figure 7. Model of NPC architecture. Shown are the cytoplasmic, central, and nuclear rings. The lumenal space/pore membrane connects the INM and ONM at the NPC. Cytoplasmic and nuclear filaments are also depicted; the latter forms a basket structure closed by a distal ring. (Adapted from Blobel and Woznaik, 2000)

The component nuclear pore proteins of the NPC have been termed nucleoporins or nups and are present in two copies per spoke, which are located symmetrically on either

axis of the NPC midplane (Mazzanti, Bustamante, and Oberleithner, 2001; Miller and Forbes, 2000; Rout et al., 2003). Using octagonally symmetric architecture few nups can create the massive structure of the NPC. There are three classes of nups from which the NPC is constructed. The first class of nups contains integral membrane proteins, which localize to the pore membrane domain and are termed poms (Rout et al., 2003; Suntharalingam and Wente, 2003). Poms anchor the NPC to the NE. It has been hypothesized that poms aid in the formation of the NPC, however, little is known of their exact function (Rout and Aitchison, 2001). The second class of nups is the structural proteins giving the NPC form and integrity. These nups form the central and lateral channels, which are the passages for trafficking proteins. The central channel allows proteins 50 kDa in size to freely diffuse and the lateral channel allow membrane proteins a passage from the ONM into the INM, see figure 7 (Pante and Kann, 2002; Talcott and Moore, 1999). These nups provide the framework to support the third class of nups located on both sides of the NPC (Rout et al., 2003). The third class contains the binding sites for the transport factors, which is a related group of proteins called the FG nups (Rout et al., 2003). They have been named the FG nups because they contain multiple copies of a Phe-Gly motif separated by hydrophilic residues (Rout and Aitchison, 2001; Suntharalingam and Wente, 2003). The FG nups account for almost half of the NPC mass with approximately 200 copies in each NPC (Blobel and Wozniak, 2000; Cronshaw et al., 2002; Rout et al., 2000). These FG nups exist on the nuclear and cytoplasmic filaments that extend into the nucleus and cytoplasm, repectively (Denning et al., 2003). These proteins function as docking sites for importins/karyopherins, facilitating protein translocation into and out of the nucleus (Gant, Goldberg, and Allen, 1998; Hinshaw, Carragher, and Milligan, 1992; Rout et al., 2000; Suntharalingam and Wente, 2003). These proteins and processes are highly conserved throughout many of the currently studied eukaryotic organisms (Allen et al., 2001; Denning et al., 2001; Hetzer, Walther, and Mattaj, 2005; Rout et al., 2003). Importin/karyopherin function will be discussed in detail in the protein trafficking section which follows.

4. Nuclear Protein Trafficking

Three models for nuclear protein translocation have been proposed in the literature; these are, "diffusion:retention" requiring an interaction with membranes (Ellenberg et al., 1997; Smith and Blobel, 1993; Soullam and Worman, 1993; Soullam and Worman, 1995), free diffusion requiring proteins to be soluble (Guo and Amemiya, 2005; Talcott and Moore, 1999), and nuclear localization signal mediated protein-protein interaction transport (Kalderon et al., 1984a; Kalderon et al., 1984b; Rout et al., 2003).

4.1 Soluble Protein Nuclear Trafficking

Once translated soluble proteins traffic to the nucleus by one of two methods, free diffusion or NLS mediated (Rout et al., 2003; Suntharalingam and Wente, 2003; Talcott and Moore, 1999). Nuclear trafficking of soluble proteins that do not interact with the NPC are hypothesized to translocate by free diffusion. Free diffusion is regulated by the protein's size due to the diameter of the central channel of the NPC; the approximate size limit for free diffusion through the nuclear pore is 40 kDa (Suntharalingam and Wente, 2003; Talcott and Moore, 1999). Free diffusion is an energy independent process. The protein's molecular mass is not as important to free diffusion as the protein's functional size, which can be altered by dimerization, complex formation, or even transient associations with other cellular factors (Talcott and Moore, 1999). An example of a freely diffusing protein is the green-fluorescent protein (GFP), approximately 27 kDa in size. GFP has been found to freely diffuse in both vertebrate cells and in baculovirus infected insect cells (Ogawa et al., 1995; Reilander, Haase, and Maul, 1996).

There are many examples of soluble protein nuclear trafficking, for example there are several herpes simplex virus-1 (HSV-1) soluble regulatory proteins. One is ICP0, which localizes to small dense nuclear structures called promelocytic leukemia proteins (PML) just before the onset of DNA synthesis with ICP22 and p60 (Bruni et al., 1999; Jahedi et al., 1999). ICP0 is a promiscuous transactivator, which freely diffuses into the nucleus. The protein requires a zinc ring finger and an N-terminal 180aa domain for this

diffusion to occur. After the onset of viral DNA replication ICP0 is translocated into the cytoplasm where it interacts with elongation factor EF-1δ and is phosphorylated, see figure 8 (Bruni et al., 1999; Jahedi et al., 1999).

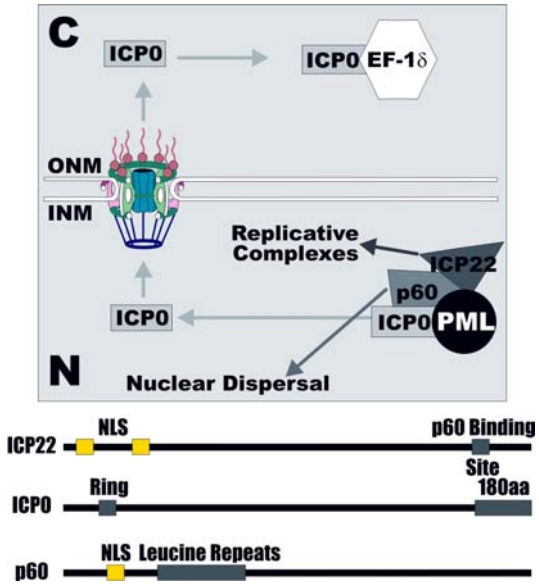


Figure 8. Nuclear trafficking of ICP22, ICP0 and p60. ICP0, a promiscuous transactivator, localizes to nuclear dense bodies termed promelocytic leukemia proteins (PML). Nuclear localization of ICP0 occurs by free diffusion; however it requires the zinc ring finger and N-terminal 180aa domains. ICP0 co-localizes with p60 and ICP22 at these PML early in HSV-1 infection. ICP 22 localizes to the nucleus via its' two NLS, after the onset of viral DNA replication it re-localizes to replicative complexes where it is required for late gene expression. ICP0 is translocated into the cytoplasm, where phosphorylation occurs and it interacts with elongation factor EF-1δ. The translocation of ICP0 and ICP22 results in the nuclear dispersal of p60 a relatively new protein of unknown function, hypothesized to localize to the nucleus via it putative NLS (Bruni et al., 1999; Jahedi et al., 1999). A cartoon schematic of the proteins, with the relevant domains denoted, is below the trafficking cartoon (Adapted from Bruni et al., 1999; Jahedi et al., 1999).

Several soluble proteins traffic through the NPC by a regulated transport, which requires a specific interaction with the NPC or an intermediate carrier. Proteins utilizing a receptor/carrier for nuclear trafficking contain a targeting signal and are typically too large to freely diffuse through the pore, greater than 40 kDa in size. This targeting signal allows for an interaction between the receptor/carrier, which then interacts directly with the components of the NPC. Targeting signals directing proteins to the nucleus are referred to as nuclear-localization signals (NLSs). In the absence of the

receptor/carrier, these nucleoproteins have no affinity for the NPC (Hetzer, Walther, and Mattaj, 2005; Rout et al., 2003).

The most common NLS is the KRKK amino acid sequence and variations there of, which are found in many nucleoproteins (Kalderon et al., 1984a; Kalderon et al., 1984b). Example of a soluble protein with a KRKK signal sequence is SV-40 Large T antigen (Kalderon et al., 1984a). Two HSV-1 soluble proteins, ICP22 and p60, localize to the nucleus via NLS transport, see figure 8. ICP 22 localizes to the nucleus via its' two NLS, after the onset of viral DNA replication ICP22 localizes to replicative complexes where it is required for late gene expression. The translocation of ICP0 and ICP22 results in the nuclear dispersal of p60 a relatively new protein of unknown function, hypothesized to localize to the nucleus via it putative NLS (Bruni et al., 1999; Jahedi et al., 1999).

The receptor/carrier function is performed by the protein super-family of karyopherins/importins (Gorlich and Kutay, 1999; Mosammaparast and Pemberton, 2004; Pemberton and Paschal, 2005; Riddick and Macara, 2005; Talcott and Moore, 1999). These receptor/carrier proteins will be discussed and for uniformity of language, the importin nomenclature will be used. These various nomenclatures exist because of coincidental discovery by multiple laboratories (Wente, 2000). NLS mediated import requires energy, physiological temperatures, a NLS, and soluble transport machinery (Gorlich and Kutay, 1999; Mosammaparast and Pemberton, 2004; Pemberton and Paschal, 2005; Riddick and Macara, 2005). There are three steps in NLS mediated nuclear import: (1) docking at the NPC, (2) translocation, and (3) nuclear deposition of the cargo, see figure 9 (Kaffman and O'Shea, 1999; Pemberton and Paschal, 2005; Rout et al., 2003).

Docking at the NPC begins as the cargo protein containing the NLS interacts with the importin proteins. This importin protein belongs to the importin-β family of proteins, which is characterized by a RanGTP binding domain at the N-terminus. Interactions with importin-β is in some cases direct, in others mediated by the NLS generated protein-protein interaction with importin-α (Gorlich and Kutay, 1999;

Kaffman and O'Shea, 1999; Pemberton and Paschal, 2005; Quimby and Corbett, 2001; Rout et al., 2003). The importin-cargo complex then docks with the cytoplasmic fibers, which are composed of FG nups. Translocation through the pore traverses a 200-300 Å distance delivering the cargo to the nucleus. Although this process is least understood two hypotheses have been suggested: (1) the importin-cargo complex could be translocated by "hopping" between the FG nups or (2) movement through the pore occurs by random non-directed diffusion with an irreversible point committing the complex to nuclear diffusion (Bayliss, Corbett, and Stewart, 2000; Gorlich and Kutay, 1999; Kaffman and O'Shea, 1999; Pemberton and Paschal, 2005; Rout et al., 2003). Once the importin-cargo complex has reached the nucleus, the cargo is deposited by being displaced from the importin by Ran. Ran in its GTP-bound form, which is found inside the nucleus, binds with more affinity to the importin releasing the cargo. The importin-Ran complex is then recycled through the NPC, where the GTP is hydrolyzed

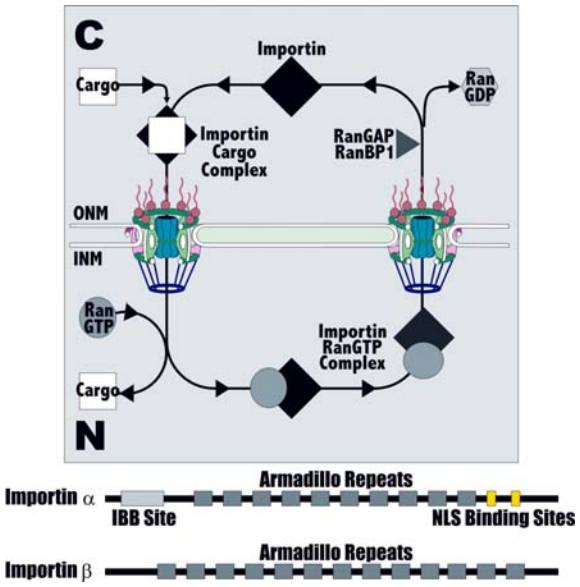


Figure 9. NLS mediated nuclear trafficking via importin protein complexes. Importin α interacts with the cargo protein via the NLS binding sites denoted in the cartoon schematic of the protein. Once importin α interacts with the cargo protein the new complex can interact with importin β via importin α beta binding site (IBB). Importin b is then responsible for interacting with the nups of the NPC. Once through the NPC, the importin cargo complex is disrupted, depositing the cargo in the nucleus, by the higher binding affinity of ran-GTP for the importins. The importin ran-GTP complex is recycled into the cytoplasm where the ran is hydrolyzed freeing the importins for the next round of import (adapted and simplified from Kaffman and O'Shea 1999; Rout and Aitchison 2001; Rout et al. 2003; Pemberton and Paschal, 2005).

and the importin is released (Gorlich and Kutay, 1999; Kaffman and O'Shea, 1999; Pemberton and Paschal, 2005; Rout et al., 2003).

4.2 Membrane Protein Trafficking

Membrane proteins either bind to one leaflet or completely span a lipid bilayer while maintaining contact with the surrounding aqueous medium on one or both sides of the membrane and the lumen. There are two types of membrane proteins; integral membrane and membrane associated. Integral membrane proteins typically span the membrane with a transmembrane segment or a lipid anchor (von Heijne, 1996). Membrane associated proteins, also known as peripheral membrane proteins, typically bind to one side, one leaflet, of the membrane or associate by noncovalent interaction with other membrane proteins. Membrane associated proteins interact with a membrane either directly or indirectly. A direct interaction with the membrane is mediated by an amphiphilic α-helices and/or a lipid anchor, and an indirect interaction occurs via a protein-protein interaction (Kaffman and O'Shea, 1999; von Heijne, 1996).

Membrane proteins integrate in the membrane in either a co- or post-translational manner at the translocons in the ER. The co-translational integration is where the membrane protein is being translated by the ribosome at the same time that the protein is being integrated at the translocon. Because of this membrane proteins are not typically translated on cytosolic free ribosomes, but instead at ER bound ribosomes. Post-translational insertion occurs after the protein is completely translated, typically from cytosolic free ribosomes.

Co-translational insertion occurs as the ribosome bound nascent chain is targeted to the translocation site, termed the translocon, in the ER membrane by binding the signal recognition particle (SRP) (Clemons et al., 2004; Johnson and van Waes, 1999; Rapoport et al., 2004). The SRP then targets its membrane receptor in a GTP-dependent manner (Johnson and van Waes, 1999; Keenan et al., 2001; Wilkinson, Regnacq, and Stirling, 1997). Co-translational insertion can occur in a SRP independent manner, however, this is a rare event (Kalies and Hartmann, 1998).

There are two different models for the construction of the translocon and translocation of the nascent protein based primarily on two different laboratories work, the Johnson lab and the Rapoport lab. The eukaryote translocon is composed of minimum of four proteins as it can be successfully reconstituted by these four proteins; Sec61α, Sec61β, and Sec61γ (known as heterotrimeric Sec61), and TRAM (translocation-associated membrane protein) (Johnson and van Waes, 1999). The Johnson lab proposes that the ER lumen side of the translocon is sealed prior to binding by the protein BiP (Johnson and van Waes, 1999). However, the Rapoport lab proposes that the Sec61 complex forms an hourglass pore shape; as such the pore residues fit like a gasket around the translocating polypeptides, hindering ions and other small molecules from passing. Rapoport's group propose that it is this action and not BiP that seals the translocons pore (Clemons et al., 2004; Rapoport et al., 2004).

The resulting complex of RNC (ribosome-nascent chain) with the SRP binds to the translocon to begin integration (Johnson, 2005). Once the nascent chain is about 70 amino acids in length the Johnson proposes that BiP is released from the lumen side of the translocon exposing the nascent chain to the ER lumen (Johnson, 2005). When the transmembrane (TM) sequence of the protein is being synthesized by the ribosome the translocon is open to the ER lumen but sealed to the cytosol by the bound ribosome. Once the TM sequence is synthesized the lumenal end of the pore is either directly or indirectly resealed by BiP. After a few more residues are added to the nascent chain the ribosome seal is broken to allow the cytoplasmic domain of the protein to enter the cytosol. When the synthesis of the protein is complete the TM sequence of the protein leaves the BiP sealed translocon as in traffics through the ER with termini in the cytosol and lumen of the ER (Johnson, 2003; Johnson, 2005; Johnson and van Waes, 1999).

However, the Rapoport proposes based on a 3D crystal structure of a non-integrating translocon without an attached ribosome that the translocon takes on a clam shell shape and as the signal sequence is translated it intercalates between two of the transmembrane domains of the Sec61 complex, thus opening the clam shelf. This destabilizes the interactions of the plug helix that keeps it in the center of the Sec61 complex and creates

the open pore. The front of the clam shell provides a lateral gate for the hydrophobic transmembrane domains to exit into the lipid phase, which would occur sequentially as they are synthesized (Clemons et al., 2004; Rapoport et al., 2004).

Regardless of the models recent crosslinking studies from the Summers and Johnson's labs suggest that membrane protein sorting to the INM for some viral and cellular proteins is an active process involving specific non-nuclear proteins (Saksena et al., 2004). These studies found that the first transmembrane sequence of integral membrane proteins directed to the INM, photocrosslinked to TRAM, where as non-INM directed integral membrane proteins did not. Because few transmembrane sequences of non-INM directed membrane proteins photocrosslink to TRAM, the authors concluded that the INM-directed transmembrane sequences occupy different sites within the translocon from the non-INM directed transmembrane sequences (Saksena et al., 2004). Therefore protein sorting to the INM begins with protein translocation in the ER, a process mediated by protein-protein interactions, which has been shown in McCormick et al., 2003.

Post-translational insertion occurs after protein synthesis is complete. Proteins using this mode have a less hydrophobic signal sequence and may therefore escape interacting with SRP during synthesis. Little is known of the membrane targeting mechanism of post-translational translocation, but a mechanism has been determined in *S. cerevisiae*. In yeast many of the proteins that function in co-translational translocation have been identified as components of post-translational translocation. In post-translational translocation, the channel partners are another membrane protein complex (Sec62/63 complex) and BiP, a member of the HSP 70 family of ATPases. These proteins form a tetramer which binds with Sec61 to form a seven-component Sec complex (Johnson, 2005; Kalies and Hartmann, 1998; Osborne, Rapoport, and van den Berg, 2005; Rapoport et al., 2004; Wilkinson, Regnacq, and Stirling, 1997).

The hypothesized mechanism for post-translational translocation is a ratchet method. Basically, a polypeptide in the translocon can slide either direction, but binding to BiP inside the ER lumen prevents back movement into the cytosol, resulting in translocation.

There are five stages to this mechanism. First, before translocation the polypeptide loses all bound cytosolic chaperones, allowing it to move into the translocon. Second, the polypeptide is targeted to the translocon, for translocation. Third, BiP binds to the polypeptide as it enters the lumen. As additional portions of the polypeptide enter the lumen, additional BiP molecules bind to it, inhibiting back movement, ratcheting the polypeptide further into the translocon. Fourth, Brownian motion moves the polypeptide forward. And finally fifth, BiP is released upon exchange of ADP for ATP (Johnson, 2005; Kalies and Hartmann, 1998; Osborne, Rapoport, and van den Berg, 2005; Rapoport et al., 2004; Wilkinson, Regnacq, and Stirling, 1997).

4.2.1 Membrane Associated Protein (Peripheral) Nuclear Trafficking

Membrane associated proteins most often interact in a post-translational manner with the ER or other membrane systems. This is achieved by several mechanisms including protein-protein interactions, amphipathic helices, and lipid anchors, all of which are possible membrane interactions of E26 as it contains no predictable transmembrane domain. There are several examples in the literature where protein-protein interactions account for a membrane interaction and/or localization, including Young Arrest (YA), otefin, and the HSV protein U_L31 (Goldberg et al., 1998; Liang and Baines, 2005; Liang et al., 2004; Mani et al., 2003; Reynolds et al., 2001). These examples will be discussed.

An amphipathic helix is a structural motif, which has been found in many classes of globular and lipid-associating proteins. The key feature of an amphipathic helix is the presence of two clearly defined faces of the helix, one hydrophobic which interacts with the acyl chains of the phospholipids and the other hydrophilic which is oriented towards the aqueous phase (Rosseneu et al., 1992). Having this arrangement allows amphipathic helical protein to insert in the out leaflet of a lipid bi-layer. The amphipathic helix-loop-helix (HLH) motif has been identified in some developmental regulators and in genes coding for eukaryotic DNA-binding proteins, while not directly relevant to membrane proteins this might apply to the soluble form of E26. This motif enables proteins to

dimerize, and a basic region near this motif to specifically contact DNA (Phoenix et al., 2002; Rosseneu et al., 1992).

There are many types of lipid modifications that anchor proteins to a membrane system, most are beyond the scope of this dissertation as they do not appear to apply to the E26 membrane interaction, however palmitoylation will be discussed as E26 contains 5 cysteine amino acids which are possible site of palmitoylation. Protein palmitoylation is the addition of fatty acids to a protein through an N-amide or a thioester bond. A thioester bond is also known as S-acylation which is unique in that it is the only reversible lipid modification (Linder and Deschenes, 2003). This lipid covalent linking to cysteine amino acids anchors proteins to the membrane as the lipid is inserted into the bi-layer of the membrane system. Palmitoylation is thought to occur by a palmitoytranferase (PAT) or acyltransferase in an enzymatic reaction, however there is some contradictory evidence as spontaneous palmitoylation can occur in the presence of palmitoyl-CoA (Dietrich and Ungermann, 2004). Palmitoylation can occur anywhere in the cell, but predominantly occurs in the ER. Recently three proteins have been identified that are believed to be PATs. There is no consensus sequence for palmitoylation; however the common features of most palmitoylated proteins are a membrane targeting sequence near the target cysteines that consist of positive charges, adjacent lipid anchors, or transmembrane domains. Cysteines with proximal polar or charged side chains can increase the potential to form a thiolate by as much as six orders of magnitude by decreasing the pK_a of the target cysteine (Mossner, Iwai, and Glockshuber, 2000). The cysteine located at amino acid 129 in E26 is flanked by charged amino acids, figure 4. Protein-protein interactions have also been theorized to influence protein stability, which would increase accessibility of the sulphydryl group or change the local pK_a of the target cysteine increasing the chances of palmitoylation (Bhattacharyya and Wedegaertner, 2000; Dietrich and Ungermann, 2004). E26 has several putative protein interactions including ODV E66, FP25K, IE-1, and actin (Beniya, Braunagel, and Summers, 1998; Braunagel et al., 2004; Imai et al., 2004). The interaction with E66 could initiate or promote E26's membrane association.

Palmitoylation has also been shown to redirect proteins in the cell. When these proteins become palmitoylated they are redirected from their primary location in the cell to a secondary location. In the case of the 37 kDa phospholipid scramblase 1 protein, palmitoylation has been shown to redirect scramblase 1 from the nucleus to the plasma membrane. Scramblase 1 is directed to the nucleus via a non-classical NLS, which interacts with importin-α (Ben-Efraim et al., 2004; Chen et al., 2005).

Trafficking of membrane associated proteins occurs by the same mechanisms of soluble and integral membrane protein trafficking, free diffusion, NLS mediated, or "diffusion:retention" (For a full explanation of the "diffusion:retention" model please see section 4.2.2). The membrane associated proteins most often interact with the membrane after trafficking to their final locations. Examples of a membrane associated proteins with a functional KRKK NLS sequences are the cytidine 5'-triphosphate:phosphocholine cytidylyltransferase (CTP:CCT), nuclear lamins, otefin,

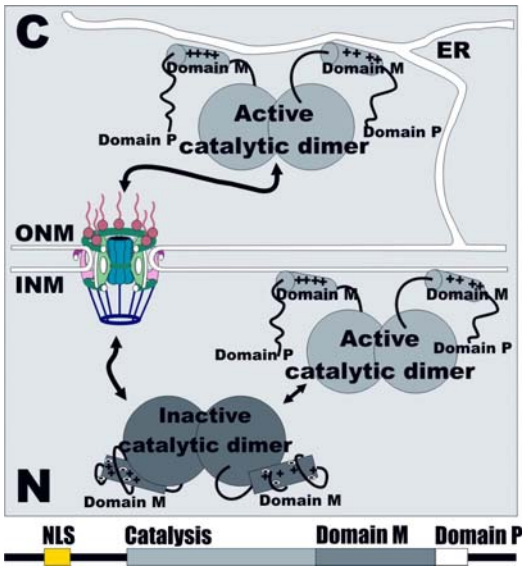


Figure 10. Cellular trafficking of CTP:CCT. CTP:CCT catalyzes the formation of cytidine 5'-diphosphate choline (CDP choline), which is the head-group donor in the synthesis of phospatidylcholine. CTP:CCT localizes to the nucleus via an N-terminal NLS and alternates between a soluble inactive form and a membrane bound active form. Binding of the membrane domain activates CTP:CCT by relieving an inhibitory constraint in the catalytic domain. The membrane binding domain of CTP:CCT responds to changes in the physical properties of phosphatidylcholine deficient membranes. CTP:CCT binds to the outer leaflet of the ER and INM via an amphipathic helix found in domain M of the cartoon schematic of the protein domains. Domain P is a phosphorylation site (Adapted from Cornell and Northwood, 2000; DeLong et al., 2000; Xie et al., 2004; Bogan et al., 2005).

and Young Arrest (YA) protein (Cornell and Northwood, 2000; Dechat et al., 1998; Donnelly and Elliott, 2001; Goldberg et al., 1998; Worman and Courvalin, 2000).

CTP:CCT is a 367 amino acid protein, which exhibits amphitropism, proteins whose activities are regulated between an inactive soluble form and an active membrane-lipid-bound form, see figure 10. Binding of the membrane domain activates CTP:CCT by relieving an inhibitory constraint in the catalytic domain. The membrane binding domain of CTP:CCT responds to changes in the physical properties of phosphatidylcholine deficient membranes. CTP:CCT is soluble, yet becomes membrane bound in the cytoplasm and nucleus (DeLong, Qin, and Cui, 2000). The interaction with membranes occurs by an amphipathic helix, allowing the protein to associate with the outer leaflet of the INM. When in the membrane bound state, CTP:CCT catalyzes the formation of cytidine 5'-diphosphate choline (CDP choline), which is the head-group donor in the synthesis of phospatidylcholine (Bogan et al., 2005; Cornell and Northwood, 2000; Xie et al., 2004).

The nuclear lamins traffic to the nucleus using a NLS and once there bind to proteins of the INM (Broers et al., 1999; Gruenbaum et al., 2000). This interaction with the INM occurs because the lamins are prenylated, specifically farnesylated, at their C-terminus once in the nucleus, lipid anchoring them to the inner nuclear membrane (Hofemeister, Weber, and Stick, 2000). The exception is lamin C, which is never prenylated (Worman and Courvalin, 2000).

Otefin and Young's Arrest (YA), *Drosophilia* proteins, are two other proteins of the INM. These proteins like the others traffic to the nucleus via a NLS. YA is a 696 amino acid hydrophilic protein that lacks any decernable transmembrane domain or known membrane-targeting motif similar in this aspect to E26, however once in the nucleus it interacts with chromatin and laminas of cleavage-stage embryos in a cell cycle dependent manner (Liu and Wolfner, 1998; Lopez and Wolfner, 1997; Mani et al., 2003). Otefin is a 45 kDa protein that contains a large hydrophilic domain and a single C-terminal 17 amino acid hydrophobic domain. The C-terminal hydrophobic sequence is essential for targeting otefin to the nuclear periphery, however amino acids between

173 and 372 are required for efficient targeting of otefin to the nuclear envelope. Amino acids between 35 and 172 are required for further stabilization of the otefin-nuclear envelope interaction (Ashery-Padan et al., 1997a; Ashery-Padan et al., 1997b; Goldberg et al., 1998; Liu and Wolfner, 1998; Lopez and Wolfner, 1997; Padan et al., 1990).

Two viral proteins of HSV-1, U_L34 and U_L31, traffic together to the INM. U_L34 is a 30 kDa integral membrane protein with a 22 amino acid transmembrane domain. This protein localizes to the nucleus where it is required for envelopment of nucleocapsids at the nuclear membrane (Yamauchi et al., 2001). U_L34 is believed to traffic to the nucleus via lateral diffusion. U_L34 also acts to traffic U_L31, a 32 kDa protein, to the nuclear envelope. U_L34 it interacts with the U_L31 in the cytoplasm after it is inserted into the ER and then the complex of U_L34 and U_L31 traffic to the nucleus (Reynolds et al., 2001). Thus U_L31 traffics to the nuclear envelope via a protein-protein interaction as demonstrated by the absence of U_L34, where U_L31 is more susceptible to proteaosome degradation and no longer localizes to the nuclear rim, but accumulates in the nucleoplasm (Liang and Baines, 2005; Liang et al., 2004; Reynolds et al., 2001; Ye et al., 2000). Transiently expressed U_L34, which is membrane associated, is sufficient to target U_L31 to the nuclear rim in uninfected cells (Reynolds et al., 2001; Yamauchi et al., 2001). U_L31 has also been suggested to have roles in both viral DNA synthesis and DNA cleavage and packaging (Liang and Baines, 2005). U_L34 and U_L31 trafficking could be similar in mechanism to ODV E66 and E26 as the putative interaction between the E66 and E26 could direct the membrane association targeting this form of E26 to ODV envelopes located in the nucleus.

4.2.2 Integral Membrane Protein Nuclear Trafficking

The prevailing hypothesis states that “diffusion:selective retention” occurs as IMPs interact with the endoplasmic reticulum (ER) and traffic to the inner nuclear membrane via lateral diffusion (Smith and Blobel, 1993; Soullam and Worman, 1995). IMPs transmembrane segments are inserted into the membrane in either a co- or post-translational event. Once inserted in the ER, the protein traffics by laterally diffusing

through the contiguous membrane of the ER and the ONM. Nuclear trafficking progresses as the integral membrane protein in the ONM traffics through the 10 nm lateral channels of the NPC and into the INM. Trafficking through the lateral channel is limited by the size of the cytosolic portion of the protein; however, larger proteins can traffic through these channels depending on the orientation of the trafficking protein in the membrane (Talcott and Moore, 1999). Thus proteins with cytosolic domains larger that 45-70 kDa should be excluded from the INM as they should not pass through the size restrictive lateral channel. Coincidentally, most of the INM proteins studied have cytosolic domains smaller that this limitation supporting the model (Ellenberg et al., 1997; Lin et al., 2000; Ostlund et al., 1999). However, one group of INM proteins, the Nesprins, recently identified contains cytosolic domains up to 1 mDa in size. Note, that studies on this family of proteins is rapidly progressing, which suggests that there are several different isoforms and the INM isoform is yet to be identified (Apel et al., 2000; Mislow et al., 2002a; Mislow et al., 2002b; Zhang et al., 2005; Zhang et al., 2001). Once the integral membrane protein reaches the INM it is retained via immobilization through binding with immobilized nuclear components and/or other resident INM proteins. Proteins lacking affinity for nuclear components should continue to diffuse by this model not effectively concentrating to detectible levels. A considerable amount of data supports that binding nuclear components is required for accumulation in the INM, recently there has been a few papers that suggest that binding is not required and that sorting of these proteins to the INM can begin in the translocon (Braunagel et al., 2004; Hofemeister and O'Hare, 2005; Ohba et al., 2004; Rolls et al., 1999; Saksena et al., 2004; Smith and Blobel, 1993).

Lamin B receptor (LBR) and ODV E66 are model proteins in our studies for integral membrane protein localization to the inner nuclear membrane via lateral diffusion (Braunagel et al., 1999; Braunagel et al., 2004; Ellenberg and Lippincott-Schwartz, 1999; Ellenberg et al., 1997; Rosas-Acosta, Braunagel, and Summers, 2001; Saksena et al., 2004). ODV E66 has previously been discussed in the baculovirus section, 1.3.2 ODV Envelope, of this document. LBR has also been studied in HSV-1 infected cells,

where it traffics normally to the INM (Scott and O'Hare, 2001). Many other integral membrane proteins are speculated to traffic by lateral diffusion including nurim, emerin, MAN1, UNC-84 and LAPs (Gruenbaum et al., 2000; Rolls et al., 1999; Tews, 1999; Worman and Courvalin, 2000).

MATERIALS AND METHODS

1 Cells and viruses

*Sf*9 cells, cloned from *Spodoptera frugiperda* IPLB-Sf21-Ae cells, were infected with wild type *Ac*NPV (E2 strain) and various constructed mutant viruses. Infections were performed at various multiplicities of infection (moi) from 0.5-20 as described by Summers and Smith, 1987. Inoculation of the cells with the virus is defined as time zero. Viral inocula were removed after 1 hour of absorption.

1.1 Cloning Techniques

During the following cloning experiments many basic cloning protocols were performed. For brevity, a summary of those basic techniques will follow, for complete details protocols were followed from Ausubel et al., 1992. Complete clone maps are presented in Appendix B for all cloning strategies and clones. DNAs used in these experiments were restriction enzyme digested with Promega and New England Biolabs (NEB) restriction endonucleases. DNA fragment isolation was performed using 0.5–2.0% agarose gels via electrophoresis. Isolated DNAs used in the following cloning strategies were purified from the agarose using BioRad's Gene Clean or Qiagen's QiaQuick gel purification kits. Once the DNAs were gel purified, the fragments were ligated together using Promega T4 DNA ligase overnight at 16 ^{0}C. One microliter of the ligation mix was then electrotransformed in to 50 μl of electro-competent *E.coli* DH5α cells using a BioRad Gene Pulser II electrophorator set at 25 μF, 200 Ohms, 1.8kV, with a 1 mm gap cuvette. The transformed mixture was then plated for isolation on LM plates containing ampicillin (50 μg/ml) as a selection agent. Individual colonies were grown in liquid medium, from which the amplified resulting vector construct was isolated, and sequence confirmed utilizing the Gene Technology Laboratory (GTL, Texas A&M University, College Station, TX) service facility.

1.2 Cloning Strategies

The goals of the cloning strategies are to identify the regions of E26 that are

responsible for directing E26 to intranuclear microvesicles, identify regions of E26 that interact with nuclear membranes, identify possible protein interactions that might assist E26 in its nuclear trafficking, and to get a better understanding of the membrane associated protein that the *da26* gene produces during infection. A genetic approach was employed deleting regions of the *da26* gene for investigations using in vitro translations, transient expression in uninfected and infected cells, and recombinant virus production. These experiments where then used to identify characteristics of the E26 protein relevant to the goals stated above.

1.3 Cloning Strategies for Construction of Mutant Viruses

Three strategies were employed sequentially for the development of regional domain deletions of the *da26* gene.

The first strategy (1.3.1 – Knockout) was based on the conclusions of O'Reilly et al., 1990 and was an attempt to remove the *da26* gene and insert the β-glucuronidase gene into the *da26* locus as a color selector for the identification of the recombinant virus. Then truncations of the *da26* gene were to be inserted in to the *da26* locus to determine what function each of the different regions of the gene had on trafficking the protein to the nucleus. This strategy failed as the *da26* gene was found to be essential. The clones for this strategy are described in appendix B14-17.

The second strategy (1.3.2 – Insertion) employed a special recombination vector, constructed for this purpose that allowed two of the *da26* promoters to be shifted to a non-coding region of the virus genome with the full or truncated *da26* gene, termed regional domains, and appropriate epitope tags. The epitope tags were required to differentiate the products from the second copy of the *da26* gene from the native copy. This strategy also failed; it was concluded that a trans-acting factor must be missing for transcription to occur. The clones for this strategy are described in appendix B18-50.

The third strategy (1.3.3 – Polh Promoter) employed the commercially available pBAC-gus vector and the intermediate constructs built in the previous strategy, resulting

in placing the *da26* gene and truncations under the polyhedrin promoter. The clones for this strategy are described in appendix B51-58.

The evolution of the cloning strategies for this project used many of the preceding clones in the subsequent strategies. As such for the ease of the reader the global sequential strategy is listed in figure 11, below. The individual details of each strategy will be presented in the following sections.

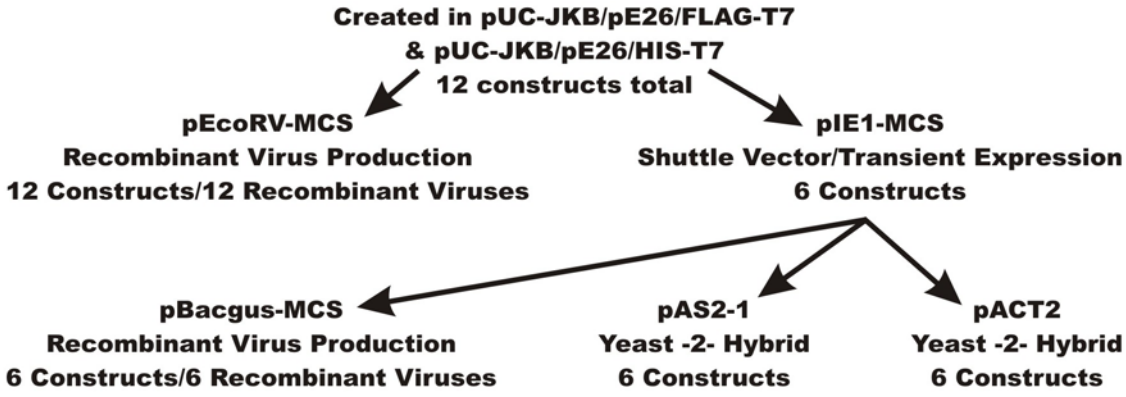


Figure 11. Sequential cloning of the BV/ODV E26 regional domains. The *da26* gene was truncated to produce several regional domains that were used in many different attempts to study E26 localization and membrane association. This is the global strategy of the evolution of the subsequent constructs.

1.3.1 Strategy One: Knockout

In order to knock out or remove the *da26* gene from the viral genome the PstI-G fragment of the genome was utilized, which had been previously placed into a pUC18 vector. As this construct will allow for homologous recombination in the E26 native locus, it was altered to remove the bulk of the *da26* gene. The whole gene was not removed as the c-terminal region contains the promoter for *da16*. The knockout was produced by a two step PCR method where the upstream and downstream regions of the *da26* gene were PCR amplified with a homologous region on the c-terminus or n-terminus respectively. The PCR reaction utilized four oligonucleotides: forward, E26-S1 and reverse, E26-CS1, and also forward, E26-CS2, and reverse E26-S2, see table 1.

These oligonucleotides will insert a small MCS in the E26 locus containing 3 restriction sites, including *Sac*I, *Sma*I, and *Hind*III. The DNA was amplified using an initial denaturation step for 2.5 min at 95 ^{0}C followed by 30 cycles of 20 sec at 95 ^{0}C, 1 min 30 sec at 68 ^{0}C, 45 sec at 72 ^{0}C, and after the thirty cycles for full extension a 7 min period at 72 ^{0}C. The PCR products were electrophorated onto a 1.5% agarose gel, from which they were cut and gel purified with the Gene Clean III Kit as used in the second stage PCR. The second stage PCR used an initial denaturation step for 4 min at 95 ^{0}C followed by 10 cycles of 20 sec at 95 ^{0}C, 1 min 30 sec at 68 ^{0}C, 1 min 15 sec at 72 ^{0}C. The E26-S1 and E26 S2 oligos were then added and the PCR continued with 25 cycles of 20 sec at 95^{0}C, 1 min 30 sec at 68^{0}C, 1 min 15 sec at 72^{0}C, and after the thirty cycles for full extension a 7 min period at 72 ^{0}C. The resulting PCR product had the *da26* gene removed from the ATG start to the *da16* promoter, the 417th nucleotide of the *da26* gene. The PCR product and the pUC18/PSTI-G vector were digested with *Nco*I and *Bgl*II. The PCR product was ligated into the digested vector and then electrophorated into DH5α cells from which the amplified DNA was isolated. The clone named pUC18/ΔE26 KO is described in appendix B14.

Table 1
Oligonucleotides used for two stage PCR deletion of the *da26* gene and insertion of a new multiple cloning site into pUC18/PSTI-G.

Name	5’ ----Sequence---- 3’
E26 S1	GGCCTTGGAAGCCGGGATACC
E26 S2	CTGACATTTAAACAGATCTCAGTATC
E26 CS1	GAGCTCTCCCGGGTAAGCTTTTCGATGTTTCGCCTTTGAACGTC
E26 CS2	AAGCTTACCCGGGAGAGCTCGTAAGGAGACGTTTGGCCGAGC

In order to use the *Sma*I site inserted into the new MCS the *Sma*I site contained in pUC18 had to be removed. As such the pUC18 vector was digested with *Eco*RI and *Sma*I. The digestion mix was phenol:chloroform extracted and the DNA was isolated by ethanol washes. The *Eco*RI site was then blunted by the exonuclease activity of Mung

Bean Nuclease (Promega) for 30 minutes at 30 ^{0}C. A second phenol:chloroform extraction was performed with an associated ethanol wash. The resulting blunt ended DNA was ligated together overnight to create ΔpUC18 (EcoI-SmaI), described in appendix B15.

The pUC18/ΔE26 KO and ΔpUC18 (EcoI-SmaI) were then used to create ΔpUC18 (EcoI-SmaI)/ΔE26 KO, thus making the *Sma*I site found in the MCS *da26* gene locus unique. This was done by digesting both clones with *Pst*I. *Pst*I restriction sites flank both ends of the PSTI-G (ΔE26 KO) genome library fragment, which is contained in the pUC18/ΔE26 KO vector, thus digestion with *Pst*I liberates the altered PSTI-G (ΔE26 KO) insert. The ΔpUC18 (EcoI-SmaI) contains one copy of the *Pst*I site, which is located in its MCS. The altered PSTI-G (ΔE26 KO) library fragment was ligated into the digested ΔpUC18 (EcoI-SmaI) vector and then electrophorated into DH5α cells from which the amplified DNA was isolated. The clone named ΔpUC18 (EcoI-SmaI)/ΔE26 KO is described in appendix B16.

In order to detect the recombinant virus the β-Gus gene was inserted into the E26 native locus for a color selector during the plaque purification stages of the virus production. The resulting virus is similar to the one constructed in O'Reilly et al., 1990. The β-Gus gene was digested from pUC18-β-Gus (commercially available) with *Sma*I and *Sac*I and agarose gel purified using the Gene Clean III kit. The ΔpUC18 (EcoI-SmaI)/ΔE26 KO vector was also digested with *Sma*I and *Sac*I and agarose gel purified using the Gene Clean III kit. The β-Gus gene was ligated into the vector and then electrophorated into DH5α cells from which the amplified DNA was isolated. The clone named ΔpUC18 (EcoI-SmaI)/ΔE26 KO/β-Gus is described in appendix B17.

Ultimately this strategy failed because purification of the recombinant virus from the recombination vector was not possible due to the fact that the *da26* gene was found to be essential.

1.3.2 Strategy Two: Insertion of second gene copy with promoters

A homologous recombination vector was constructed to aid in the production of recombinant viruses; this vector allows a second epitope tagged copy of BV/ODV E26 gene with mutations and native promoters (-38 and -262) to be place into the viral genome. The construction of a recombination vector, pEcoRV-MCS, would allow for insertion of the *da26* gene and promoters in a non-coding region of the viral genome up

Table 2
Oligonucleotides used for PCR amplification of the homologous recombination regions and multiple cloning site generation for EcoRV-MCS vector.

Name	5' ----Sequence---- 3'
EcoRV MCS 1	GCGTTGACTCGCTGCACCTCGAGCAGTTCG
EcoRV MCS 2	TCTAGAGCTCCAGATCTGCAGCGGCCGCCCGGGGAATTCCAGTAAG TTAGGTATTTATTTGCAACTATATATG ATAGACATTTTCC
EcoRV MCS 3	CCCGGGCGGCCGCTGCAGATCTGGAGCTCTAGATGCCATGGATATC ACTAGTGGAGATAATTAAAATGATAAATCTCGC
EcoRV MCS 4	GTC CAGCCATCGGTTCGGAAAAACAACCC

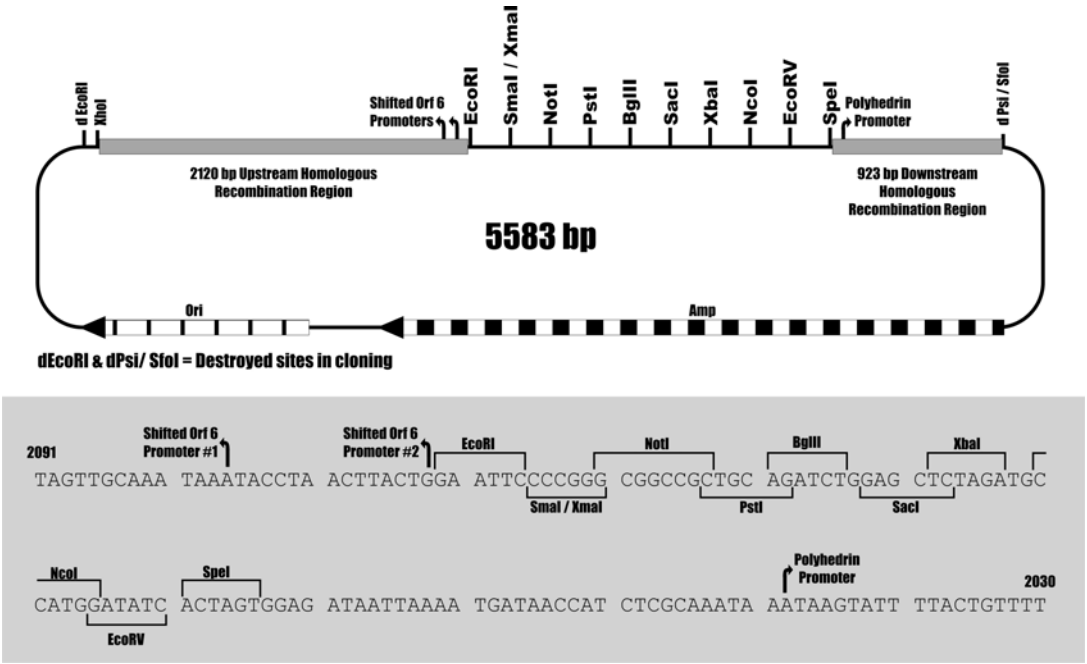


Figure 12. pEcoRV-MCS homologous recombination vector. Note the unique multiple cloning sites with the homologous recombination regions up- and downstream. Note the shifted Orf6 promoters.

stream the polyhedrin gene. Construction of this vector occurred in stages. The first stage was to generate the upstream region by PCR for insertion into pUC 18. The upstream region was PCR amplified using two oligonucleotides: forward, EcoRV MCS 1 and reverse, EcoRV MCS 2, table 2. The DNA was amplified using an initial denaturation step for 2 min 30 sec at 95 ^{0}C followed by 30 cycles of 2 min 30 sec at 95 ^{0}C, 1 min 30 sec at 57 ^{0}C, 1 min at 72 ^{0}C, and after the thirty cycles for full extension a 10 min period at 72 ^{0}C. The PCR product was digested with *Xho*I and *Pst*I and the pUC18 vector was digested with *Sfo*I and *Pst*I. The PCR product was ligated into the digested vector and then electrophorated into DH5α cells from which the amplified DNA was isolated. Once sequence confirmed, the second stage of the construct was initiated, as the downstream region was PCR amplified using two oligonucleotides: forward, EcoRV MCS 3 and reverse, EcoRV MCS 4, see table 2. The DNA was amplified using an initial denaturation step for 2 min 30 sec at 95 ^{0}C followed by 30 cycles of 2 min 30 sec at 95 ^{0}C, 1 min at 55 ^{0}C, 3 min at 72 ^{0}C, and after the thirty cycles for full extension a 10 min period at 72 ^{0}C. The downstream PCR product and the upstream/pUC18 vector were digested with *Pst*I and *Sna*BI and then ligated into the digested vector. This mixture was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed, see figure 12. The clone named pEcoRV-MCS is described in appendix B37.

A series of truncations in the da26 gene were constructed and termed regional domains, figure 13. These truncations were produced in an attempt to identify the regions responsible for the membrane association and nuclear localization of E26. This required the truncations to be epitope tagged to allow for the differentiation of the E26 regional domain from the E26 produced by the infection. This utilized the T7 epitope and antisera to this epitope, which was placed on the c-terminus of the resulting proteins.

Construction of the regional domains (truncations/mutations) in the second copy of the *da26* gene required many intermediate constructs to achieve the final clones. First, a modified pUC 19, pUC-JKB, was made with a new multiple cloning sites. Second, epitope tag sequences were added to the pUC-JKB construct for delineation of native

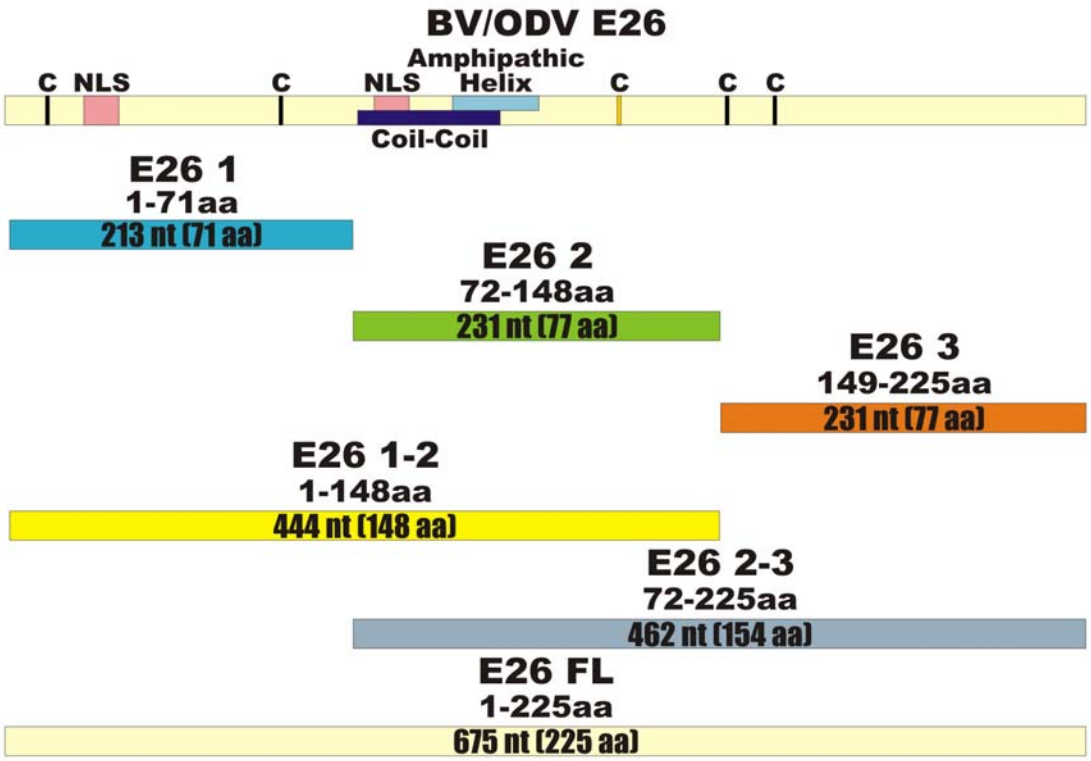


Figure 13. Regional domains of BV/ODV E26. The *da26* gene was truncated to produce these regional domains. For comparison the full length gene with the putative domains have been included above. The domains include cysteine amino acids (C) possible palmitoylation sites, nuclear localization signal (NLS), coil-coil domains regions of protein-protein interactions, and amphipathic helix possible membrane association domains.

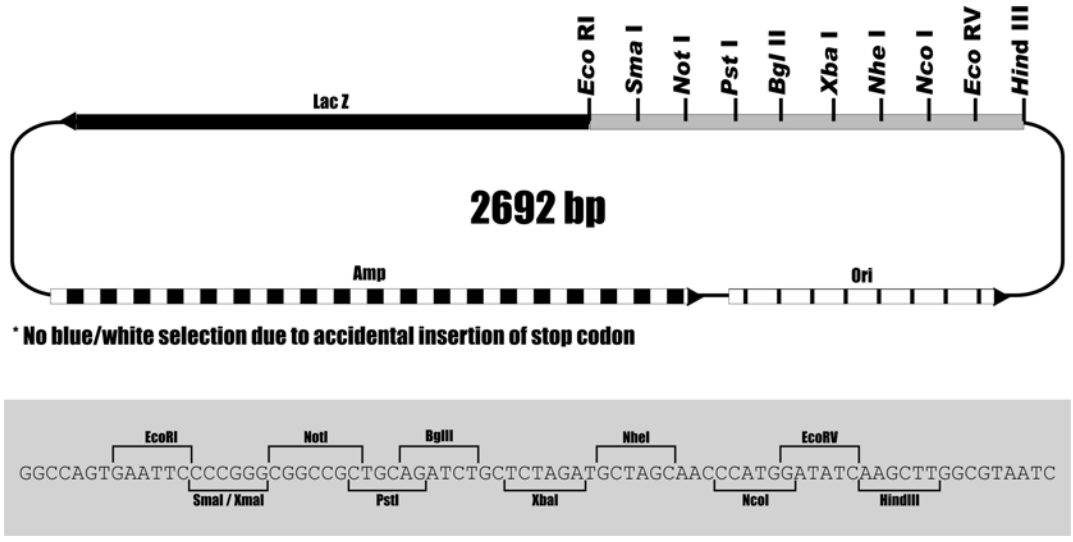
E26 from the additional recombinant copy (Jarvik and Telmer, 1998). Third, the *da26* promoters, -38 and -262, were cloned into this vector, pUC-JKB-Tag. Fourth, the E26 regional domains were cloned into the pUC-JKB-pE26-Tag vector. Lastly, the completed promoter-E26 regional domain-Tag gene was removed and cloned into the homologous recombination vector, pEcoRV-MCS for recombinant virus production.

The modification of pUC19 was produced through the addition of annealed oligonucleotides. The pUC 19 vector was digested with *Eco*RI and *Hin*dIII allowing the ligation of the new multiple cloning site (MCS) between these digested sites using two oligonucleotides: forward, JKB-For and reverse, JKB-Rev, see table 3. The new MCS was required for the addition of enough unique restriction enzyme sites for future clone constructs. The two oligonucleotides were added together in equal molar ratios and

Table 3
Oligonucleotides used for creation of unique multiple cloning sites were inserted into pUC19.

Name	5’ ----Sequence---- 3’
JKB-For	AATTCCCCGGGCGGCCGCTGCAGATCTGCTCTAGATGCTAGCAACCCATG GATATCA
JKB-Rev	GGGGCCCGCCGGCGACGTCTAGACGAGATCTACGATCGTTGGGTACCTAT AGTTCGA
Kpn-For	CTAGCTAACTGGTACCTTGACCATGGATATCA
Kpn-Rev	AGCTTGATATCCATGGTCAAGGTACCAGTTAG

A



B

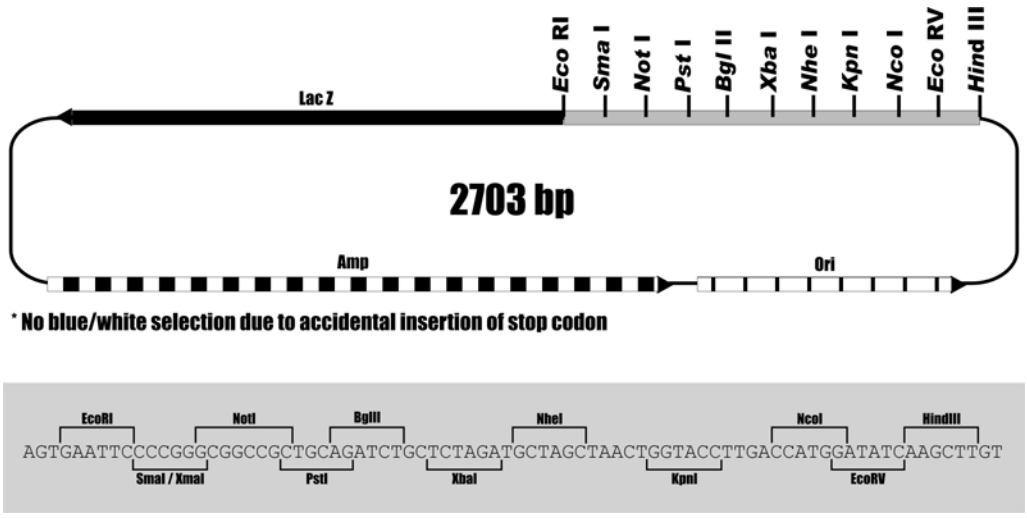


Figure 14. pUC-JKB (A) and pUC-JKB Extended Kpn (B) vector maps. These two vectors allow for intermediate cloning to be achieved before the end result is cloned into the EcoRV-MCS homologous recombination vector. Note the similar MCS to the EcoRV-MCS vector. There is no red/white selection with this pUC based vector as the new MCS inserted a stop codon.

heated to 95 ^{0}C and cooled 0.1 ^{0}C per sec until the solution reached 4 ^{0}C. These annealed oligonucleotides were added to a known amount of digested and gel purified pUC18 DNA and ligated together using T4 DNA ligase from Promega overnight at 16 ^{0}C. The ligated DNA was then electro-transformed and plated for isolation on LM-Amp plates. Individual colonies were grown in liquid medium, from which the amplified resulting vector was isolated, and sequence confirmed, see figure 14A. The clone was named pUC18/19-JKB, and is described in appendix B18.

During the cloning process it was determined the addition of a *Kpn*I site was needed. This site was added by the use of two oligonucleotides: forward, Kpn-For, and reverse, Kpn-Rev, see table 3. The two oligonucleotides were added together in equal molar ratios and heated to 95 ^{0}C and cooled 0.1 ^{0}C per sec until the solution reached 4 ^{0}C. These annealed oligonucleotides were added to a known amount of digested and gel purified pUC18 DNA and ligated together using T4 DNA ligase overnight at 16 ^{0}C. The ligated DNA was then electro-transformed and plated for isolation on LM-Amp plates. Individual colonies were grown in liquid medium, from which the amplified resulting vector was isolated, and sequence confirmed, see figure 14B. The clone named pUC18/19-JKB-Ext KpnI is described in appendix B19.

Table 4
Oligonucleotides used for creation of an epitope region, which were inserted into pUC-JKB extended Kpn.

Name	5’ ----Sequence---- 3’
Flag For	CTAGCCCGGGCGCCGCGCACTACTTAATTAACCGAGCATGCAGCTCTAGCTC TAGCTCTAGCTCTAGCTCTGACTACAAAGACGATGACGATAAATAATAAAAT AAATTAAAC
Flag Rev	CATGGTTTAATTTATTTTATTATTTATCGTCATCGTCTTTGTAGTCAGAGCTAG AGCTAGAGCTAGAGCTAGAGCTGCATGCTCGGTTAATTAAGTAGTGCGCGGC GCCCGGG
His For	CTAGCCCGGGCGCCGCGCACTACTTAATTAACCGAGCATGCAGCTCTAGCTC TAGCTCTAGCTCTAGCTCTCACCATCACCATCACCATCACCATTAATAAAATA AATTAAAC
His Rev	CATGGTTTAATTTATTTTATTAATGGTGATGGTGATGGTGATGGTGAGAGCTA GAGCTAGAGCTAGAGCTAGAGCTGCATGCTCGGTTAATTAAGTAGTGCGCGG CGCCCGGG
T7 For	TAACATGG CCAGCATGACTGGC GGTCAGCAAATGGGCGCATG
T7 Rev	CGCCCATTTGCTGACCGCCAGTCAT GCTGGCCATGTTAAT

Once the pUC18/19-JKB-Ext KpnI was made, the in-frame fusion epitope tag sequence was then added. As it could not be predicted which type of epitope tag would perform best under all types of experimental conditions in virally infected cells, three different epitope tags were used; T7, Flag, and His (Jarvik and Telmer, 1998). Two tags were placed in each construction, either T7-Flag or T7-His, to give the greatest flexibility to all future experiments. These constructions were produced through the addition of annealed oligonucleotides. pUC-JKB extended was digested with *Nhe*I and *Nco*I allowing ligation of the epitope tag between these digested sites using two oligonucleotides: forward, Flag-For for Flag, or His-For for His; reverse, Flag-Rev for Flag, or His-Rev for His, see table 4. The two oligonucleotides were added together in equal molar ratios and heated to 95 ^{0}C and cooled 0.1 ^{0}C per sec until the solution reached 4 ^{0}C. These annealed oligonucleotides were added to a known amount of digested and gel purified pUC18 DNA and ligated together using T4 DNA ligase overnight at 16 ^{0}C. The ligated DNA was electrotransformed and plated for isolation on LM-Amp plates. Individual colonies were grown in liquid medium, from which the amplified resulting vector was isolated, and sequence confirmed, see figure 15A. The clones named pUC18/19 FLAG Tag and pUC18/19 HIS Tag are described in appendix B20 and 21, respectively.

The T7 epitope tags were then added to each of these clones, pUC-JKB-His and pUC-JKB-Flag, with annealed oligonucleotides. The vector-tag constructs were digested with *Pac*I and *Sph*I allowing ligation of the T7 Tag using two oligonucleotides: forward, T7-For; reverse, T7-Rev, see table 4. The two T7 oligonucleotides were added together in equal molar ratios and heated to 95 ^{0}C and cooled 0.1 ^{0}C per sec until the solution reached 4 ^{0}C. These annealed oligonucleotides were added to a known amount of digested and gel purified pUC-JKB-HIS and pUC-JKB-FLAG DNA and ligated together using T4 DNA ligase overnight at 16 ^{0}C. The ligated DNA was electrotransformed and plated for isolation on LM-Amp plates. Individual colonies were grown in liquid medium, from which the amplified resulting vector was isolated, and

sequence confirmed, see figure 15B. The clones named pUC18/19 FLAG-T7 Tag and pUC18/19 HIS-T7 Tag are described in appendix B22 and 23, respectively.

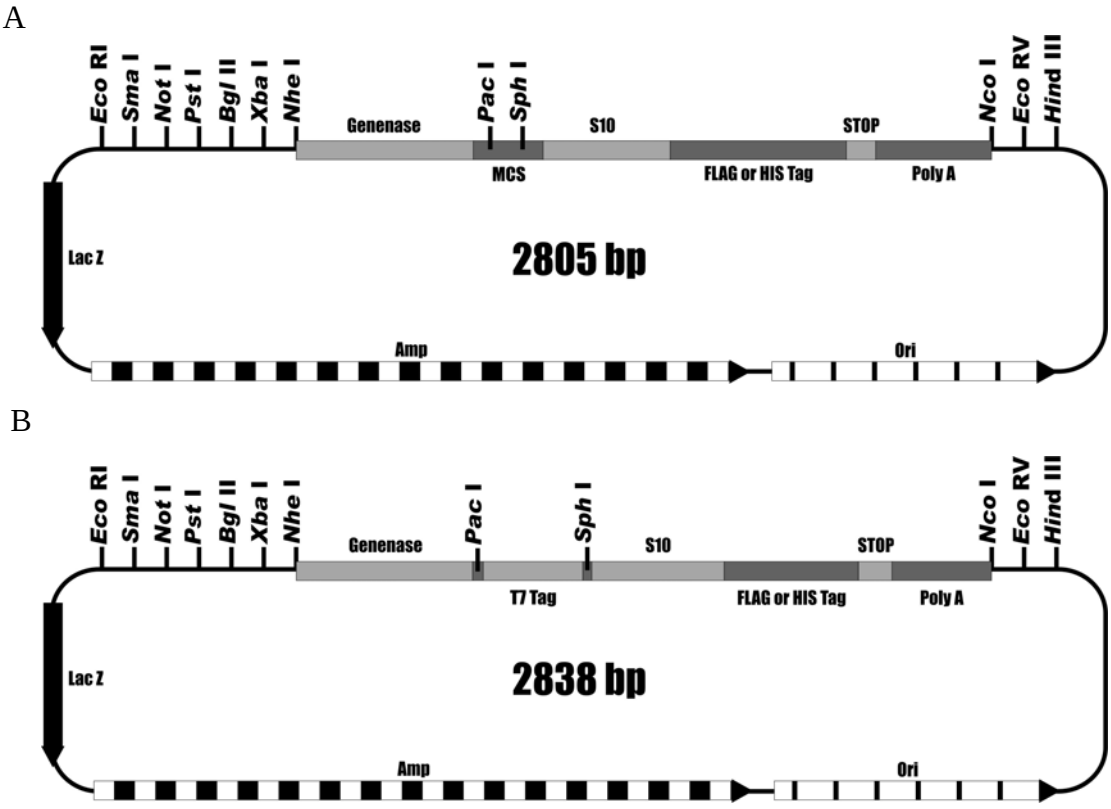


Figure 15: pUC-JKB-Tag (A) and pUC-JKB-Tag2 (B) vector maps. These two intermediate clones were constructed allowing for the attachment of an epitope tag sequence. Figure 15A has either the FLAG or HIS epitope tags inserted with a linker arm spacing them from the insertion area of the gene of interest. This vector also has a genenase cleavage sequence to remove the epitope tag. Figure 15B in the same construct shown in Fig 15A with the addition of a second epitope tag, a T7 tag.

Table 5
Oligonucleotides used for PCR amplification of the *da26* gene promoter region and insertion into pUC-JKB-Tag vector.

Name	5' ----Sequence---- 3'
pE26 For	CACTGCAGGATATAGACATGACATCAGTCG
pE26 Rev	GATGCTAGCGAGCTCAATGGTACCTATGGGCCCTTCGATGTTTCGCCTTT GAAC

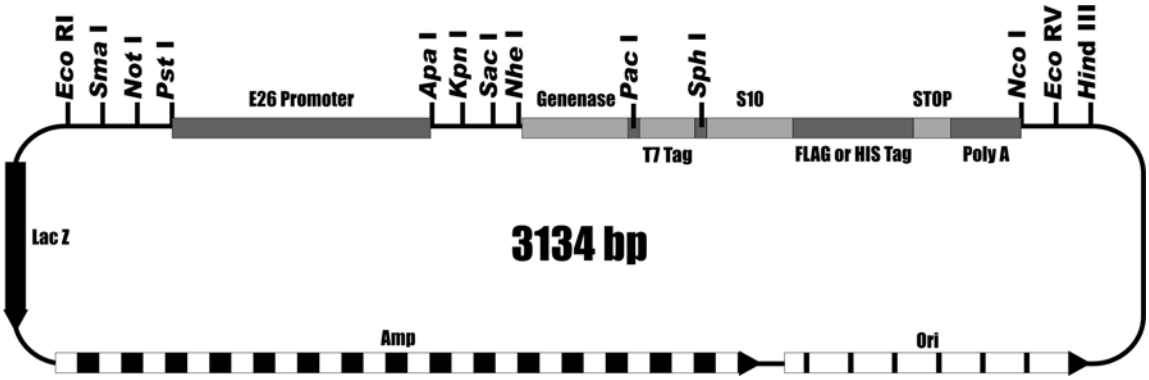


Figure 16. pUC-JKB-pE26-Tag vector map. This is an intermediate clone with the addition of the *da26* promoters. These promoters will allow for similar levels of expression of the recombinant regional domains to those of native E26.

With the epitope tags in place the *da26* promoters, -38 (CATTA) and -262 (GACATC), were then added to the vector to allow for native expression of the E26 regional domains in the mutant viruses. This was achieved with a PCR strategy allowing amplification of the promoters from the viral genome using two oligonucleotides: forward, pE26-For; reverse, pE26-Rev, see table 5. The DNA was amplified using an initial denaturation step for 2 min 30 sec at 95 ^{0}C followed by 30 cycles of 2 min 30 sec at 95 ^{0}C, 1 min 30 sec at 57 ^{0}C, 1 min at 72 ^{0}C, and after the thirty cycles for full extension a 10 min period at 72 ^{0}C. The PCR product and the pUC-JKB-Tag vector were digested with *Pst*I and *Nhe*I to allow for ligation of the promoters into the vector and generation of the new MCS to be used for insertion of *da26* gene regional domains in frame with the epitope tag sequences. The PCR product was ligated into the digested vector and electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed, figure 16. The clones named pUC18/19/pE26/FLAG-T7 Tag and pUC18/19/pE26/HIS-T7 Tag are described in appendix B24 and 25, respectively.

Regional domain constructs were generated by PCR. For this strategy, the *da26* gene was divided into 3 relatively equal portions, figure 17. The oligonucleotides in table 6 were used in the generation of the regional domains.

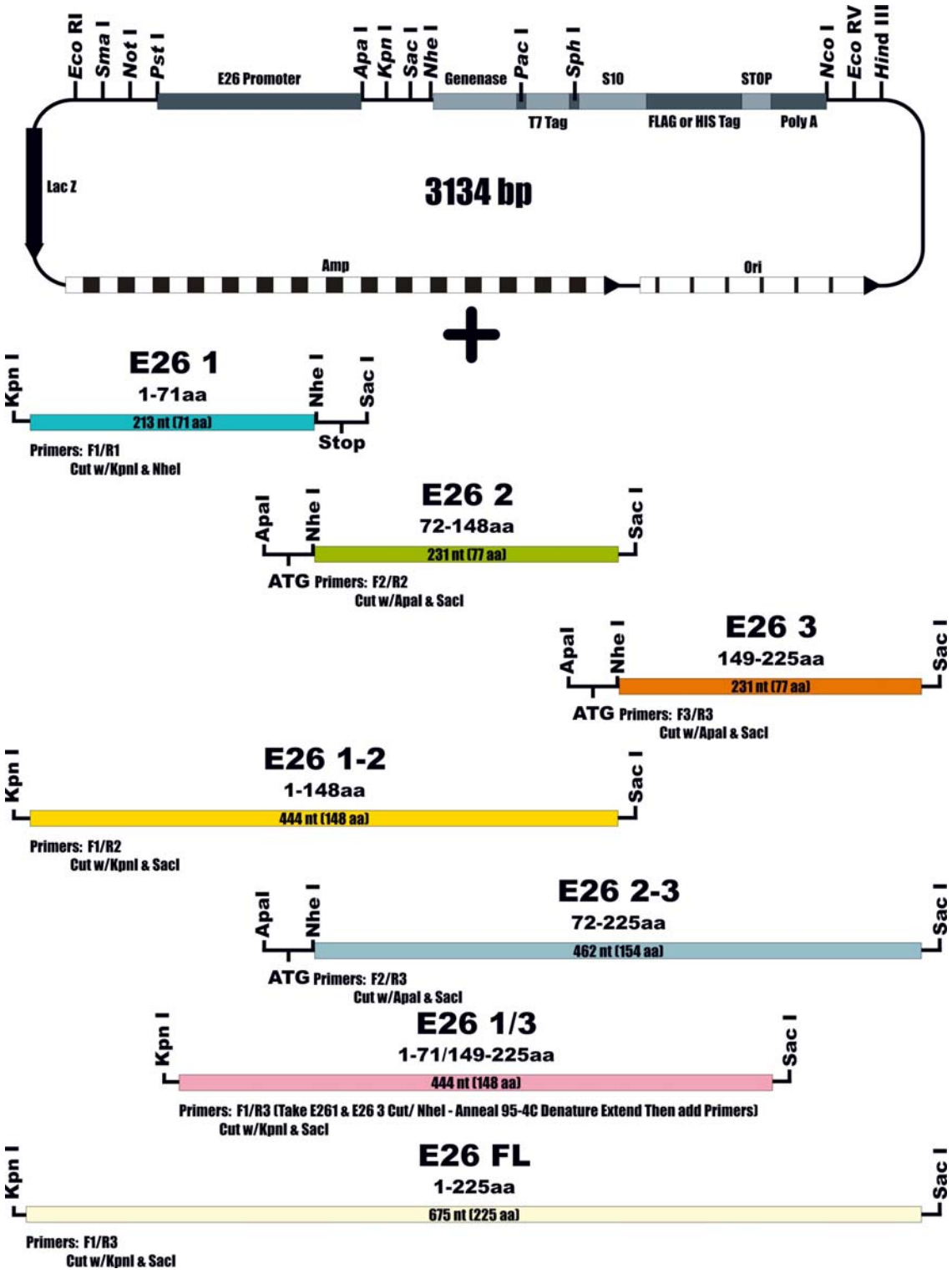


Figure 17. Regional domain cloning strategies. Regional domain cloning strategies are displayed in this figure. All regional domains of E26 were PCRed from the viral genome using the primers denoted above; the sequence of these primers is described in table 8. Note the E26 protein has been split into almost equal thirds for the use in the trafficking studies to follow.

Table 6
Oligonucleotides used in PCR amplification cloning strategies for the generation of E26 domains and the insertion of those domains into pUC-JKB-pE26-Tag.

Name	5' ----Sequence---- 3'
F1	GAGGTACCATGGAGTCTGTTCAAACGCG
R1	CCTAGCCGTTATACGCTCGAGTTAGCTAGCGAAATTCAAGTGCGTGTTACG
F2	CGTATAACGGCTAGGGGGCCCATGGCTAGCAATAAGATACAGTCTGTACATAA
R2	GACTCGAGGGCCAAACGTCTCCTTACAA
F3	CGTATAACGGCTAGGGGGCCCATGGCTAGCGAGCTGTGCACATTGTACAAC
R3	GACTCGAGATAGGCGTTAATATCACTTTGAG

E26 Domain #1

The E26 regional domain #1 clones, figure 17, were generated with F1 and R1 oligonucleotides shown in table 6. The DNA was amplified using an initial denaturation step for 2 min 30 sec at 95 ^{0}C followed by 30 cycles of 30 sec at 95 ^{0}C, 1 min 30 sec at 58 ^{0}C, 1 min at 72 ^{0}C, and after the thirty cycles for full extension a 10 min period at 72 ^{0}C. The PCR product and the pUC-JKB-pE26-Tag vector were digested with *Kpn*I and *Nhe*I to allow for ligation of the regional domain into the vectors. The PCR products were ligated into the digested vectors and electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clones were named pUC18/19/pE26/E26 1/FLAG-T7 Tag and pUC18/19/pE26/E26 1/HIS-T7 Tag, and are described in appendix B26 and 27, respectively.

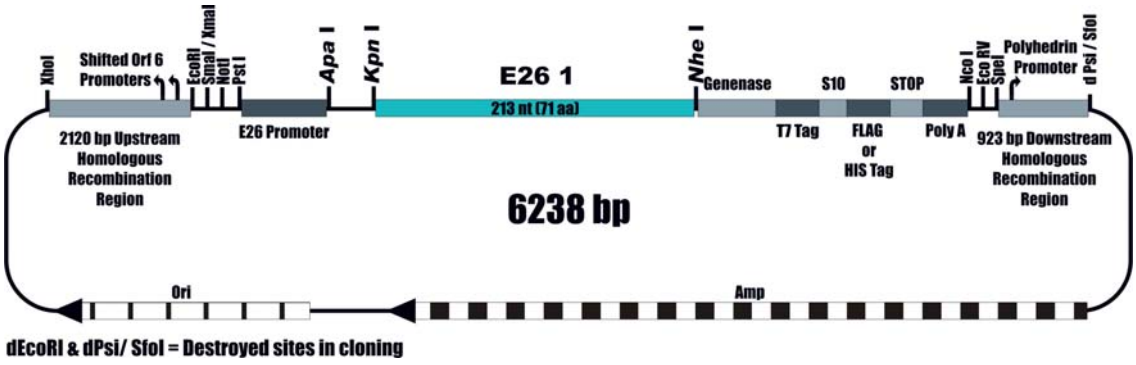


Figure 18. E26 regional domain #1 in the homologous recombination vector. Regional domain 1 will examine the role of the C-terminal region in trafficking of the E26 protein. Note the epitope tags that will be used to differentiate the recombinant from the native.

The regional domain constructs were then digested with *Pst*I and *Eco*RV to isolate the newly constructed E26 regional domains with promoters and epitope tag sequences, see figure 17. These new regional domains were then ligated, using the same restriction sites, into the EcoRV-MCS recombinant virus constructs for virus production. These new constructs amplified and sequence confirmed as before, see figure 18. The clones named pEcoRV-MCS/E26 1/FLAG-T7 Tag and pEcoRV-MCS/E26 1/HIS-T7 Tag are described in appendix B39 and 40, respectively.

E26 Domain #2

The E26 regional domain #2 clones, figure 17, were generated with F2 and R2 oligonucleotides shown in table 6. The DNA was amplified using an initial denaturation step for 2 min 30 sec at 95 ^{0}C followed by 30 cycles of 30 sec at 95 ^{0}C, 1 min 30 sec at 58 ^{0}C, 1 min at 72 ^{0}C, and after the thirty cycles for full extension a 10 min period at 72 ^{0}C. The PCR product and the pUC-JKB-pE26-Tag vectors were digested with *Apa*I and *Sac*I to allow for ligation of the regional domain into the vectors. The PCR product was ligated into the digested vectors and electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clones named pUC18/19/pE26/E26 2/FLAG-T7 Tag and pUC18/19/pE26/E26 2/HIS-T7 Tag are described in appendix B28 and 29, respectively.

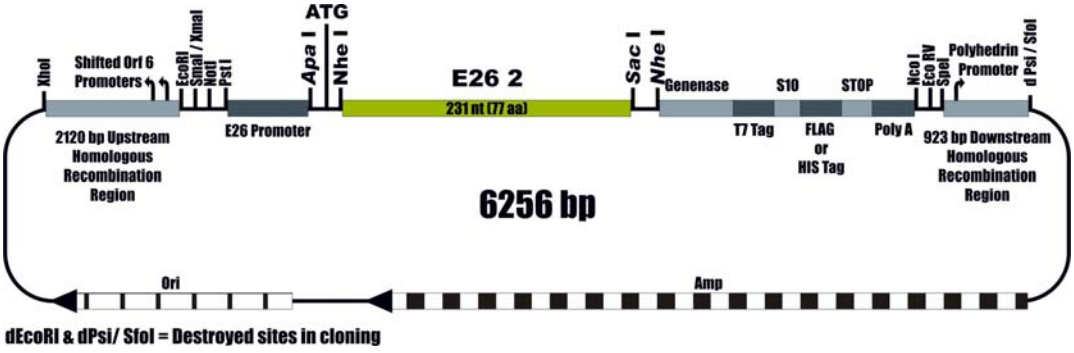


Figure 19. E26 regional domain #2 in the homologous recombination vector. Regional domain 2 will examine the role of the central region in trafficking of the E26 protein. Note the epitope tags that will be used to differentiate the recombinant from the native.

The regional domain constructs were digested with *Pst*I and *Eco*RV to isolate the newly constructed E26 regional domain with promoters and epitope tag sequences, see figure 17. These new regional domains were then ligated, using the same restriction sites, into the EcoRV-MCS recombinant virus constructs for virus production. These new constructs were amplified and sequence confirmed as before, see figure 19. The clones named pEcoRV-MCS/E26 2/FLAG-T7 Tag and pEcoRV-MCS/E26 2/HIS-T7 Tag are described in appendix B41 and 42, respectively.

E26 Domain #3

The E26 regional domain #3 clones, figure 17, were generated with F3 and R3 oligonucleotides shown in table 6. The DNA was amplified using an initial denaturation step for 2 min 30 sec at 95 ^{0}C followed by 30 cycles of 30 sec at 95 ^{0}C, 1 min 30 sec at 58 ^{0}C, 1 min at 72 ^{0}C, and after the thirty cycles for full extension a 10 min period at 72 ^{0}C. The PCR product and the pUC-JKB-pE26-Tag vectors were digested with *Apa*I and *Sac*I to allow for ligation of the regional domain into the vectors. The PCR product was ligated into the digested vector and electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clones named pUC18/19/pE26/E26 3/FLAG-T7 Tag and pUC18/19/pE26/E26 3/HIS-T7 Tag are described in appendix B30 and 31, respectively.

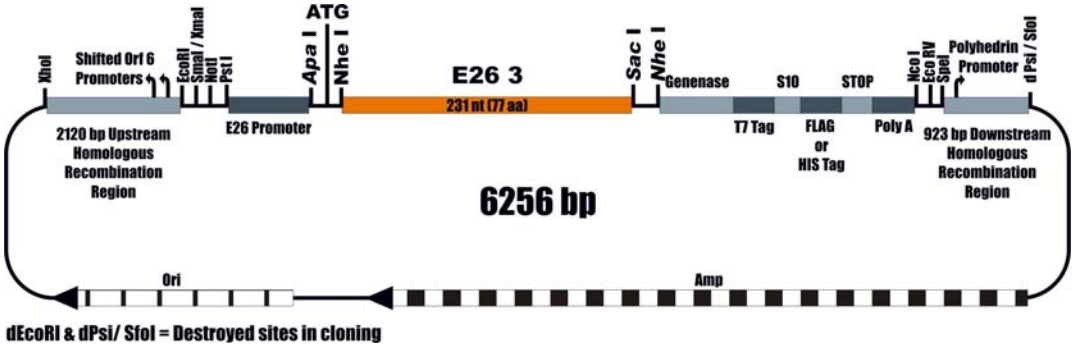


Figure 20. E26 regional domain #3 in the homologous recombination vector. Regional domain 3 will examine the role of the N-terminal region in trafficking of the E26 protein. Note the epitope tags that will be used to differentiate the recombinant from the native.

The regional domain constructs were digested with *Pst*I and *Eco*RV to isolate the newly constructed E26 regional domain with promoters and epitope tag sequences, see figure 17. These new regional domains were then ligated, using the same restriction sites, into the EcoRV-MCS recombinant virus constructs for virus production. These new constructs were amplified and sequence confirmed as before, see figure 20. The clones named pEcoRV-MCS/E26 3/FLAG-T7 Tag and pEcoRV-MCS/E26 3/HIS-T7 Tag are described in appendix B43 and 44, respectively.

E26 Domain #1-2

The E26 regional domain #1-2 clones, figure 17, were generated with F1 and R2 oligonucleotides shown in table 6. The DNA was amplified using an initial denaturation step for 2 min 30 sec at 95 ^{0}C followed by 30 cycles of 30 sec at 95 ^{0}C, 1 min 30 sec at 58 ^{0}C, 1 min at 72 ^{0}C, and after the thirty cycles for full extension a 10 min period at 72 ^{0}C. The PCR product and the pUC-JKB-pE26-Tag vectors were digested with *Kpn*I and *Sac*I to allow for ligation of the regional domain into the vectors. The PCR product was ligated into the digested vectors and electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clones named pUC18/19/pE26/E26 1-2/FLAG-T7 Tag and pUC18/19/pE26/E26 1-2/HIS-T7 Tag are described in appendix B32 and 33, respectively.

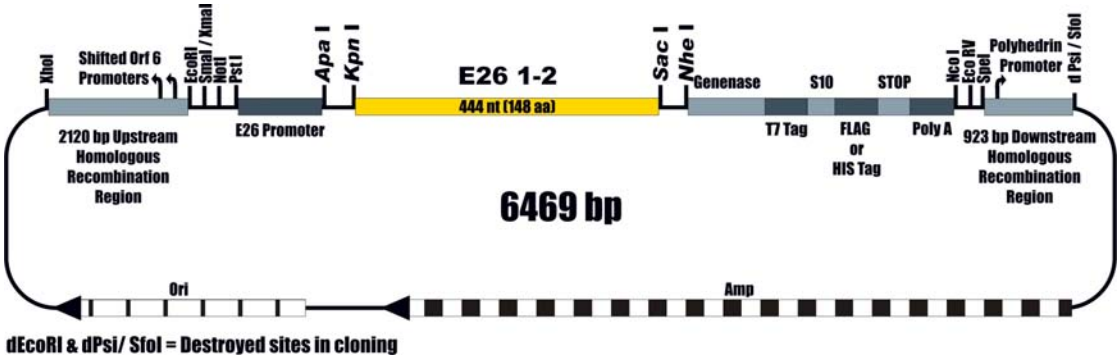


Figure 21. E26 regional domain #1-2 in the homologous recombination vector. Regional domain 1-2 will examine the role of the C-terminal and central regions in trafficking of the E26 protein. Note the epitope tags that will be used to differentiate the recombinant from the native.

The regional domain constructs were digested with *Pst*I and *Eco*RV to isolate the newly constructed E26 regional domain with promoters and epitope tag sequences, see figure 17. These new regional domains were then ligated, using the same restriction sites, into the EcoRV-MCS recombinant virus constructs for virus production. These new constructs were amplified and sequence confirmed as before, see figure 21. The clones named pEcoRV-MCS/E26 1-2/FLAG-T7 Tag and pEcoRV-MCS/E26 1-2/HIS-T7 Tag are described in appendix B45 and 46, respectively.

E26 Domain #2-3

The E26 regional domain #2-3 clones, figure 17, were generated with F2 and R3 oligonucleotides shown in table 6. The DNA was amplified using an initial denaturation step for 2 min 30 sec at 95 ^{0}C followed by 30 cycles of 30 sec at 95 ^{0}C, 1 min 30 sec at 58 ^{0}C, 1 min at 72 ^{0}C, and after the thirty cycles for full extension a 10 min period at 72 ^{0}C. The PCR product and the pUC-JKB-pE26-Tag vectors were digested with *Apa*I and *Sac*I to allow for ligation of the regional domain into the vectors. The PCR product was ligated into the digested vectors and electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clones named pUC18/19/pE26/E26 2-3/FLAG-T7 Tag and pUC18/19/pE26/E26 2-3/HIS-T7 Tag are described in appendix B34 and 35, respectively.

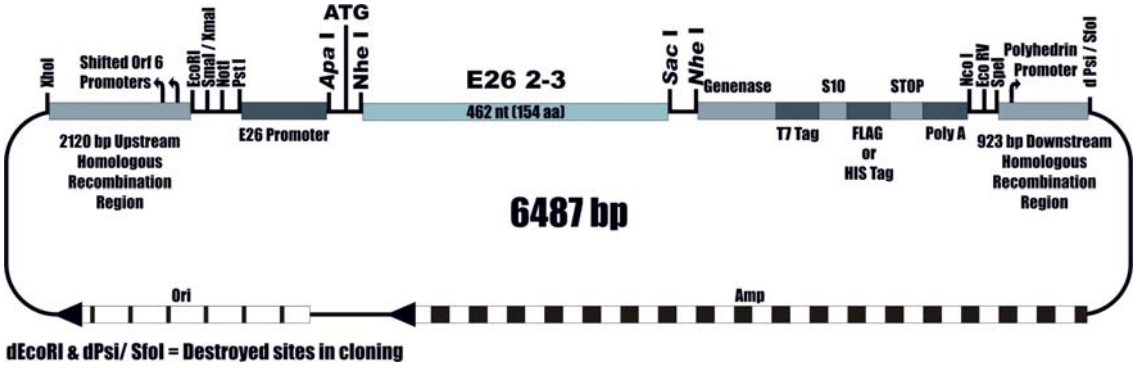


Figure 22. E26 regional domain #2-3 in the homologous recombination vector. Regional domain 2-3 will examine the role of the central and N-terminal regions in trafficking of the E26 protein. Note the epitope tags that will be used to differentiate the recombinant from the native.

The regional domain constructs were digested with *Pst*I and *Eco*RV to isolate the newly constructed E26 regional domain with promoters and epitope tag sequences, see figure 17. This new regional domain was then ligated, using the same restriction sites, into the EcoRV-MCS recombinant virus constructs for virus production. These new constructs were amplified and sequence confirmed as before, see figure 22. The clones named pEcoRV-MCS/E26 2-3/FLAG-T7 Tag and pEcoRV-MCS/E26 2-3/HIS-T7 Tag are described in appendix B47 and 48, respectively.

E26 Domain FL

The complete *da26* gene, figure 17, was PCR generated with the F1 and R3 oligonucleotides, shown in Table 6. The DNA was amplified using an initial denaturation step for 2 min 30 sec at 95 ^{0}C followed by 30 cycles of 30 sec at 95 ^{0}C, 1 min 30 sec at 58 ^{0}C, 1 min at 72 ^{0}C, and after the thirty cycles for full extension a 10 min period at 72 ^{0}C. The PCR product and the pUC-JKB-pE26-Tag vectors were digested with *Kpn*I and *Sac*I to allow for ligation of the regional domain into the vectors. The PCR product was ligated into the digested vectors and electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clones named pUC18/19/pE26/E26 FL/FLAG-T7 Tag and pUC18/19/pE26/E26 FL/HIS-T7 Tag are described in appendix B36 and 37, respectively.

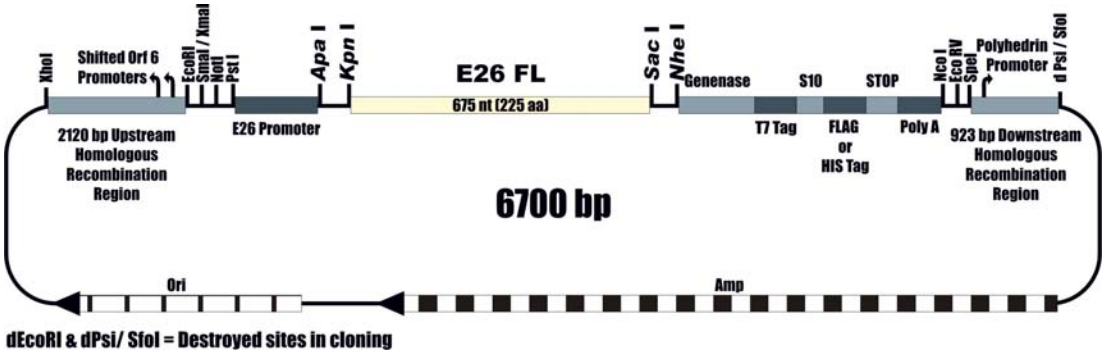


Figure 23. E26 regional domain FL in the homologous recombination vector. The full length protein will examine the trafficking of the protein in the presence of the epitope tags to confirm it traffics normally. Note the epitope tags that will be used to differentiate the recombinant from the native.

The regional domain constructs were then digested with *Pst*I and *Eco*RV to isolate the newly constructed E26 regional domain with promoters and epitope tag sequences, see figure 17. This new regional domain was then ligated, using the same restriction sites, into the EcoRV-MCS recombinant virus constructs for virus production. These new constructs were amplified and sequence confirmed as before, see figure 23. The clones named pEcoRV-MCS/E26 FL/FLAG-T7 Tag and pEcoRV-MCS/E26 FL/HIS-T7 Tag are described in appendix B49 and 50, respectively.

Recombinant genes for the construction of mutant viruses were constructed in a homologous recombination vector called pEcoRV-MCS, described above (figure 12, page 45, appendix B38). This vector contains a 2.1 kb upstream and a 0.9 kb downstream homologous recombination region for the *Ac*NPV genome. These recombination regions are separated by a multiple cloning site, which has been placed in a non-coding region of the viral genome near the polyhedrin gene. Upon transfection of pEcoRV-MCS DNA with Bac-Pak 6 DNA, homologous recombination of the vector with BacPak 6 occurs resulting in production of a polyhedra positive recombinant virus (Summers and Smith, 1987).

Recombination occurred and viable mutant viruses were isolated. These viruses however either did not transcribe or translate the recombinant epitope tagged genes. It was concluded from preliminary investigations that trans-acting factors were missing that were required by the two *da26* promoters, which were used.

1.3.3 Strategy Three: Polyhedrin (Polh) Promoter

The third strategy employed the commercially available pBAC-gus vector and the pIE1-E26 regional domain constructs built in the in vitro translation and transient expression strategy (1.4, page 63), resulting in placing the *da26* gene and truncations under the polyhedrin promoter. This required an alteration to the pBAC-gus vector to allow for cut and paste insertion of the constructs listed in the previous section and addition of a single T7 epitope on the c-terminus of the protein. The pBACgus-1 vector was digested with *Bam*HI and *Sph*I to allow for insertion of a new MCS, which was

generated by annealed oligonucleotides, see table 7. The two MCS oligonucleotides were added together in equal molar ratios and heated to 95 ^{0}C and cooled 0.1 ^{0}C per sec until the solution reached 4 ^{0}C. These annealed oligonucleotides were added to a known amount of digested and gel purified pBACgus-1 DNA and ligated together using T4 DNA ligase overnight at 16 ^{0}C. The ligated DNA was electrotransformed and plated for isolation on LM-Amp plates. Individual colonies were grown in liquid medium, from which the amplified resulting vector was isolated, and sequence confirmed. The clone named pBACgus-MCS is described in appendix B51.

Table 7
Oligonucleotides used for insertion of MCS into pBACgus-1 vector.

Name	5' ----Sequence---- 3'
pBACgus MCS For	GATCTCCATGGCAGGGCCCGAATTCGGTACCCTCGAGGGATCCTAATAAA ATAAATTAAACGCATG
pBACgus MCS Rev	CGTTTAATTTATTTTATTAGGATCCCTCGAGGGTACCGAATTCGGGCCCTG CCATGGA

To generate pBACgus-E26#1, the pIE1-E26#1 clone (appendix B8) was used as an intermediate and was digested with *Nco*I and *Xho*I. The new vector pBACgus-MCS was also digested with *Nco*I and *Xho*I and ligated to the insert. The ligation product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. A small sequence was removed from a non-essential region of the MCS of the final construct; this should not affect the function of the clone, for details see the clone named pBACgus-E26#1 in appendix B52.

To generate pBACgus-E26#2, the pIE1-E26#2 clone (appendix B9) was used as an intermediate and was digested with *Nco*I and *Xho*I. The new vector pBACgus-MCS was also digested with *Nco*I and *Xho*I and ligated to the insert. The ligation product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clone named pBACgus-E26#2 is described in appendix B53.

To generate pBACgus-E26#3, the pIE1-E26#3 clone (appendix B10) was used as an intermediate and was digested with *Nco*I and *Xho*I. The new vector pBACgus-MCS was also digested with *Nco*I and *Xho*I and ligated to the insert. The ligation product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. A small sequence was removed from a non-essential region of the MCS of the final construct; this should not affect the function of the clone, for details see the clone named pBACgus-E26#3 in appendix B54.

To generate pBACgus-E26#1-2, the pIE1-E26#1-2 clone (appendix B11) was used as an intermediate and was digested with *Nco*I and *Xho*I. The new vector pBACgus-MCS was also digested with *Nco*I and *Xho*I and ligated to the insert. The ligation product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. A small sequence was removed from a non-essential region of the MCS of the final construct; this should not affect the function of the clone, for details see the clone named pBACgus-E26#1-2 in appendix B55.

To generate pBACgus-E26#2-3, the pIE1-E26#2-3 clone (appendix B12) was used as an intermediate and was digested with *Nco*I and *Xho*I. The new vector pBACgus-MCS was also digested with *Nco*I and *Xho*I and ligated to the insert. The ligation product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. A small sequence was removed from a non-essential region of the MCS of the final construct; this should not affect the function of the clone, for details see the clone named pBACgus-E26#2-3 in appendix B57.

To generate pBACgus-E26 FL, the pIE1-E26#FL clone (appendix B13) was used as an intermediate and was digested with *Nco*I and *Xho*I. The new vector pBACgus-MCS was also digested with *Nco*I and *Xho*I and ligated to the insert. The ligation product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. Two small sequences were removed from a non-essential region of the MCS and the T7 epitope sequence of the final construct; this required additional cloning to generate the necessary construct, for details see the clone named pBACgus-E26#FL (NO T7 TAG) in appendix B57.

To generate pBACgus-E26 FL-T7, the pBACgus-E26 FL (NO T7 TAG) clone (appendix B57) was used as an intermediate and was digested with *Xho*I and *Bam*HI for oligonucleotide insertion of the need T7 epitope sequence, table 8. The two T7 oligonucleotides were added together in equal molar ratios and heated to 95 ^{0}C and cooled 0.1 ^{0}C per sec until the solution reached 4 ^{0}C. These annealed oligonucleotides were added to a known amount of digested and gel purified pBACgus-1 DNA and ligated together using T4 DNA ligase overnight at 16 ^{0}C. The ligated DNA was electrotransformed and plated for isolation on LM-Amp plates. Individual colonies were grown in liquid medium, from which the amplified resulting vector was isolated, and sequence confirmed. The clone named pBACgus-E26 FL-T7 is described in appendix B58.

Table 8
Oligonucleotides used for insertion of T7 epitope into pBACgus-E26 FL (NO T7 TAG) vector.

Name	5' ----Sequence---- 3'
T7 For	TCGAGATGGCCAGCATGACTGGCGGTCAGCAAATGGGCG
T7 Rev	CTACCGGTCGTACTGACCGCCAGTCGTTTACCCGCCTAG

1.4 Cloning Strategies for in vitro Translation and Transient Expression

In vitro translation clones were developed to determine how the *da26* gene is transcribed and translated in infected and uninfected in vitro environments. In vitro translation strategies were employed in an attempt to understand if newly produced E26 can interact with either dog or insect microsomal membranes. These experiments went further to determine how the newly produced E26 protein interacts with infected and uninfected *Sf9* microsomal membranes. The goals of these experiments were designed to answer the following questions. Can E26 interact with a membrane in the absence of infection? Does the E26 membrane interaction require other viral or soluble cellular factors? Is the timing of infection relevant to the E26 membrane interaction? If an interaction between E26 and the microsomes is possible other experiments could be

performed to determine which amino acids of E26 are responsible for the interaction. Two cloning constructs were generated for use in in vitro translation experiments; *da26* was placed under the control of the Sp 6 and T7 promoters in the pGEM vector system (Promega P2151 and P2161). For a detailed cloning map of these constructs reference appendix, B1 and B2.

The *da26* gene was PCR generated and placed in the pGEM3z/UTR vector using the *Xba*I and *Hin*dIII restriction sites. The PCR reaction utilized two oligonucleotides: forward, E26-N-term-XbaI and reverse, E26-N-HIS-R, see table 9. The DNA was amplified using an initial denaturation step for 2 min 30 sec at 95 ^{0}C followed by 30 cycles of 30 sec at 95 ^{0}C, 1 min 30 sec at 52 ^{0}C, 45 sec at 72 ^{0}C, and after the thirty cycles for full extension a 7 min at 72 ^{0}C. The pGEM3z/UTR was digested with *Xba*I and *Hin*dIII. The PCR product was ligated into the digested vector and then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clone named pGEM3z/UTR/E26 is described in appendix B1.

Table 9
Oligonucleotides used for PCR amplification of the *da26* gene and insertion into pGEM3z/UTR.

Name	5' ----Sequence---- 3'
E26-N-term-XbaI	GCTCTAGATGGAGTCTGTTCAAACGCG
E26-N-HIS-R	GCGAAGCTTTAATAGGCGTTAATATCACTTTG

The *da26* gene and the UTR region were digested with *Eco*RI and *Hind*III from pGEM3Z/UTR/E26 and isolated from an agarose gel using the Gene Clean III kit. This insert was then ligated into pGEM4z, which was digested with *Eco*RI and *Hind*III. The ligated product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clone named pGEM4z/UTR/E26 is described in appendix B2.

Several clones were generated for use in the transient expression studies. These experiments were performed to determine how the *da26* gene is expressed in the absence

of infection. Can the E26 protein interact with membranes and are multiple forms of the protein produced in the absence of infection? To extend these studies a T7 epitope tag was added to the c-terminus of the *da26* gene so that the transfected cells could be infected. The addition of the T7 tag would allow for the differentiation of the transiently expressed form from the form or forms expressed by the viral infection. These experiments took the approach of recovery of function or interacting partners as E26 is not expressed in the absence of infection as it is a viral protein. These studies were furthered through the use of confocal microscope to determine where E26 locates in an uninfected cell.

The *da26* promoters, -38 a.a. (CATTA) and -262 (GACATC), were initially utilized in the pUC19/pE26-E26 and pEGFP/pE26-E26 constructs as it was believed that the uninfected cell would recognize the *da26* promoters and function like the IE-1 promoter which is capable of producing transcripts, which results in protein production in an uninfected cell. The *da26* promoters were not so capable. For information about the *da26* promoters, reference figure 28, page 85. For a detailed cloning map of these constructs reference appendix, B4 and B5.

Table 10
Oligonucleotides used for PCR amplification of the *da26* gene and insertion into pUC19.

Name	5' ----Sequence---- 3'
E26-GFP-F	CGCAAGCTTCCATCAAATTCACAGAACG
E26-N-HIS-R	GCGAAGCTTTAATAGGCGTTAATATCACTTTG

The *da26* gene and promoters (-38 and -262) were PCR generated and placed in the pUC19 vector using the *Hin*dIII restriction site. The PCR reaction utilized two oligonucleotides: forward, E26-GFP-F and reverse, E26-N-HIS-R, see table 10. The DNA was amplified using an initial denaturation step of 2 min 30 sec at 95 ^{0}C followed by 30 cycles of 30 sec at 95 ^{0}C, 1 min 30 sec at 52 ^{0}C, 45 sec at 72 ^{0}C, and after the thirty cycles for full extension a 7 min period at 72 ^{0}C. The PCR product and pUC19

were digested with *Hin*dIII. The PCR product was ligated into the digested vector and then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clone named pUC19/E26 (no stop codon) is described in appendix B4.

The *da26* gene and promoters (-38 and -262) were PCR generated and placed in the pEGFP vector (Clonetech 6077-1) using the *Hin*dIII and *Bam*HI restriction sites. This was done to achieve an auto-fluorescent E26 fusion protein of a size exceeding the free diffusion limit of the NPC to confirm regulated nuclear trafficking. The PCR reaction utilized two oligonucleotides: forward, E26-GFP-F and reverse, E26-GFP-R, see table 11. The DNA was amplified using an initial denaturation step for 2 min 30 sec at 95 ^{0}C followed by 30 cycles of 30 sec at 95 ^{0}C, 1 min 30 sec at 54 ^{0}C, 45 sec at 72 ^{0}C, and after the thirty cycles for full extension a 7 min period at 72 ^{0}C. The PCR product was digested with *Hin*dIII and *Bam*HI and the pEGFP vector was digested similarly. The PCR product was ligated into the digested vector and then electrophorated into DH5α cells from which the amplified DNA was isolated. The clone named pEGFP/E26 is described in appendix B5.

Table 11
Oligonucleotides used for PCR amplification of the *da26* gene and insertion into pEGFP.

Name	5' ----Sequence---- 3'
E26-GFP-F	CGCAAGCTTCCATCAAATTCACAGAACG
E26-GFP-R	GCGGATCCATAGGCGTTAATATCACTTTG

It was found that the -38 and -262 *da26* promoters did not produce and transcripts and thus no protein in uninfected cells. As these constructs did not produce protein the pIE1-4/E26 construct was produced for a series of transfection experiments to determine the how E26 is produced in an uninfected cell for comparison with E26 produced during infection, appendix B6.

Since the above constructs apparently were not transcribed or translated the *da26* gene was PCR generated and placed in the pIE1-4 vector (Novagen 69091-3) using the *Bam*HI and *Bgl*II restriction sites. The PCR reaction utilized two oligonucleotides: forward, E26-N-HIS-F and reverse, E26-BglII, see table 12. The DNA was amplified using an initial denaturation step for 4 min at 95 ^{0}C followed by 30 cycles of 30 sec at 95 ^{0}C, 1 min 30 sec at 58 ^{0}C, 45 sec at 72 ^{0}C, and after the thirty cycles for full extension a 10 min period at 72 ^{0}C. The PCR product and pIE1-4 were digested with *Bam*HI and *Bgl*II. The PCR product was ligated into the digested vector and then electrophorated into DH5α cells from which the amplified DNA was isolated. The clone named pIE1-4/E26 is described in appendix B6.

Table 12
Oligonucleotides used for PCR amplification of the *da26* gene and insertion into pIE1-4.

Name	5' ----Sequence---- 3'
E26-N-HIS-F	CGCAAGCTTCCATCAAATTCACAGAACG
E26-BglII	GCGAAGCTTTAATAGGCGTTAATATCACTTTG

To further the transfection studies and to allow the cells transiently expressing the *da26* gene to be infected, a series of truncations in the da26 gene were constructed and termed regional domains, figure 13. These truncations were produced in an attempt to examine the membrane association and nuclear localization of E26. This required the truncations to be epitope tagged to allow for the differentiation of the transiently expressed E26 or truncation there of from the E26 produced by the infection, appendix B7-13. This utilized the T7 epitope and antisera to this epitope, which was placed on the c-terminus of the protein.

These regional domains, figure 13, have been previously produced for a recombinant virus production (PCR generation details in the previous section); as such they were cut and ligated into a modified pIE1 vector with an altered multiple cloning site (MCS). The pIE1-MCS clone was developed by inserting a new MCS using complementary

oligonucleotides, see table 13. The pIE1-4 vector was digested with *Sac*II and *Bgl*II and the annealed oligos were ligated into it overnight. The oligos were annealed by placing equal molar oligonucleotides were placed in a PCR machine heating to 95 ^{0}C and slowly cooling to room temperature at 0.1 ^{0}C per second. These annealed oligos were then added in an approximately 3 molar excess to the vector for overnight ligation. The ligated product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clone named pIE1-MCS is described in appendix B7.

Table 13
Oligonucleotides used for insertion of a new multiple cloning site into pIE1-4.

Name	5' ----Sequence---- 3'
F-pIE1-MCS	GCTACCGCGGCCATGGCAGGGAAAATAGGTACCTTGAGCTCCCTAGGA CGTGGTCGACATGGCCAGCATGACTGGC
R-pIE1-MCS	CGTCAGATCTGTTAACGTTTAATTTATTTTATTAGGATCCCTCGAGGCCC ATTTGCTGACCGCCAGTCATGCTGGCCATG

In order to generate pIE1-E26#1 domain the intermediate recombinant virus clone pUC18/pE26/E261/FLAG-T7 (appendix B26) was digested with *Kpn*I and *Nhe*I. The new vector pIE1-MCS was digested with *Kpn*I and *Avr*II, as *Nhe*I and *Avr*II will ligate together. The ligated product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clone named pIE1-E26#1 is described in appendix B8.

In order to generate pIE1-E26#2 domain the intermediate recombinant virus clone pUC18/pE26/E262/FLAG-T7 (appendix B28) and the new vector pIE1-MCS were digested with *Apa*I and *Sac*I and ligated together overnight. The ligated product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clone named pIE1-E26#2 is described in appendix B9.

In order to generate pIE1-E26#3 domain the intermediate recombinant virus clone pUC18/pE26/E263/FLAG-T7 (appendix B30) and the new vector pIE1-MCS were

digested with *Apa*I and *Sac*I and ligated together overnight. The ligated product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clone named pIE1-E26#3 is described in appendix B10.

In order to generate pIE1-E26#1-2 domain the intermediate recombinant virus clone pUC18/pE26/E261-2/FLAG-T7 (appendix B32) and the new vector pIE1-MCS were digested with *Kpn*I and *Sac*I and ligated together overnight. The ligated product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clone named pIE1-E26#1-2 is described in appendix B11.

In order to generate pIE1-E26#2-3 domain the intermediate recombinant virus clone pUC18/pE26/E262-3/FLAG-T7 (appendix B34) and the new vector pIE1-MCS were digested with *Apa*I and *Sac*I and ligated together overnight. The ligated product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clone named pIE1-E26#2-3 is described in appendix B12.

In order to generate pIE1-E26 FL domain the intermediate recombinant virus clone pUC18/pE26/E26 FL/FLAG-T7 (appendix B36) and the new vector pIE1-MCS were digested with *Apa*I and *Sac*I and ligated together overnight. The ligated product was then electrophorated into DH5α cells from which the amplified DNA was isolated and sequence confirmed. The clone named pIE1-E26 FL is described in appendix B13.

1.5 Cloning Strategies for Antibody Production

Table 14
Oligonucleotides used for PCR amplification of the *da26* gene and insertion into pMAL-CRI.

Name	5’ ----Sequence---- 3’
E26-N-HIS-F	CGCAAGCTTCCATCAAATTCACAGAACG
E26-N-HIS-R	GCGAAGCTTTAATAGGCGTTAATATCACTTTG

The *da26* gene was placed in a pMal vector for the expression and purification of the gene in bacteria for the purpose of producing antisera to the gene’s protein product. The pMal-CRI vector was digested with *Bam*HI and *Hind*III for insertion of the *da26* gene,

which was PCR generated with *Bam*HI and *Hind*III restriction sites. The PCR reaction utilized two oligonucleotides: forward, E26-N-HIS-F and reverse, E26-N-HIS-R, see table 14. The DNA was amplified using an initial denaturation step for 4 min at 95 ^{0}C followed by 30 cycles of 30 sec at 95 ^{0}C, 1 min 30 sec at 58 ^{0}C, 45 sec at 72 ^{0}C, and after the thirty cycles for full extension a 10 min period at 72 ^{0}C. The PCR product was digested with *Bam*HI and *Hind*III. The PCR product was ligated into the digested vector and then electrophorated into DH5α cells from which the amplified DNA was isolated. The clone named pMAL-cRI/E26 is described in appendix B3.

1.6 Cloning Strategies for Yeast Two Hybrid Direct Cross Studies

A series of constructs were developed for yeast two hybrid direct cross studies, using the pIE1-E26 regional domain clones as intermediate constructs. These yeast two hybrid clones were developed to investigate the E26 – FP25K interaction previously detected in the yeast system. Each of the regional domains found in pIE1-MCS (appendix B8-13) were digested with *Nco*I and *Sal*I to isolate the regional domain insert. These isolated DNAs were gel purified using Qiagen's QiaQuick kit and ligated into both pACT2 and pAS2-1 (Clonetech Matchmaker System). The pACT2 activation domain vector was digested with *Nco*I and *Xho*I as *Sal*I will ligate to *Xho*I. The pAS2-1 binding domain vector was digested with *Nco*I and *Sal*I. Twelve constructs were made and sequence confirmed and is described in appendix B 59-70. Unfortunately these yeast two hybrid experiments were never performed as other experiments took the project in a different direction. Specifically these constructs were made to identify the regional domain of E26 that is interacting with FP25k, this interaction or lack there of as found from the use of FP25K deletion virus did not alter the ability of E26 to locate to intranuclear microvesicles or interact with membranes. While the interaction with FP25k is intriguing it is outside the primary focus of the project.

2 Recombinant Virus Production

Recombinant virus production followed protocols outlined in Summers and Smith,

1987. In brief, 2 μg of recombinant DNA was co-transfected with 1 μg of Bac Pac 6 DNA into $2.5x10^5$ *Sf*9 cells by a calcium phosphate precipitation technique. The recombinant and viral DNA mixture was added to 750 μl of Grace's medium and 750 μl of transfection buffer (25mM HEPES, pH 7.1, 140 mM NaCl, 125 mM $CaCl_2$). Cells were then incubated in the presence of the DNA/transfection mixture for 4 hours. After 4 hours the DNA/transfection mixture was removed, the transfected cells were rinsed with and grown in fresh TNM-FH + 10% FBS + antibiotics. The transfected cells were incubated 5-7 days at 27 ^{0}C and frequently examined for the appearance of infection, specifically the production of polyhedra. The media containing the newly generated BV was collected and centrifuged to remove any cell debris.

The newly generated BV was purified from the background Bac Pac 6 virus by the plaque assay technique. *Sf*9 cells were seeded into a 60 x 15 mm culture plate at a density of 2.0×10^6 cells per plate and allowed to attach. Ten-fold dilutions were prepared from the transfection media containing the newly produced BV or isolated BV collected from a previous plaque assay. Transfection mixes were diluted from 10^{-3} through 10^{-5} and viruses picked from a plaque were diluted from 10^{-1} through 10^{-2}. The 1ml of diluted virus inoculums were added to each plate and allowed to infect for 1 hour. After an hour the virus inoculum was removed and the virally infected cells were overlaid with 4 mls of 1.5% low melting point (SeaPlaque) agarose made with TMN-FH + FBS. The LMP agarose was allowed to solidify and the plates were incubated in a humidified incubator for 4-7 days at 27 ^{0}C. Plaques were identified in the agarose and cored. The cored plaques were placed in 1 ml of TMN-FH + 10% FBS and the virus was allowed to diffuse into the media. Further plaque assays were performed to further purify the virus, if necessary, or the virus was scaled by passage through *Sf*9 cells.

To confirm the recombinant virus was made correctly viral DNA was isolated and tested for the correct gene insertion with PCR (Invitrogen, 2000). An aliquot, 750 μl, of the first passage of the recombinant virus was microfuged at 5000 rpm for 3 min at room temperature. The viral supernatant was removed and placed in a new microfuge tube where 750 μl of 20% polyethylene glycol (PEG) in 1 M NaCl was added. The mixture

was inverted several times to mix. The solution was then microfuged at max speed for 10 min at room temperature, after which the supernatant was discarded and the pellet was resuspended in 100 μl of disruption buffer (10 mM Tris pH 7.6, 1 mM EDTA, and 0.25% SDS). In order to degrade the viral proteins and expose the viral DNA for PCR, 10 μl of Proteinase K (20mg/ml) was added and the mixture was incubated overnight at 37 ^{0}C. The mixture was then extracted twice with an equal volume of phenol-chloroform (1:1). The upper aqueous phase was precipitated with the addition of 1/10 the total volume of 3 M sodium acetate and 2 volumes of 100% ice cold ethanol. The precipitation was centrifuged at max speed for a minimum of 30 min at 4 ^{0}C. The ethanol was removed and the DNA pellet was resuspended in 10 μl of sterile water.

Once the viral DNA was isolated, the PCR reaction could occur as normal, using 5 μl of the DNA. The two oligonucleotides used in this reaction are critical to correctly test for a properly made recombinant virus, see table 15. The forward oligonucleotide was made to a region upstream from where the recombinant gene was inserted. The reverse oligonucleotide was made to the T7 epitope. Thus, a PCR product of predicted size should occur if the gene was properly inserted into the correct region of the viral genome.

Table 15
Oligonucleotides used for PCR verification of recombinant virus production.

Name	5' ----Sequence---- 3'
EcoRV-For	CCATTGTAATGAGACGCAC
T7 PCR-Rev	CCATTTGCTGACCGCCAG

2.1 Previously Published Recombinant Viruses

Two recombinant viruses, pVLDa26 and vDa26z, produced previously by others were used in these studies. pVLDa26 is an over-expression of the E26 protein by placing a second copy of the gene under the polyhedrin promoter. This virus is reported by Benyia et al, 1998. vDa26z has an in frame insertion of the *lacZ* gene in the *da26*

gene following amino acid #118. This virus was a gift from Lois Miller and is reported by O'Reilly et al, 1990.

3 Antibody Production

The E26 antiserum 7554 was published in Benyia et al., 1998. Briefly, the *da26* gene was placed under the polyhedrin promoter and a recombinant virus for the abundant expression of E26 was produced. Polyhedrin-expressed 26 kDa protein was cut from SDS-PAGE gels, eluted, and used as antigen for preparation of antibody. Rabbits were injected every 28 days, for a total of four injections with antigen (RIBI; Hamilton, MA). To generate high affinity antisera, the second and third injections contained approximately one-half the amount of the first injection, and the final injection contained approximately one-fourth the initial amount of antigen. Ten days after the last injection, sera were collected and titer was evaluated.

This serum was found to have a weak affinity for ODV-E25. To remove this cross-reactivity the *E25 gene* was cloned into pGEX for expression in bacteria. The bacterial expressed E25 was collected and bound to a GST beads and exposed to the E26 serum to bind the epitopes which cross-react. This slurry was then tap spun to pellet the beads in order to remove the 7554 serum. The serum was removed and cross reactivity with E25 was no longer detected by Western blot analysis at 1:500 (appendix A6). This pre-absorbed 7554 serum was used in all cases where 7554 was used in this study at a dilution of 1:20,000 in Western blot analysis.

Bacterial expressed E26 was used to produce antiserum in this study by using the pMAL-cRI/E26 construct (appendix B3). E26 was expressed in bacteria according to the pMAL-cRI vector system (New England Biolabs). The E26 protein (26 kDa) was then purified using maltose resin or was cut from SDS-PAGE gels, eluted, and used as antigen for preparation of antibody. Rabbits were injected every 28 days, for a total of four injections with antigen (RIBI; Hamilton, MA). The initial injection consisted of approximately 200 μg of protein plus adjuvant. To generate high affinity antisera, the second and third injections contained approximately one-half the amount of the first

injection, and the final injection contained approximately one-fourth the initial amount of antigen. Ten days after the last injection, sera were collected and titer was evaluated. Two serums (12499 and 12500) were found to have affinity to E26 as well as a cellular protein of approximately the same size. To remove the cross-reactivity for the cellular protein, *Sf*9 cells were fixed and permeablized for 10 minutes in each of the following solutions with three 5 minute washes of 1x PBS pH 7.2 between each solution: 3.7 % paraformaldehyde, methanol, and 0.5% Triton X-100 in 1x PBS pH 7.2 in a T-25 flask. The 12499 and 12500 antisera were diluted to 1:500 in blocking solution (1x PBS pH 7.2, 3% BSA, 1% Normal Serum typically chicken, filter sterilize) and exposed to the fixed cells for 1 hour. The supernatant was removed and microfuged for 10 minutes at 4 ^{0}C to remove any cell debris and frozen. Cross reactivity with the unknown cellular protein was no longer detected by Western blot analysis at 1:500 (appendix A7). Note this pre-absorbed serum was used in all cases where either 12499 or 12500 were used in this study at a dilution of 1:1,000 in Western blot analysis.

4 Transfection/Transient Expression

Transfections for transient expression of foreign genes in *Sf*9 cells were performed following protocols outlined in Summers and Smith, 1987. In brief, 1-10 μg (typically 2.5 μg) of DNA was transfected into 1.0x10^6 *Sf*9 cells by a calcium phosphate precipitation technique. The DNA was added to 750 μl of Grace's medium and 750 μl of transfection buffer (25mM HEPES, pH 7.1, 140 mM NaCl, 125 mM $CaCl_2$). Cells were then incubated in the presence of the DNA/transfection mixture for 4 hours. After 4 hours the DNA/transfection mixture was removed, the transfected cells were rinsed with and grown in fresh TNM-FH + 10% FBS + antibiotics for the desired time post transfection, typically 48 to 72 hours.

For transfection experiments followed by infection the above protocol was followed allowing the cells to recuperate for 48 hours post transfection before infecting them with E2 wild type virus at an MOI of 20. Infections were allowed to progress for 72 to 96 hours, when the cells were isolated for subsequent experiments.

5 SDS-Page and Western Analysis

SDS-PAGE by Laemmli (1970) was performed using vertical slab gels; a 3% stacking gel was used above a 15% separating gel. Samples were denatured in 4x SDS-PAGE sample buffer for 15 min at 65 ^{0}C. After electrophoresis, SDS-PAGE gels were either Coomassie blue stained, dried and exposed to film, or transferred to PVDF membrane (Immobilon P) using the wet transfer method (Sambrook et al. 1989). Western blot analysis exposed the membrane to 3% Blotto-TTBS (3% nonfat dry milk, 50 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20, pH 7.4) for 1 hour. The membrane was then exposed to primary antibody for 2 hr to overnight. Following exposure to the primary Ab, the membrane was washed 3 times for 5 minutes in TTBS (50 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20, pH 7.4). The membrane was then exposed to a secondary Ab, appropriate to the primary Ab, which has been conjugated to either horseradish peroxidase (ABR) or alkaline phosphatase (Sigma) for 1 hr. The membrane was washed 3 times as before and exposed to either chemilumenscent reagents or color development. In the case of chemilumenscent reagents the membrane was exposed to x-ray film and the film was developed (Ausubel et al., 1992; Harlow and Lane, 1999; Sambrook, Fritsch, and Maniatis, 1989).

6 Immuno-Confocal Microscopy

*Sf*9 cells were seeded into a 6 well tissue culture plate at a density of 1x10^6 cells per well and infected with the appropriate virus at varying MOI (typically MOI 20). After 1 h p.i. the virus containing media was removed and replaced with fresh TMN-FH + 10% FBS + antibiotics. At the desired time post infection, the infected cells were washed from the 6-well plate and collected into a microfuge tube. The cells were microfuged at 2000 rpm for 5 min at 4 ^{0}C, for collection, the supernatant was removed and replaced with Grace's media, and the cell pellet was gently resuspended. The cells were pelleted again, resuspended as before in Grace's media, and then counted. An aliquot of Grace's media containing 2.1x10^5 cells was transferred to a Cytofuge concentrator and allowed to sit undisturbed for 5 min for cell attachment. The media was aspirated from the

concentrator and replaced with approximately 300 µl of 3.7% paraformaldehyde in 1x PBS pH 7.2, for 10 min for fixation. The cells were washed 3 times in 1x PBS pH 7.2 with 3-5 min incubations for each wash. The final wash was removed and the cells were permeablized in room temperature methanol for 10 min. Further permeablization was performed as the methanol was removed and replaced with 0.5% Triton X-100 in 1x PBS pH 7.2 for 10 min. The cells were wash twice as before in 1x PBS (Ausubel et al., 1992; Harlow and Lane, 1999).

Once the cells were fixed and permeablized, they were then exposed to the blocking solution (1x PBS pH 7.2, 3% BSA, 1% Normal Serum typically chicken, filter sterilize) for 1 hr at room temperature with gentle shaking. The blocking solution was removed and replaced with the primary antibody diluted in blocking solution, incubating the cells in primary Ab overnight at 4 ^{0}C. The cells were wash in 1x PBS as stated in the previous paragraph. After the final wash, the cells were exposed to the secondary Ab for 30 min to 1 hr with gentle shaking in the dark at room temperature. Typically, the secondary Ab used in these experiments consists of the Alexa conjugated secondary Ab from Molecular Probes. These antisera were pre-absorbed to fixed and permeablized *Sf*9 cells and frozen at a dilution of 1:1000. The Alexa secondary antibodies were used in these experiments at a final dilution of 1:2000 with blocking solution. The cells were again washed 3 times in 1x PBS as previously described and exposed to diluted DAPI (1:10,000 in 1x PBS pH 7.2) for 5 sec. DAPI is a fluorescent dye that labels DNA. After exposure to DAPI the cells were again washed 3 times in 1X PBS. The Cytofuge concentrator was removed from the slide and the cells were exposed to 15 µl of anti-fade from Dako (Cat S3023). The cells had a number 1.5 cover slip placed over the cells and sealed with nail hardener to prevent desiccation(Ausubel et al., 1992; Harlow and Lane, 1999).

Samples were viewed for up to 1 month from preparation date on a Zeiss Axiovert 135 microscope with a CARV nip-cow spinning disk confocal attachment. Data collection was performed using a Hamamatsu Orca or Orca ER cameras with either

Atto's CARVer or Zeiss' Axiovision software. Deconvolution software, Zeiss KS-400, was utilized on some specific images.

7 In vitro Transcription and Translation

In vitro transcription was performed using Ambion mMessage mMachine high yield capped RNA transcription kit (Cat # 1344). This kit uses RNA polymerase in conjunction with the T7 promoter for RNA production. The protocol accompanying the Ambion kit was followed for the generation of all RNA used for in vitro translation experiments. In brief, the gene of interest was place into a vector containing the T7 promoter. This construct was linearized to increase RNA yield. The transcription reaction was assembled from the following component; 10 μl of NTP/CAP, 2 μl of 10x reaction buffer, 1 μg of linear DNA, and 2 μl of enzyme. The reaction was brought to 20 μl with nuclease-free water. The transcription reaction was incubated for 2 hr at 37 ^{0}C to allow for transcription. After 2 hr the template DNA was removed from the mixture with the addition of DNAse 1, which was incubate for 15 min at 37 ^{0}C. The RNA was precipitated by LiCl method and quantified on an RNA TBE denaturing agarose gel. The RNA was stored in nuclease free water and stored at -80 ^{0}C.

In vitro translation was performed as per Promega's Rabbit Reticulocyte Lysate System instructions. The Promega's Canine Pancreatic Microsomal Membranes were also used in conjunction with the Rabbit Reticulocyte Lysate System in certain experiments. In brief, the RNA was titrated into the Rabbit Reticulocyte Lysate (RRL) system in 25 μl reaction volume. In addition to the RNA and the RRL, an amino acid mixture minus cysteine, ^{35}S Cysteine, and RNAsin were all added to the reaction. These additions add the amino acids required for protein production, a radiolabeled amino acid for identification of the translated protein, and inhibitors of RNases that might degrade the input RNA. Once the optimal quantity of RNA was found for any reaction, future experiments were conducted with that quantity. To determine translation in the presence of membranes, Canine Pancreatic Microsomal Membranes (CPMM) was also added to

the translation mixture. These experiments were Triton-X114 extracted to determine if the translated protein fractionates with the membrane or aqueous fractions.

7.1 Salt Extraction of in vitro Translated Proteins

Salt extraction of in vitro translate proteins followed protocols outlined in Borel and Simon, 1996 with modification from Mothes et al., 1997. In brief a 25 μl in vitro translation reactions translated in the presence of microsomal membranes were overlaid onto a 100 μl sucrose pads (0.5 M sucrose, 120 mM potassium acetate, 3 mM magnesium acetate) and centrifuged at 100,000 RPMs in a Beckman TL-100 ultracentrifuge using a TLA-100 rotor for 5 min at 4 ^{o}C. This was done to remove the soluble proteins and anything not associated with the microsomal membranes. The supernatant generated by this centrifugation was labeled the sucrose supernatant. The sucrose pellet fraction containing the microsomal membranes and associated proteins was resuspended in 25 μl of a low salt/sucrose buffer (250 mM sucrose, 100 mM potassium acetate, 5 mM magnesium acetate, 50 mM HEPES-KOH pH 7.9). This mixture was overlaid on to a 100 μl of a high salt/sucrose pad (250 mM sucrose, 500 mM potassium acetate, 5 mM magnesium acetate, 50 mM HEPES-KOH pH7.9) and centrifuged at 130,000 x g for 10 min at 4 ^{o}C in a TLA 100 rotor. The salt supernatant was collected, which contains membrane associated proteins. The salt pellet fraction was also collected, which contains integral membrane proteins. All volumes were normalized with the low salt/sucrose buffer and an equal volume of 4X SDS-PAGE buffer was added prior to loading on an SDS-PAGE gel.

8 Primer Extension Analysis

Primer extension techniques were used to map transcription initiation (Sambrook et al. 1989). The three oligonucleotide probes shown in the table 16 were 5' end radiolabeled with T4 polynucleotide kinase (Promega). Cellular mRNA, 3-5 μg, was probed against these radiolabeled oligonucleotides and allowed to hybridize. The oligonucleotides were extended with MMLV reverse transcriptase (SuperScript II) in the

presence of RNAsin (40U, Promega) and Actinomycin D (1.5μg, Promega). The extension products were analyzed by denaturing gel electrophoresis (Gel Conditions). The primer extension oligonucleotides were used in sequencing reactions following the Sequenase 2.0 protocol allowing for identification of the transcription initiation.

Table 16
Oligonucleotides used for primer extension experiments for the identification of the *da26* gene promoters.

Name	5' ----Sequence---- 3'
E26 Ext #1	GTCAAACTGTTCACAGAACCGACCGG
E26 Ext #2	CCCCTCTCAAAAAAACCAGGTAAATAATGG
E26 Ext #3	GTAGGCGCGTTAAACAACACGTCG

9 Triton X-114 Detergent Extractions

Phase separation of membrane proteins by Triton X-114 was performed as per Bordier, 1981 and modified by Rosenberg 1996. In brief, the Triton X-114 was prepared by adding 2 ml of Triton X-114 to 100 ml of cold TBS in a graduated cylinder. The detergent-TBS solution was thoroughly mixed and allowed to stand overnight at 37 ^{0}C. The lower layer of approximately 10-20 ml is enriched in detergent. The upper detergent depleted liquid was aspirated away and discarded. The lower layer was adjusted to 100 ml with cold TBS and thoroughly mixed. As before, the detergent-TBS mixture was allowed to stand overnight at 37 ^{0}C. Again, the upper detergent depleted liquid was removed leaving a stock solution of Triton X-114 at 11.4%, which will be used in the experiments that follow (Bordier, 1981; Rosenberg, 1996).

A cell lysate from 5x10^6 cells was prepared by adding 1 ml of the 11.4% Triton X-114 stock prepared above. The mixture was incubated with mild agitation for 1 hr at 4 ^{0}C. Cell debris was removed from the mixture by centrifugation at max speed for 10 min at 4 ^{0}C. The supernatant was removed and loaded directly onto 100 μl of sucrose cushion buffer (6% Sucrose, 0.6% Triton X-114 in TBS). The microfuged tube was placed in a heat block, allowing for the formation of the two phases, detergent and aqueous for 3 min at 37 ^{0}C. The tube was then centrifuged for approximately 5 min for

further separation of the two layers at 400 x g at 37 ^{0}C. The upper aqueous layer was transferred to a new microfuge tube and incubated on ice. The detergent layer was resuspended in 500 µl of cold TBS and incubated on ice. The detergent fraction was then taken through the incubation and centrifugation again at 37 ^{0}C. The new aqueous layer was added to the previous aqueous layer and incubated on ice. The detergent fraction containing the membrane proteins was resuspended in 2 ml of cold TBS and incubated on ice. The combined aqueous fractions were re-extracted by adding 50 µl of 11.4% Triton X-114 stock solution, which was mixed and incubated for 3 min at 37 ^{0}C. After the incubation, the aqueous layers were centrifuged as before. The aqueous phase, containing water-soluble proteins, was carefully removed from the detergent layer and incubated on ice. The detergent and aqueous fractions were run on an SDS-Page gel that was transferred to a PVDF for Western blot analysis, allowing for localization of the protein of interest in the two fractions (Bordier, 1981; Rosenberg, 1996).

10 Palmitoylation

Virally infected cells were metabolically labeled with [^{3}H] palmitate and immuno-precipitated with the E26 antisera 7554 to determine if the E26 protein incorporates the radiolabel and is thus lipid anchored to the membrane. Protocols were followed from Zhang et al., 2003. In brief, 2x10^6 *Sf*9 cells were infected at an MOI of 20 allowing the infection to progress to 34 h p.i. At 34 h p.i. the infected cells were metabolically starved by incubating in Grace's media minus FBS for 1.5 hours. Then 800 µl of Grace's media plus 0.5% FBS and 500 mCi of [^{3}H] palmitate was added to the infected-starved cells for 8 hours. These cells were then collected for immunoprecipitation.

The radiolabeled cells were lysed by exposing them to 700 µl of RIPA buffer (150 mM NaCl, 1% NP40, 0.5% Deoxycholic Acid, 0.1% SDS, 50 mM Tris pH8, 10 µg/ml leupeptin) and agitating for one hour on a rotation spindle. The lysed cells were then centrifuged at 16,000 x g for 10 minutes to remove the insoluble material. Antisera were then added (25 µl); GP64 (pos control), pre-immune (neg control), and E26 7554 and the mixture was incubated for 1 hour at 4 ^{0}C. Next, 50 µl of Immuno Pure Plus (G)

immobilized protein G beads were added and the mixture was again incubated for 1 hour at 4 ^{0}C. The bound protein was then pelleted for collection by centrifugation at 16,000 x g for 10 min. The pelleted beads were washed twice with RIPA buffer with the beads being resuspended in 40 μl of 1x disruption buffer (125 μM Tris-HCL, 1% SDS, 2.5% mercaptoethanol, 10% glycerol, 0.2% bromophenol blue). Proteins liberated from the beads were separated through a 15% SDS-PAGE gel, which was fixed in 25% isopropanol – 10% acetic acid solution for 10 min. An Enhancer solution was added to help with the detection of the tritium label for 30 min according to the manufactures instructions. The film was then dried and exposed to x-ray film.

11 MALDI-TOF Mass Spectrometry

The proteins from a total cell extract of 18 h p.i. *Sf*9 cells were separated on a 15% SDS-PAGE. The separated proteins were stained with Coomassie blue, subjected to in-gel trypsin digestion and then analyzed using matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS (Mortz et al., 1994). The 28 and 26 kDa regions of the gel were excised from the gel and further cut into 8 pieces, 3 pieces in the 28 kDa region and 5 pieces in the 26 kDa region. The separated proteins in these gel fragments were subjected to in-gel trypsin digestion and analyzed by using a matrix assisted laser desorption/ionization time of flight MSMALDI-TOF was performed on an Applied Biosystems Voyager-DE STR MALDI-TOF mass spectrometer. The databases were searched using the Profound (http://prowl.rockefeller.edu/cgi-bin/ProFound) or Mascot (http://www.matrixscience.com/cgi/index.pl?page=../home.html) search engines.

RESULTS

1 Recombinant Virus Production

Through the course of this dissertation a major effort was placed on making recombinant viruses containing truncations/mutations in the *da26* gene, table 17. Three different recombinant strategies were attempted with only limited success. The first recombinant strategy was based on the conclusions of O'Reilly et al., 1990. The conclusions in O'Reilly et al., 1990 were found to be incorrect in the process of this study. They reported that the *da26* gene was not essential, however in their studies they were working with a virus with either an incomplete recombination event or contaminated with a wild type helper virus that was beyond the detectable limits at the time the paper was published. The second recombinant virus strategy used these truncation/mutation regional domains in a non-coding region of the viral genome with 2 of the 4 *da26* promoters. However, an incomplete understanding of the *da26* promoters yielded 12 viruses that do not express recombinant protein. The third recombinant virus strategy did yield a virus used in these studies, however several of the regional domain viruses still failed to express recombinant protein. This resulted in the construction of 6 recombinant viruses with only one used in these studies.

Table 17
Tally of the attempts at regional domain recombinant virus production. Several attempts were made to construct recombinant viruses containing truncations/mutations "regional domains" in the *da26* gene. These attempts yielded few productive results.

Recombinant Virus Strategy	Failed Viruses	Functional Viruses
Strategy 1: Knockout	1	0
Strategy 2: Insertion	12	0
Strategy 3: Polh Promoter	5	1

2 Multiple Forms of BV/ODV E26

Benyia et al., 1998 reported during viral infection that the *da26* gene produces multiple forms of the protein of 34, 28, 26, and 18 kDa sizes as detected by antisera to

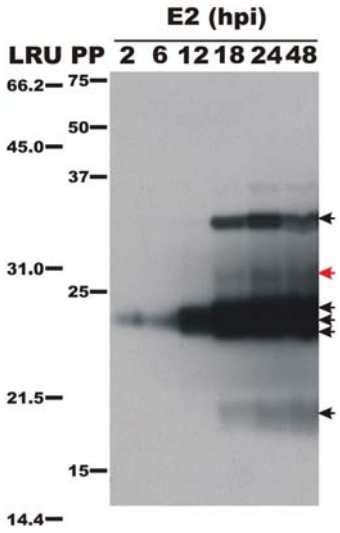


Figure 24. Temporal Time Course Western blot. Western blot of E26 from cell extracts of wild type infected cell extracts (5µg/lane) using pAb E26 7554 (1:20,000). This technique detects as many as four immuno-reactive bands to the E26 antisera at 34 (top arrow), 28 (red arrows), 26 (middle three arrows), and 18 (bottom arrow) kDa. The Western blot was optimized to detect the 28 and 26 kDa forms of E26. The 28 and 26 kDa forms were presented in *Benyia et al. 1998* as the 26 kDa broad diffuse banding pattern resulting from multiple protein species. LRU = Low range unstained marker, PP = Precision prestained marker, E2 = AcNPV E2 strain infected Sf9 cells, hpi = hours post infection. Protein sizes were originally determined by unstained standards, most experiments in this dissertation utilize prestained standards, and as such molecular weights were established and kept for consistency, see appendix A1 for a more detailed explanation.

E26 (7554): they identified 3 forms of E26 migrating at or near 26 kDa. Figure 24 is a similar Western blot to the one presented in Benyia et al., 1998 to confirm these results. Each of these 26 kDa forms was immuno-selected and Benyia found that each immuno-selected sera cross-reacted with each of the other protein forms, thus it was concluded they were the same protein. Through the course of this project an alternative denaturant, DTT, was tested and was found to more fully denature the 3 band smear at 26 kDa into two bands, supporting Benyia et al., 1998 (appendix figures A2 and A3).

To compare the effects of infection and possibly the effects other viral proteins on the production and localization of the different forms of E26, the *da26* gene was placed into the pIE1-4 vector for transient expression of E26 in uninfected cells to analyze the immunoreactivity of the different E26 protein products (pIE1-4/E26, appendix B6). A Western blot analysis of the cells transiently expressing the *da26* gene detects a single 28 kDa band, figure 25 lane Tr Sf9. A 28 kDa form of the protein is detectable during

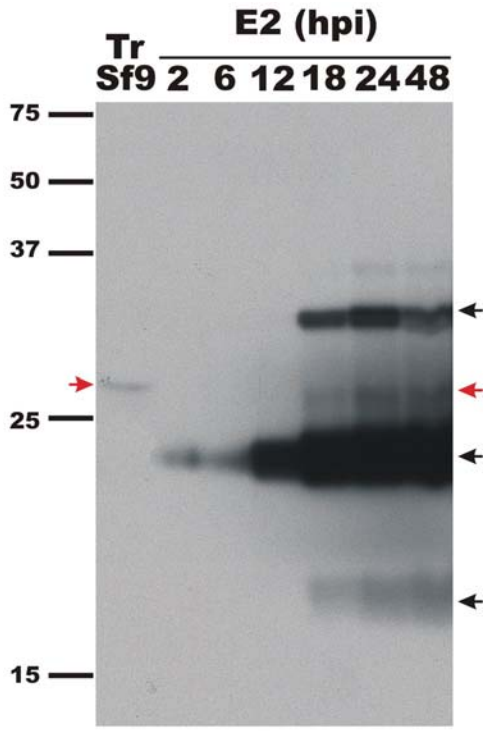


Figure 25. Temporal Time Course Western blot II. Western blot of E26 from cell extracts transiently expressing E26 (20μg/lane) compared to a temporal time course wild type infected cell extracts (5μg/lane) using pAb E26 7554 (1:20,000). This technique detects as many as four immuno-reactive bands to the E26 antisera at 34 (top arrow), 28 (red arrows), 26 (middle two arrows), and 18 (bottom arrow) kDa. The Western blot was optimized to detect the 28 and 26 kDa forms of E26. The 28 and 26 kDa forms were presented in *Benyia et al. 1998* as the 26 kDa broad diffuse banding pattern resulting from multiple protein species. Identification of the transient form as a single 28 kDa species has not been previously identified (red arrow). Tr Sf9 = Transfected Sf9 cells, E2 = AcNPV E2 strain infected Sf9 cells, hpi = hours post infection. Protein sizes were originally determined by unstained standards, most experiments in this dissertation utilize prestained standards, and as such molecular weights were established and kept for consistence, see appendix A1 for a more detailed explanation.

viral infection as early as 18 h p.i., right hand red arrow. The Western blot presented in figure 25 was optimized to detect the 28 and 26 kDa forms of E26.

To confirm that the *da26* gene produces at least two of the multiple forms detected by the 7554 antiserum MALDI-TOF mass spectrometry analysis was performed on an 18 h p.i. E2 infected total cell extract. This analysis was performed by Dr. Bill Russell in the TAMU/Laboratory for Biological Mass Spectrometry (LBMS) under the direction of Dr. Dave Russell. The proteins in this extract were separated on a 15% SDS-PAGE gel allowing two regions to be cut from the gel corresponding to 28 and 26 kDa. These regions were then trypsin digested for analysis by mass spectrometry. Amino acid

Table 18
MALDI-TOF identification of amino acid fragments from an 18 h p.i. cell extract separated on a 15% SDS-PAGE. Two amino acid fragments corresponding to computer predicted trypsin digested fragments of E26 were identified in each of the corresponding molecular weight regions of the SDS-PAGE gel. Total sequence coverage of E26 from these fragments for each individual region was 7%.

Da26 Product	Position	Amino Acid Sequence
28 kDa	66-73	NTHLNFNK
	186-193	VIVYENSR
Total Sequence Coverage	7%	

26 kDa	66-73	NTHLNFNK
	114-122	NISKPAHWK
Total Sequence Coverage	7%	

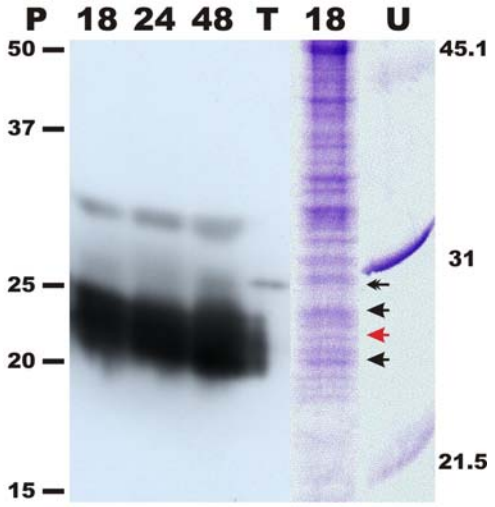


Figure 26. Maldi-TOF analysis of an 18 h p.i. E2 infected total cell extract. Mass spectrometry was performed on an 18 h p.i. E2 infected total cell extract separated on a 15% SDS-PAGE gel (25µg/lane). The double arrow region was excised for the 28 kDa form and the area between the single black arrows was excised and cut into 5 pieces according to the Coomassie labeling. Masses corresponding to E26 amino acid fragments were found in both the 28 and 26 kDa regions of the gel. In the 26 kDa region in the middle band, band 3 as denoted by the red arrow.

fragments were identified with molecular weights corresponding to computer predicted, trypsin digested, E26 amino acid fragments. Two of these E26 amino acid fragments were identified in each the 28 and 26 kDa regions of the SDS-PAGE gel denoted by the double headed arrow and the red arrow, figure 26 and table 18.

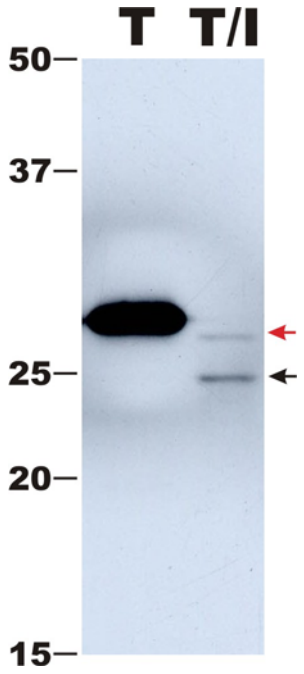


Figure 27. Western blot of E26 from cells transiently expressing E26 and then virally infected. A c-terminal T7 epitope sequence was added to the *e26* gene which was then place under the pIE-1 promoter. This construct was then transiently expressed in uninfected cells producing a single protein of approximately 28 kDa (red arrow) as detected in the total cell extract (10µg/lane). When these cells transiently expressing E26-T7 were then infected and examined by Western blot analysis two protein products were detected by the T7 antiserum at approximately 28 (red arrow) and 26 kDa (black arrow) as detected from total cell extract collected (25µg/lane). T = Transfected Sf9 cell extracts, T/I = Cells transiently expressing E26 that were then wild type infected

To reaffirm the existence of the 28 and 26 kDa species during infection a transient expression followed by infection experiment was performed using pIE1-E26 FL-T7 (pIE1-E26 FL, appendix B13). The T7 epitope with restriction sites used in its addition adds 23 amino acids to the c-terminus of the E26 protein. As seen in figures 25 and 26, the *da26* gene when transiently expressed produces a 28 kDa protein in uninfected cells. The E26-T7 fusion construct when transiently expressed in uninfected *Sf*9 cells also produces a single protein product of approximately 28 kDa, see figure 27 lane T. To confirm the effects of infection on the expression of the *da26* gene, cells transiently expressing the E26-T7 fusion were infected with wild type virus. These cells were transfected with E26-T7, allowed to recover for 48 h post transfection, and then infected with E2 virus. Transfected-infected cells were then collected at 72 h p.i. and prepared for Western blot analysis using the T7 antiserum to detect the fusion proteins produced

by the transient expression/infection process. Notice after infection a protein approximately 2 kDa smaller than the higher molecular weight protein is produced, figure 27. Therefore, viral infection alters the protein produced by the *da26* gene, resulting in the production of 28 and 26 kDa forms. Recall that the two proteins produced are larger than the 28 and 26 kDa forms of native E26 due to the addition of the T7 epitope, which adds 23 amino acids.

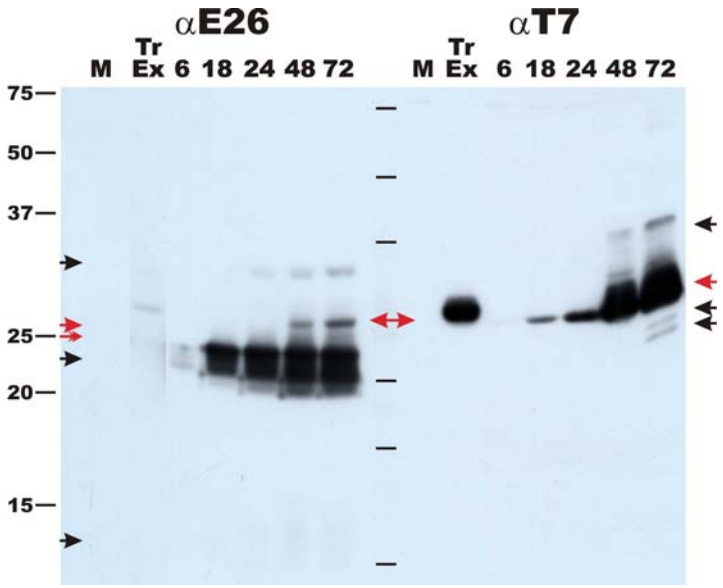


Figure 28. Western blot of total cell extracts from cells infected with polh E26-T7. Western blot comparing the different protein detected by the E26 7554 and T7 antisera. The 7554 antiserum detects the native E26 as well as some slightly higher molecular weight bands corresponding to the detection by the T7 antiserum. The recombinant protein E26-T7 expressed by the polyhedra promoter produces multiple molecular weight proteins as detected by the T7 antiserum. The ~28 kDa molecular weight transiently expressed protein is not detected as strongly by the E26 7554 antiserum as by the T7 antiserum; note the double ended red arrow depicting this band. The double headed arrow depicts the 28 kDa form produced by the native gene and the single headed red arrow depicts the ~28 kDa form produced by the E26-T7 gene placed under the polyhedron promoter. The native gene produces the 34 (top left black arrow), 28 (double headed red arrow), 26 (middle left black arrow), and 18 (lower left black arrow) kDa. The E26-T7 gene produces the ~34 (top right black arrow), ~28 (right red arrow), and ~26 (middle right black arrow) kDa. The 18 kDa form equivalent is not detected. The reason the bands are referred to as approximately X kDa is due to the size increase created by the addition of the T7 epitope.

To further investigate the roll infection plays on the expression of the E26 protein the recombinant virus, polh E26-T7, was created and used to infect *Sf*9 cells for time course analysis by Western blot using T7 antiserum, figure 28. In this virus the T7 epitope adds

15 amino acids to the c-terminus of the E26 protein (pBACgus-E26 FL-T7, B58). Detection of multiple E26-T7 protein products was possible late in infection. Recall that the polh promoter is a late promoter of baculovirus with protein expression beginning at approximately 18 h p.i. The recombinant protein is detected as early as 18 h p.i. and by 48 h p.i. multiple E26-T7 protein forms are detected by the T7 antiserum. When compared to the proteins expressed by *da26* as detected by the E26 7554 antiserum, weak detection of the recombinant E26-T7 protein is noted (large head red arrow, lane 48 and 72 h p.i.), which could be indicate less binding efficiency of the 7554 to the recombinant protein as equal protein amounts were loaded in each lane of the SDS-PAGE gel. Even when the recombinant E26-T7 protein is transiently expressed in uninfected cells only weak detection of the fusion is noted by the 7554 antiserum; however the T7 antiserum strongly detects the recombinant protein as noted when the two Tr Ex lanes are compared. The weak detection of the 28 versus 26 kDa forms is probably due to lower binding efficiencies of the serum; indicate few shared epitope in the polyclonal antisera; or a possible masking of epitopes by altering the protein folding due to the addition of the T7 epitope.

As the *da26* gene produces multiple forms of related proteins during infection and these forms appear to be expressed at later time points of infection; investigation into the promoters used to transcribe the *da26* gene was performed. Due to the fact that the multiple forms of related proteins are detected late in infection the identity of the late promoter responsible for production of the late forms of E26 is critical, as the previously identified *da26* promoter CATTA, 38 nucleotides upstream of the protein's ATG start, was classified as an early promoter. Also this information was needed for production of recombinant viruses developed in an attempt to study the regional domains of E26.

Primer extension was used to precisely locate the 5' end of the transcripts containing the *da26* gene. This analysis used three different reverse strand oligonucleotides to identify promoters at various lengths from the initiation site of the *da26* gene. Four promoters producing transcripts containing the *da26* gene were identified at various lengths from the initiation site including two that are over 780 nucleotides from the *da26*

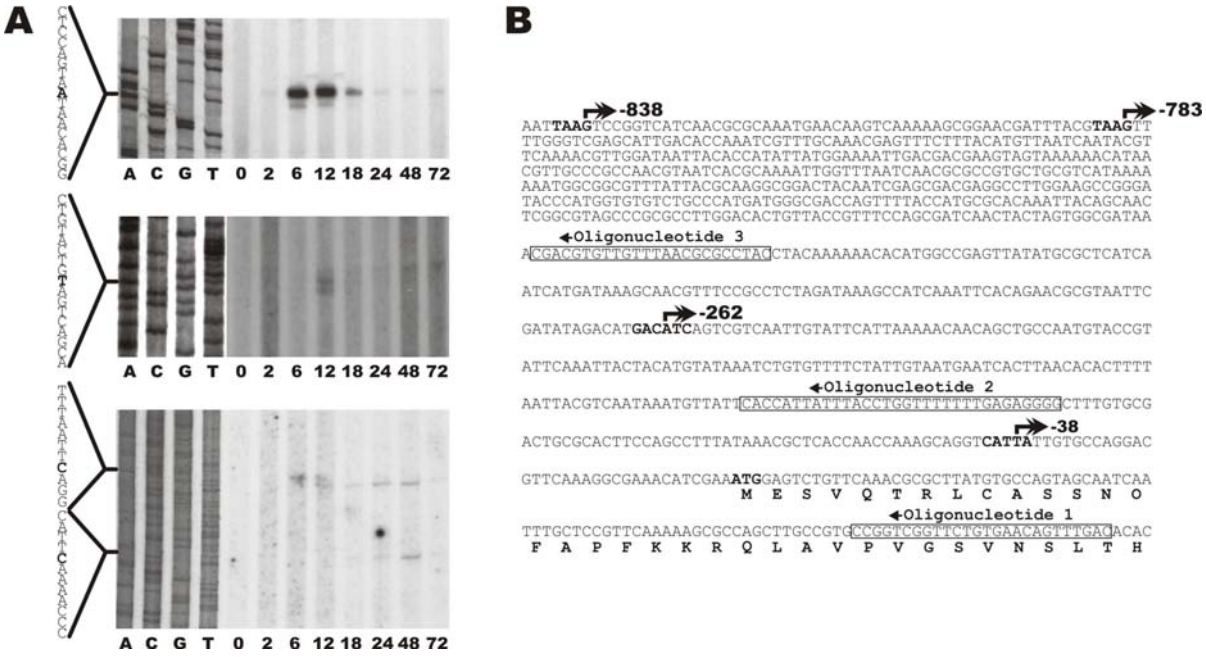


Figure 29. **Primer extension.** Three different reverse strand oligonucleotides were used to identify the 5' ends of transcripts containing the *e26* gene. Four promoters producing transcripts containing the *e26* gene were identified at various lengths from the initiation site including two that are over 780 nucleotides from the *e26* methionine initiation. These are the CATTA promoter, 38 nucleotides upstream of the initiation site; the GACATC promoter, 262 nucleotides upstream; the TAAG promoter, 783 nucleotides upstream; and the TAAG promoter, 838 nucleotides upstream. The *e26* gene is transcribed by the CATTA promoter as early as 2 h p.i. and is maximally produced between 6-12 h p.i. The other three promoters' produces transcripts at much lower level predominately at later times during infection starting at 12 h pi and continuing throughout the 72 h pi time point of this experiment.

methionine initiation, see figure 29. The CATTA promoter, 38 nucleotides upstream of the initiation site, was previously identified by Guarino and Summers, 1988. Three additional promoters were identified for the *da26* gene including GACATC, 262 nucleotides upstream; TAAG, 783 nucleotides upstream; and TAAG, 838 nucleotides upstream. The *da26* gene is transcribed by the CATTA promoter as early as 2 h p.i. and is maximally produced between 6-12 h p.i. The other three promoters' produces transcripts at much lower levels, predominately at later times during infection starting at 12 h pi and continuing throughout the 72 h pi time point of this experiment. Other promoter factors such as cis or trans-acting elements may yet be identified as in an unrelated experiment when the first two promoters (-38 and -262) of the *da26* gene were

shifted to a non-coding region of the viral genome with the *da26* gene, no recombinant protein was produced from a viable infection.

The data presented above addresses the conclusions of Imai et al., 2004 which states that the protein identified as BV/ODV E26 is not encoded by *da26*, but is encoded by another gene. The authors found that the E26 antisera 7554 was unable to detect or weakly detected ORF8, but exhibited a strong cross reactivity with another slightly smaller protein. Recall that *orf8* is a homolog of the *Ac*NPV *da26* gene from *Bm*NPV, which has a 97% identity and a 98% similarity to the *Ac*NPV *da26* gene. The transfection/infection and polhE26-T7 infection studies, figure 27 and 28, using the T7 epitope demonstrate that the *da26* gene does indeed produce at least the 28 and 26 kDa forms of E26 and the mass spectrometry data, figure 26, confirms this finding. To further examine this issue a Western blot analysis was performed to compare the antisera used to detect the ORF8 protein with the E26 antiserum 7554. An antiserum derived from bacterial expressed *da26*, 12500, was also used in this analysis to help explain the Imai et al., 2004 results. The membrane associations of these two forms will be addressed shortly as this point was also questioned in the Imai et al., 2004, publication.

Antibodies produced to protein expressed by the *da26* gene in bacterial cells (αOrf8 and α12500) detect a single protein on a Western blot at approximately 28 kDa (right red arrow figure 30). However, antibodies produced to protein expressed by the *da26* gene in *Ac*NPV infected insect cells (7554) detect the multiple protein forms of *da26* on a Western blot. These multiple forms include 34, 28, 26, and 18 kDa forms (left arrows: black, red, black, black, respectively figure 30). The forms are detected late in infection as opposed to the single 28 kDa form which is detected as early as 6 h p.i. in the Western blot in figure 30. The data presented in figures 27 and 28 demonstrate that viral infection alters the protein expressed by the *da26* gene, therefore protein expressed in the absence of infection does not produce the 26 kDa form, figures 25-28, and sera derived from protein expressed in the absence of infection is unlikely to detect the 26 kDa form. Also of interest the sera derived from bacterially expressed protein are used in a Western blot at a dilution of 1:1000, with sporadic results given at this dilution by

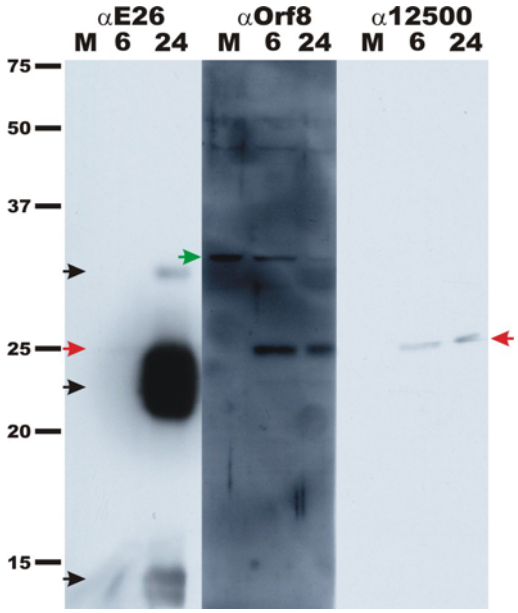


Figure 30. Western blot of total cell extracts comparing αE26 7554, αOrf8, and α12500 antisera. Western blots comparing the different protein detected by antisera produced against virally and bacterially expressed E26. *Ac*NPV E2 produced E26 was used to generate αE26 7554 (1:20,000) in rabbits. Bacterially expressed *Bm*NPV Orf 8 (E26 homology) was used to generate αOrf8 (1:1000) by Imai et al. 2004 in rats and bacterially expressed *Ac*NPV E26 was used to generate α12500 (1:2000) in rabbits. The protein produced in bacteria that were used for antibody production detect a 28 kDa protein (red arrow), however the virally produced protein used for antibody production detects multiple forms at 34 (top black arrow), 28 (red arrow), 26 (middle black arrow), and 18 (lower black arrow) kDa. The green arrow denotes cross reactivity of αOrf8 with some cellular protein as it appears in the uninfected mock lane.

the Orf8 serum. The antiserum produced to protein expressed during infection detects both the 28 and 26 kDa forms and is used at a 1:20,000 dilution. If the sera derived from protein expressed in bacterial has fewer epitopes to the shared domains of the 28 and 26 kDa forms they would be less immuno-reactive to the 26 kDa form than the serum derived from protein collected from infected cells. However, the inverse would not be true as the serum derived from protein collected from infected cells expresses both forms of the protein and is more immuno-reactive as it must be extensively diluted for use.

3 BV/ODV E26 Membrane Association

Previous studies have been conflicting, indicating that E26 is a membrane protein and/or a soluble protein. Imai et al., 2004, state that they were unable to detect ORF8,

an E26 homolog, as an envelope protein of either BV or ODV in *Bm*NPV. In order to address this issue and determine the ability of E26 to associate with membranes several studies were performed, which indicate that the 28 kDa form of E26 is soluble and that the 26 kDa form can be membrane associated. These results are consistent with the Imai et al., 2004 data when you consider that they were unable to detect the 26 kDa form and therefore were only detecting and studying the 28 kDa form.

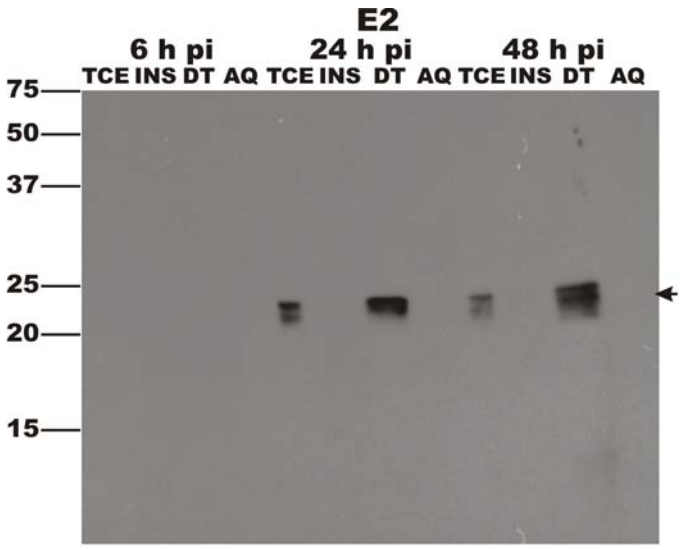


Figure 31. Triton X-114 extraction of infected total cell extracts. E2 infected cells were extracted with triton x-114 detergent to fractionate the proteins into three fractions, insoluble (INS), detergent (DT), and aqueous (AQ). These fractions were then used in a Western blot to determine how E26 fractionates under these conditions. Samples were optimized to view the 28 and 26 kDa proteins detected by the E26 7554 antiserum (1:20,000), however only the 26 kDa form (black arrow). The total cell extracts (TCE) were also placed on these Western blots for a positive control (5μg/lane). E26 fractionates into the detergent fraction of the wild type infected cells and 24 and 48 h p.i.

Wild type infected cells were extracted with triton X-114 detergent to fractionate the cellular proteins into three fractions; insoluble, detergent, and aqueous. This protocol determines if a protein is considered to be a membrane or soluble protein. These fractions were then examined in a Western blot to determine where E26 fractionates with these extraction conditions (figure 31). Samples volumes were loaded on the SDS-PAGE gels to optimize the detection of the 28 and 26 kDa proteins with the E26 7554

antiserum. The total cell extracts (TCE) were used as positive controls. E26 was not detected using these conditions at 6 h p.i. as denoted in the first four lanes of the Western blot shown in figure 31. At 24 and 48 h p.i., the 26 kDa form was detected in the TCE lanes as well as the detergent fraction lanes (DT) but was not detected in the insoluble (INS) or aqueous (AQ) fraction lanes (figure 31). Therefore, the 26 kDa form is membrane associated during and after 24 h p.i.

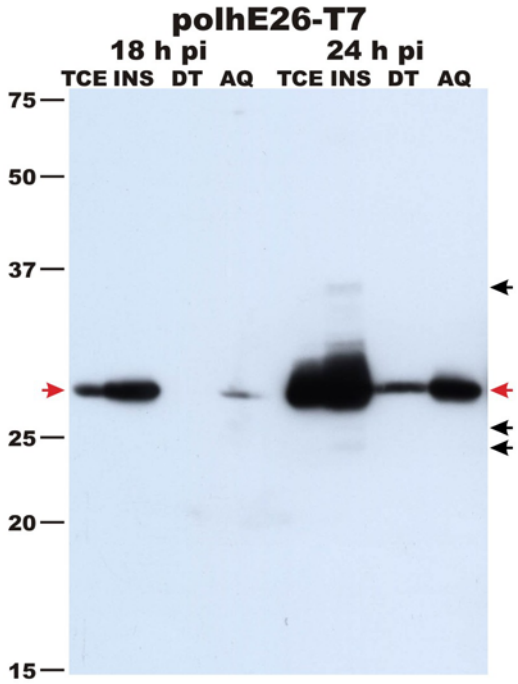


Figure 32. Triton X-114 extraction of polh E26-T7 infected total cell extracts. polh E26-T7 infected cells were extracted with triton x-114 detergent to fractionate the proteins into three fractions, insoluble (INS), detergent (DT), and aqueous (AQ). These fractions were then used in a Western blot to determine how E26 fractionates under these conditions using T7 antiserum. The total cell extracts (TCE) were also placed on these Western blots for a positive control (5µg/lane). The ~28 kDa form of E26 fractionates (left red arrow) predominantly into the insoluble fraction early, 18 h p.i. However, with the progression of infection, 24 h p.i. and beyond, E26 fractionates, into the insoluble, detergent, and aqueous fractions. Specifically the ~28 kDa form (right red arrow) fractionates into the insoluble, detergent, and aqueous fractions. The other forms, ~34 and ~26 are detected at low levels in the insoluble fraction at 24 hpi as denoted by the black arrows.

To further study the membrane association of E26, the recombinant virus polh E26-T7 was constructed placing a second copy of the *da26* gene containing a c-terminal T7 epitope tag under the polh promoter. The T7 epitope adds 15 amino acids to the c-

terminus of the protein (pBACgus-E26 FL-T7, B58). Cells were infected with polh E26-T7, harvested at the corresponding time post infection and extracted with triton X-114 detergent to fractionate the proteins into three fractions; insoluble, detergent, and aqueous. Western blot was then used to determine where E26-T7 fractionates, figure 32. The total cell extracts were used as a positive control. The bulk of E26 fractionates into the insoluble fraction initially after the recombinant protein is expressed at 18 h p.i. with some protein detected in the aqueous fraction. At 24 h p.i. and later, E26 fractionates between the insoluble, detergent, and aqueous fractions. This indicates that the membrane association of the 26 kDa form is a temporal phenomenon and that the membrane association occurs independently from the size differences exhibited by other forms of the protein.

To determine how E26 is interacting with membranes in the absence of a predictable transmembrane segment, several protein modifications known to result in membrane association were examined: these included protein-protein interactions, amphipathic helices, and lipid anchors. Recall that by cross-linking using a BS^3 (a lysine-lysine cross linker) E26 is in close proximity to the INM-SM of E66 in the microsome, E26 contains putative amphipathic helices, and cysteines, which are potential sites for the thioester bonds in palmitoylation. Due to the nature of triton X-114 extractions, only proteins tightly associated with the membrane, integral or lipid anchored, will typically fractionate into the detergent fraction (Bordier, 1981).

Because E26 is tightly associated with the membranes in the triton X-114 extractions, palmitoylation studies were performed to determine if it is lipid anchored to the membrane, figure 33. Cells were infected with wild type virus. At 34 h p.i. the cells were starved and palmitic acid labeled with ^3H added. The cells were then collected and protein immunoprecipitated using the 7554 E26 antiserum. As reported the baculovirus protein gp64 is palmitoylated and therefore was used as a positive control in these experiments, figure 33A (Zhang, Han, and Blissard, 2003). The immunoprecipitation using the E26 antiserum isolated one labeled protein migrating between the 20 and 25 kDa markers, see figure 33B. This band corresponds to the major 26 kDa detected in the

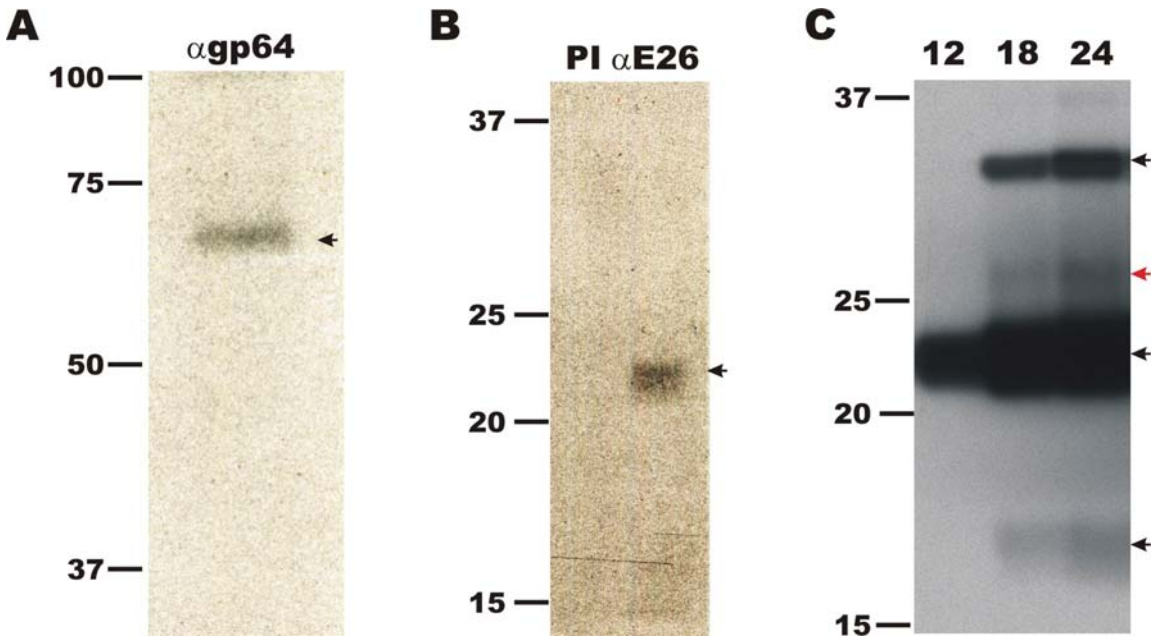


Figure 33. Detection of palmitoylated E26. Cells were infected with wild type virus. At 34 h p.i. the cells were starved and then fed palmitic acid labeled with ^{3}H. The cells were then collected and lysed for use in an immunoprecipitation using the 7554 E26 antiserum. Antibodies to gp64 were used as a positive control, see part A (black arrow denote gp64). The immunoprecipitation using the E26 antiserum isolated one labeled protein migrating between the 20 and 25 kDa markers, see part B, black arrow denote E26 26 kDa form (recall relative mobility difference between prestained and unstained standards). This band corresponds to the major 26 kDa band of E26 detected in the Western blot in part C. Top right black arrow denotes 34 kDa form, red arrow denotes 28 kDa form, middle black arrow denotes 26 kDa form, and the lower black arrow denotes the 18 kDa form.

Western blot in figure 33C. The nature of this type of experiment does not allow for the Western blot to be performed on the same gel as the immunoprecipitation (IPP). This is due to the IPP being labeled with radioactive ^{3}H, which requires extended exposure time (typically greater than 6 months) to identify the radioactive incorporation in to the protein of interest. As palmitoylation of a protein occurs at cysteine amino acids, thus anchoring the protein to the membrane via a lipid anchor, the 5 cysteine amino acids of E26 are suspect, with specific interest focused on the cysteine at amino acid 129. This cysteine is of particular interest as it is surrounded by charges which increase the likelihood that it is palmitoylated. However, any of the cysteines could be palmitoylated during infection. These cysteine amino acids, specifically the three located in the C-terminal region are conserved in the E26 homologues found in other baculoviruses, see figure 34.

Figure 34. Baculovirus E26 homologues and the conserved cysteines. The cysteine amino acids are possible sites for palmitoylation of the E26 protein. Note the high conservation of the cysteines located in the C-terminal portion of the protein.

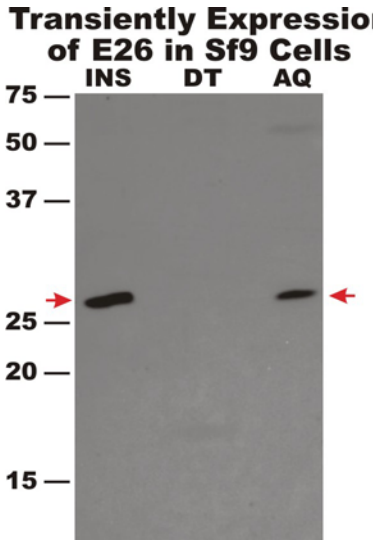


Figure 35. Triton X-114 extraction of uninfected total cell extracts. Uninfected transiently expressing E26 cells were extracted with triton x-114 detergent to fractionate the proteins into three fractions, insoluble (INS), detergent (DT), and aqueous (AQ). In uninfected cells when E26 is transiently expressed it fractionates into the insoluble and aqueous fractions and only produces a 28 kDa protein product (red arrow).

As the membrane association of the 26 kDa form has been found to be palmitoylated during infection, studies to characterize the 28 kDa form were performed to address the issues of membrane association stated in the Imai et al., 2004 paper. Recall that their antiserum derived from bacterially expressed protein only detected the 28 kDa form. I've shown that the 28 kDa form is the only form detected in cells transiently expressing the *da26* gene, figures 25-27, therefore these cells were extracted with triton X-114 detergent to fractionate the proteins into three fractions; insoluble, detergent, and aqueous. These fractions were tested by Western blot to determine the distribution of E26 in these fractions, figure 35. The 28 kDa form was detected in the insoluble and aqueous fractions instead of the detergent fraction as found for the 26 kDa form during infection, figure 35 lanes INS and AQ. Therefore the 28 kDa is a soluble protein in uninfected cells, which is consistent with the results in Imai et al., 2004 as this is the only form they were detecting.

To further understand this, cells were transfected with the *da26-T7* gene and then infected to determine the effects of infection on the membrane association of the 28 kDa

form. The *da26* gene was placed in frame with a T7 epitope tag at the c-terminus of the gene and the fusion was placed under the pIE1 promoter in the vector pIE1-4 (pIE1-E26 FL, B13). The addition of the c-terminal T7 epitope adds 23 amino acids to the E26 protein. This was done to allow for expression of an epitope tagged form of E26 in order to discriminate it from the other forms of E26 produced during infection from the native gene locus. One set of *Sf* 9 cells was transfected with the *da26-T7* gene (pIE1-E26 FL, B13) and tested by Western analysis using antisera to the T7 epitope as a positive control, figure 36 Transient Expression lanes. Another set of cells was transfected with *da26-T7*, allowed to recover for 48 h post transfection, and then infected with E2 virus. Transfected-infected cells were then collected at 72 h p.i. and prepared for Western blot analysis using the T7 antiserum to detect the fusion proteins produced by the transient expression/infection process, figure 36 Transient Expression/Infection lanes. As shown in figure 36 the *da26-T7* gene produces one protein product migrating at approximately 28 kDa and this form fractionates into the insoluble and aqueous fractions consistent with the data in figure 35. However, when *da26-T7* transfected cells were infected with E2 virus 48 h after the cells were transfected and analyzed by Western blot analysis, five protein products were detected in the AQ lane by the T7 antisera including 2 predominant bands at approximately 28 and 26 kDa and as many as 3 minor bands, see figure 36. The 3 minor bands are visible on the original Western blot film and depending on the printing of this reproduction are only slightly visible. The 28 kDa and 26 kDa forms were detectable in the insoluble lane. Therefore the 28 kDa form expressed from *da26* is a soluble protein in infected and uninfected cells and the alteration or expression of the 26 kDa form during infection is independent of its membrane association. This is consistent with a possible post-translational modification of the protein, specifically with palmitoylation which is a reversible post-translational lipid anchor. This observation is consistent with the observations of Imai et al., 2004 in that they were only detecting the 28 kDa soluble form of E26/ORF8 which is why they were unable to identify it as a *Bm*NPV ODV or BV envelope protein.

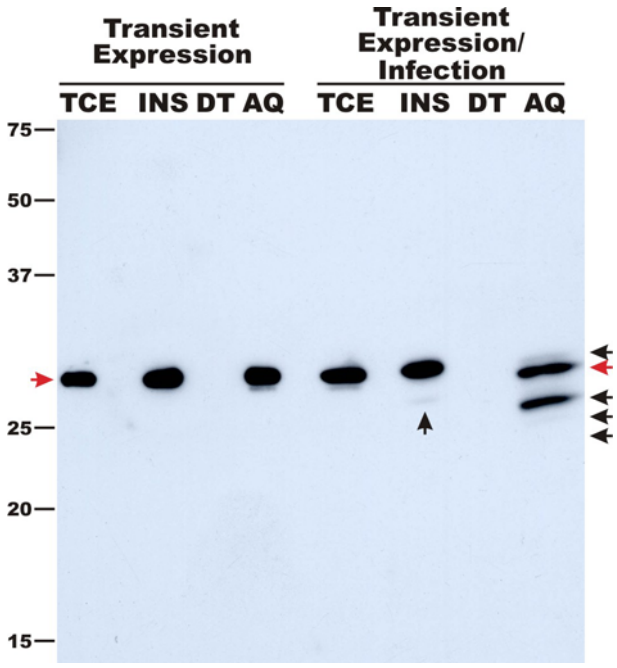


Figure 36. Triton X-114 extraction of cells transiently expressing E26-T7 and cells that are transiently expressing E26-T7 that have then been infected. E26-T7 fractionates as E26 did in the previous figure, 35, in uninfected cells (Red arrow on left). However when the cells that are transiently expressing E26-T7 are infected with wild type virus and then fractionated with triton X-114 multiple forms are detected. Multiple forms of E26 are detected in the aqueous lane indicating that the E26-T7 protein is altered during infection, arrows on right. Red arrow on right denotes 28 kDa, top black arrow is slightly larger than 28 kDa, the lower three black arrows on the right could represent the 26 kDa equivalents. Note the vertical black arrow in the insoluble lane pointing to the 26 kDa equivalent in that lane.

Previous crosslinking studies identified E26 in close proximity to the in vitro translated INM-SM of E66 in wild type infected insect microsomal membranes and in vitro from infected enriched nuclei membranes (Braunagel et al., 2004). Microsomal membranes are prepared from cell extracts and are made predominantly of endoplasmic reticulum. The enriched nuclei factions contain intact nuclei with attached ER and are in vitro translation competent. The INM-SM of E66 contains a transmembrane segment that has been demonstrated to insert into these microsomes in a co-translational translocation fashion (Saksena et al., 2004). The crosslink of the INM-SM and E26 was initially identified by mass spectrometry from in vitro translations performed using the enriched nuclei preparation. This was confirmed by in vitro translating the INM-SM in infected *Sf*9 microsomal membranes, crosslinking (amine-amine), and extracting proteins in close proximity by IPP using sera to the epitope tagged INM-SM. E26 was

identified in these IPP by Western blot analysis using the 7554 E26 antiserum. Because E26 was found to associate with membranes during infection and found in these microsomes a series of in vitro translations were performed to determine if newly produced E26 could associate with microsomal membranes in vitro.

First, E26 RNA was translated in vitro with canine pancreatic microsomal membranes (CPMM) to determine if the protein will associate with the membranes in the absence of the other insect and viral proteins as it is the standard membrane system used in these types of studies. In vitro translations with infected and uninfected *Sf*9 cell microsomal membranes will be presented shortly. The CPMMs were collected from in vitro translated E26 reactions by pelleting them through a sucrose cushion. This allowed the proteins associated with the CPMMs to be salt extracted to determine if in vitro translated E26 associates with the canine membranes. In vitro translations (totals) were placed on the Western blot as a positive control for the in vitro translation of E26 protein. The protein detected in the sucrose supernatant is the soluble form, which is not incorporated into the membranes, note that a single ~28 kDa form of E26 is detected; this could correlate to the same 28 kDa soluble form found in infected and uninfected cells. The sucrose pellet contains the CPMMs and any protein associated with these membranes. Very little E26 is detected in the sucrose pellet fraction. When the CPMMs are exposed to a high salt cushion and centrifuged the integral membrane proteins fractionate with the CPMMs into the salt pellet fraction and the membrane associated protein fractionate into the salt supernatant fraction. The small portion of E26 found to fractionate with the CPMMs after the initial sucrose pellet, which removed the soluble protein, is fractionated into the salt supernatant at barely detectable levels, figure 37. Therefore the ~28 kDa form, produced by in vitro translation of *da26* in the presence of CPMMs is predominantly a soluble protein.

To further the above experiments and determine the effects of cellular and viral integral membrane proteins on the membrane association of E26, in vitro translation studies were performed with uninfected and infected *Sf*9 microsomal membranes (UIMM and IIMM respectively). The *da26* RNA was in vitro translated in the presence

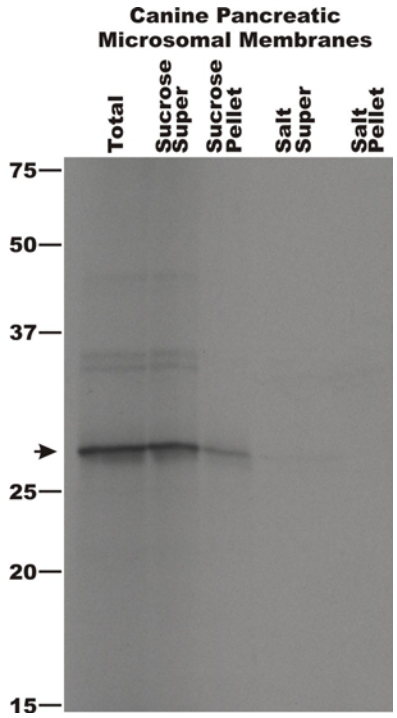


Figure 37. Salt extractions of in vitro translated E26 in the presence of canine pancreatic microsomal membranes. E26 RNA was in vitro translated in the presence of canine pancreatic microsomal membranes and then salt extracted to determine if the newly produced E26 is incorporated into the membranes. The sucrose supernatant is the E26 not incorporated into the membranes (black arrow). The sucrose pellet contains the membrane associated form of E26. When salt is applied the E26 associating with the membranes fractionates into the supernatant fraction, however this is a minimum of the total E26 produced.

of these *Sf*9 microsomal membranes and then salt extracted to determine if the in vitro translated E26 is incorporated into insect membranes in the absence of the soluble cellular and viral components. In vitro translations (totals) were placed on the Western blot as a positive control for the in vitro translation of E26 protein, figure 38 Total. The E26 detected in the sucrose supernatant is the soluble form of the protein, which is not incorporated into the membranes; note that a single ~28 kDa form is detected in the sucrose supernatant of the uninfected microsomal membranes, which could correlate to the same 28 kDa soluble form found in infected and uninfected cells. The sucrose supernatant of the infected microsomal membranes contains 5 other protein bands, 2 of larger molecular weight and 3 of smaller molecular weight. This could indicate that the modification of E26 into its multiple forms is capable of occurring in the presence of infected microsomal membranes and the proteins that associate with them or this could

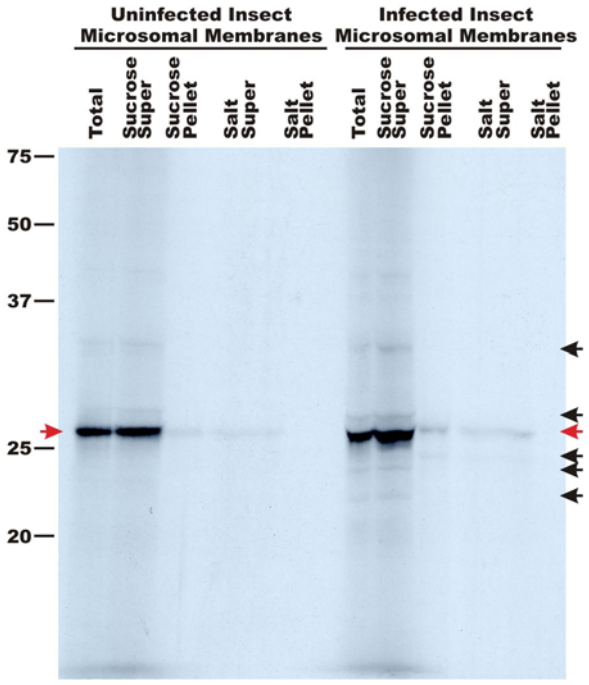


Figure 38. Salt extractions of in vitro translated E26 in the presence of uninfected and infected microsomal membranes. E26 RNA was in vitro translated in the presence of uninfected and infected microsomal membranes and then salt extracted to determine if the in vitro translated E26 is incorporated into the membranes. Salt extraction protocols were followed from Borel and Simon, 1996 with modifications from Mothes et al., 1997. The protein contained in the sucrose supernatant is soluble protein, therefore not incorporated into the membranes and thus removed before salt extracting the remainder. The sucrose pellet contains the forms interacting with membranes. When salt is cushion is applied and centrifuged the membrane associated forms fractionate into the salt supernatant and the integral membrane proteins fractionate into the salt pellet. The E26 associating with the infected microsomal membranes fractionates into the supernatant fraction in the broad band detected above. Slightly more E26 associates with the infected microsomes over the uninfected; however this is a very small portion of the total. Note the appearance of additional forms of E26 in the infected microsomes that are not visible in the uninfected microsomes. Top arrow on right is 34 kDa, red arrow on right is 28 kDa, lower three arrows correspond to the 26 kDa smear.

be partial degradation and random incorporation of the radioactive label that identifies these proteins. The sucrose pellet contains the UIMM and IIMMs in the respective experiments and any protein associated with these membranes. Very little or no E26 is detected in the sucrose pellet fraction of the UIMMs; however a small amount of E26 is detected in the sucrose pellet fraction of the IIMMs. When the UIMM and IIMMs are exposed to a high salt cushion and centrifuged the integral membrane proteins fractionate with the membrane in the salt pellet fraction and the membrane associated protein fractionate into the salt supernatant fraction. As very little or no E26 was detected in the UIMM sucrose pellet, no E26 protein should be identified in either the

salt pellet or the salt supernatant, which is the case and indicates that E26 when in vitro translated in the presence of UIMMs is a soluble protein of ~28 kDa. The small portion of E26 found to fractionate with the IIMMs after the initial sucrose pellet, which removed the soluble protein, is fractionated into the salt supernatant at low levels in the broad band shown in the corresponding lane of figure 38. Therefore ~28 kDa form, expressed by in vitro translation in the presence of UIMMs is a soluble protein. The E26 expressed by in vitro translations in the presence of IIMMs is predominantly soluble with low levels detected peripherally associating with the infected microsomal membranes. The E26 lipid anchor previously identified would not be accountable for this membrane association as it would have caused the lipid anchored protein to fractionate into the salt pellet fraction. These data imply that the soluble viral and possibly soluble cellular components likely have a role in the membrane association of E26.

4 BV/ODV E26 Localization

Deciphering the localization of E26 is important (yet is difficult) as there are multiple forms of E26 detected by the antisera 7554. In an attempt to identify how and when E26 localizes to the nucleus in infected and uninfected cells immuno-confocal microscopy (ICM) was performed. E26 localization during infection has been well studied after 6 h p.i. however the earlier time points during infection have not been examined. An extensive time course analysis of E26 localization as detected by αE26 7554 and ICM detects E26 in punctate regions of the cytoplasm between 1 and 4 h p.i., figure 39 top row of micrographs. By 6 h p.i. E26 is detected in discrete regions in the nuclear periphery, figure 39. In Beniya et al., E26 localized to electron dense regions of the nucleus and cytoplasmic membranes at 6 h p.i. as detected by immuno-electron microscopy (IEM). At this stage of infection the observations made in Benyia et al., 1998 are consistent with the results as are the micrographs presented in Imai et al., 2004. E26 accumulates in discrete nuclear regions between 8 and 12 h p.i. and by 12-24 h p.i. (figure 39) starts to accumulate in regions of the nucleus not labeled by the DNA dye

DAPI. It is at this 12 h p.i. time point where E26 localization presented by Imai et al., 2004 differs from my results and Benyia et al., 1998. During the later stages of infection, 16-72 h p.i., E26 localized to distinct regions peripheral to but in close proximity to formation of the virogenic stroma, figure 39 bottom row. For a better clarification of the distinct regions proximal to the virogenic stroma see figure 40 page 106. The E26 localization correlates with E26 localization by IEM presented by Benyia et al., 1998; to areas enriched in microvesicles, ODV envelopes, and mature ODV.

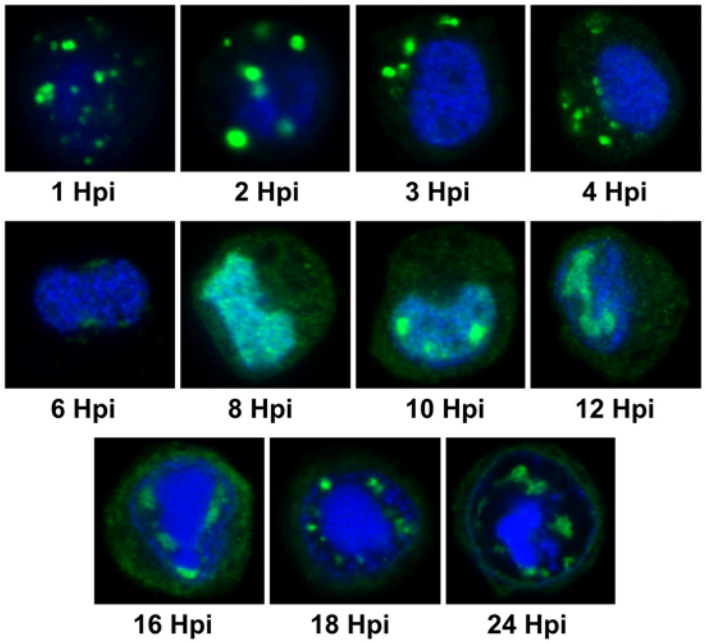


Figure 39. Immunoconfocal micrographs of wild type infected *Sf*9 cells. E26 localization during infection was determined using E26 7554 antiserum to detect E26 in fixed *Sf*9 cells. The E26 antiserum was visualized by an Alexa 488 secondary antibody, which carries a fluorophore when excited, emits detectible light at 488 nm. The emission detected is pseudo-colored green in the above micrographs. The blue color is the cellular and viral DNA as detected by the dye DAPI.

I've shown in figures 35 and 36 (pages 98 and 99, respectively) that when the *da26* gene is transiently expressed in uninfected cells, the 28 kDa form is soluble. ICM was performed to determine where the soluble 28 kDa form localizes in the cell. The 28 kDa form in expressed in cells transiently expressing the *da26* gene localizes to discrete

regions of the nucleus, denoted in green as detected by the E26 antiserum 7554. The INM's location is denoted through the use of an antiserum to a drosophila lamin, ADL 67, depicted in red and DAPI labels the DNA depicted in blue, figure 40. There are no intranuclear microvesicles produced in uninfected cells, so this localization in conjunction with the triton extraction data (figures 35 and 36) supports the conclusion that the nuclear localized 28 kDa form is soluble and yet localizes to discrete nuclear regions. This localization pattern maybe similar to proteins localized at transcription centers. E26 has been suggested to play a role in transcription by Guarino and Summers, 1988 and Imai et al., 2004.

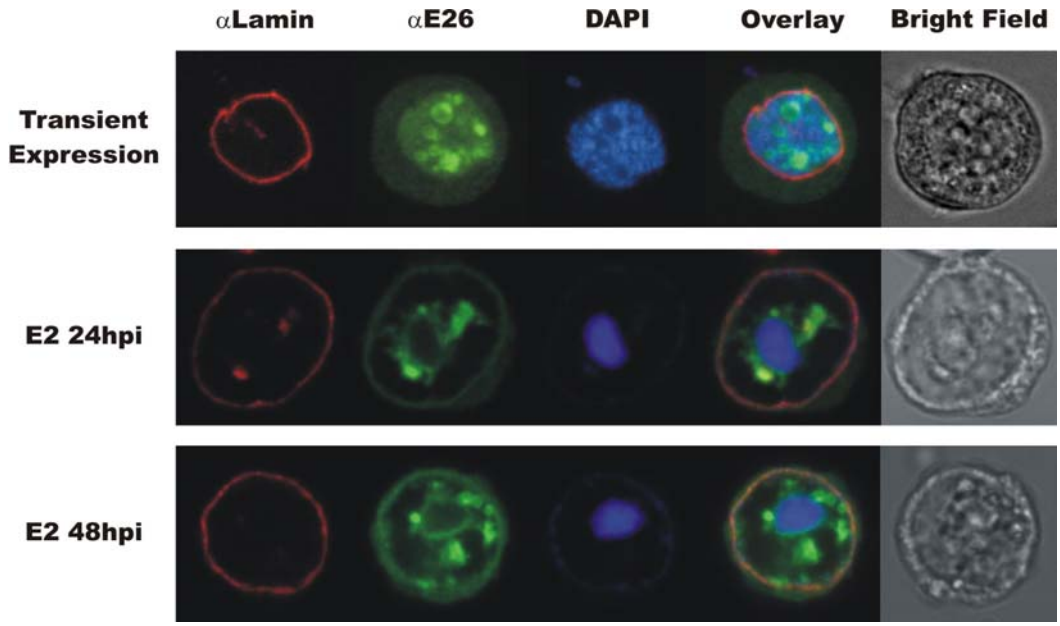


Figure 40. Immunoconfocal micrographs of uninfected cells transiently expressing the *da26* gene and 24 and 48 h p.i. infected *Sf*9 cells. E26 localization during infection was determined using E26 7554 antiserum to detect E26 in fixed *Sf*9 cells. The E26 antiserum was visualized by an Alexa 488 secondary antibody, which carries a fluorophore when excited, emits detectible light at 488 nm. The emission detected is pseudo-colored green in the above micrographs. The red pseudo-color portion of the micrographs is the lamin protein detected by antiserum with a secondary Alexa 594 antiserum. The blue color is the cellular and viral DNA as detected by the dye DAPI; in the infected cells this denotes the virogenic stroma. In uninfected cells E26 localizes to discrete regions of the nucleus.

For comparison, localization of E26 during the later stages of infection was included in figure 40 because confocal microscopy of the cells transiently expressing E26 was also performed on cells 48 h post transfection. Localization of E26 varies when comparing cells transiently expressing E26 to that of infected cells. In infected cells at 24 and 48 h p.i. E26 localizes to the peripheral regions of the virogenic stroma and discrete regions of the nucleus in close proximity to the virogenic stroma, figure 40. To identify the peripheral regions of the virogenic stroma compare the co-localization of E26 and DNA as denoted by the overlap of the green and blue areas. The E26 in these areas co-localize at least partially to areas containing viral DNA. For the localization of the E26 in close proximity to the stroma look at the intense green label in discrete regions of the nuclei. These discrete regions detected during infection can be correlated to regions enriched in microvesicles, ODV envelopes, and ODV by IEM, which are absent in the uninfected cells. E26 found in these regions is likely membrane associated as supported by the IEM in Benyia et al., 1998.

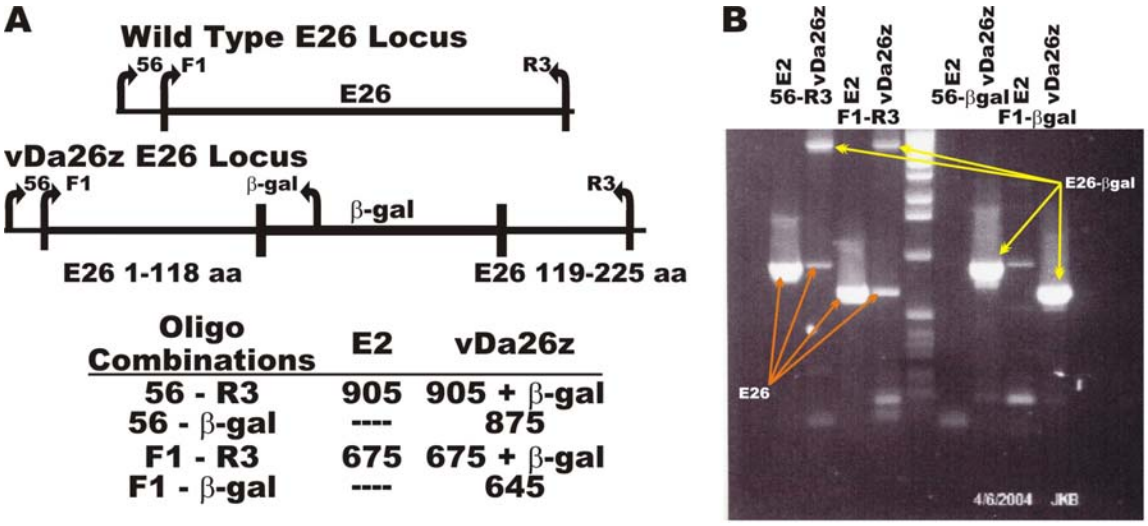


Figure 41. PCR analysis of the recombinant virus vDa26z. Viral DNA was isolated from BV of vDA26z recombinant virus supplied by O'Reilly et al. PCR analysis was then performed to determine if the E26-βgal gene fusion was placed in the correct locus of the genome. Several oligonucleotides combinations were used to determine where the fusion was placed in the viral genome see part A. When oligonucleotides combinations for the full length E26 gene were used two gene products were detected, the native level and the E26-βgal gene fusion, see part B.

O'Reilly et al., 1990 developed a recombinant virus, vDA26z, in which the *da26* gene was reported as truncated by the insertion of the *β-galactosidase* gene after E26 amino acid 118. Upon inspection of the viral DNA by PCR, I determined that a second copy of the *da26* gene existed either through an incomplete recombination event or by a wild type helper virus. After 3 additional rounds of plaque purification in an attempt to eliminate any helper virus, detection of the second copy of the *da26* gene was always present, figure 41.

Detection of the Δ1-118E26-βgal recombinant fusion protein produced from vDa26z virally infected cells by Western blot is possible when using antiserum directed against the β-galactosidase protein, figure 42. When using antiserum for E26 (7554) weak detection of the Δ1-118E26-βgal recombinant fusion protein is possible in a Western blot (data not shown), however detection of full length E26 is readily detected by this serum in the infected cells. This implies that either epitopes to the c-terminal region make up the bulk of those recognized by the 7554 serum and/or that the β-gal addition masks or alters protein folding concealing the epitopes recognized by the 7554 serum. The Δ1-118E26-βgal recombinant fusion protein is missing the c-terminal region of the E26 protein including the region containing three cysteine amino acids, however the n-terminus containing the 2 putative NLSs and amphipathic helix are present. Therefore the recombinant protein's ability to associate with membranes and its' localization were examined by triton X-114 and ICM, respectively. The 24 and 48 h p.i. wild type infected cells that do not produce Δ1-118E26-βgal fusion function as the negative control in figure 42. Δ1-118E26-βgal was found to fractionate almost entirely into the insoluble fraction, figure 42 INS lanes compared to AQ lanes. Also note that two bands were identified by the anitserum to βgal in the 48 hpi AQ lane (far right). This implies Δ1-118E26-βgal is a soluble protein and that it likely interacts with DNA which is a component of the INS fraction by triton X-114 extractions. This further suggests that either the c-terminal portion of E26 maybe responsible for the membrane association of E26 or that the addition of βgal disrupts that membrane association.

Figure 42. Triton X-114 extractions of *Sf*9 cells infected with vDA26z. Cells infected with the vDA26z virus were extracted as before with the triton X-114 detergent to determine where the E26-βgal recombinant protein fractionated to determine if it was capable of membrane association with its' increased size and loss of c-terminal region. The E26-βgal protein fractionated into the insoluble fraction at 24 h p.i. and into the insoluble and aqueous fractions at 48 h p.i. when the cells were extracted with the detergent (black arrows).

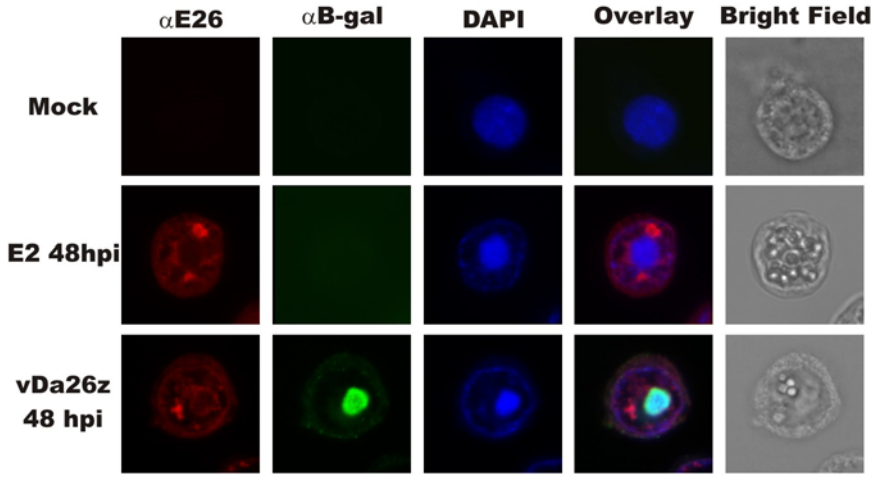


Figure 43. Localization of E26-βgal protein by immunoconfocal analysis of cells infected with vDA26z. *Sf*9 cells infected with vDa26z virus were fixed after 48 h p.i. and prepared for immuno-confocal analysis. The cells were exposed to two different antisera, αE26 7554 (rabbit) and αβ-gal (mouse) antisera. The E26 antiserum was detected with an Alexa 594 secondary against rabbit serum and the β-gal was detected with an Alexa 488 secondary against mouse serum. The E26 protein detected is pseudo-colored red, the E26-βgal protein is pseudo-colored green, and the DNA has been labeled by the dye DAPI. The E26-βgal protein localizes to the virogenic stroma while the full length protein localizes to regions that correlate to microvesicles and the peripheral regions of the virogenic stroma.

ICM was performed on cells infected with vDA26z to confirm the possible DNA association predicted by the triton-X114 extractions and to determine if the large fusion protein was also capable of nuclear localization during infection. Δ1-118E26-βgal localized to the virogenic stroma (blue) inside of the nucleus in the confocal microscopy experiments, figure 43 3rd row in green. Native E26 (red) was readily detected (7554

antiserum) as seen by comparing the αE26 lanes of the E2 and vDa26z infections. This supports the above observation that Δ1-118E26-βgal is soluble and likely associating or interacting with DNA as it co-localizes with the virogenic stroma, the site of viral DNA.

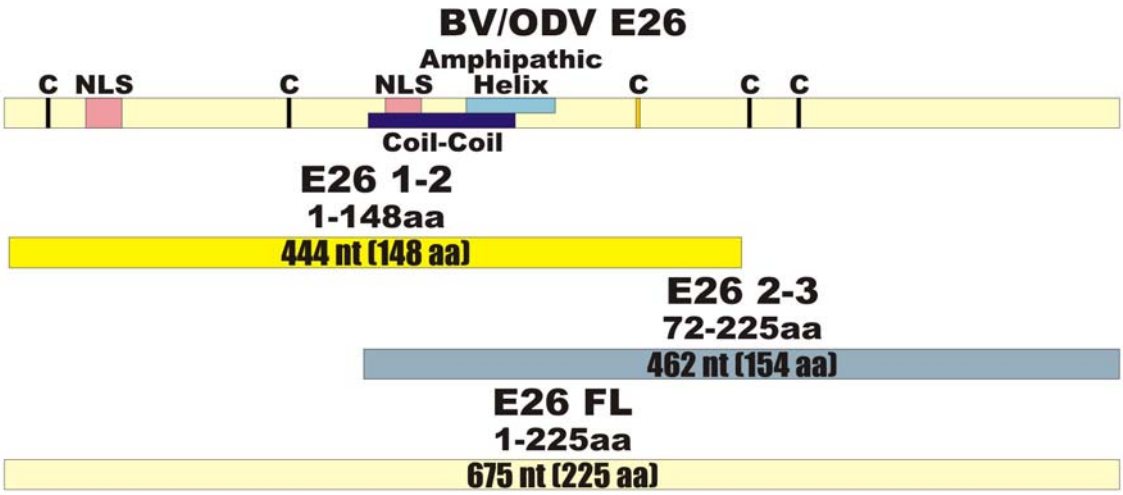


Figure 44. Regional domains used in transfection/infection studies of BV/ODV E26. The *da26* gene was truncated to produce these regional domains. For comparison the full length gene with the putative domains have been included above. The domains include cysteine amino acids (C) possible palmitoylation sites, nuclear localization signal (NLS), coil-coil domains regions of protein-protein interactions, and amphipathic helix possible membrane association domains.

To confirm the observations from the vDa26z virally infected cells suggesting that the c-terminal region of E26 is likely responsible for the membrane association and/or intranuclear microvesicles localization of E26 in wild type infected cells, transfection/infection studies were performed using two of the regional domains of E26, figure 44 (pIE1-E26 #1-2, B11 and pIE1-E26 #2-3, B12). The first two regional domains or N-terminus of E26 are contained in the pIE1-E26 #1-2 clone and account for the first 148 amino acids of the E26 protein plus a 23 amino acid addition adding a T7 epitope to the c-terminus of the truncated protein. The second two regional domains or c-terminus of E26 are contained in the pIE1-E26 #2-3 clone and account for amino acid 71 to 225 of the E26 protein plus a 23 amino acid addition adding a T7 epitope to the c-terminus of the truncated protein. These two truncated proteins expressed by the IE1

promoter were transfected along with a third construct representing the full length *da26* gene, pIE1-E26 FL (B13) into uninfected cells. After a 48 hour post transfection recovery period these cells were infected with wild type virus for 72 h and examined by ICM, figure 45.

The N-terminal region of E26 #1-2 (1-148) co-localized with the DAPI label identifying the virogenic stroma as seen in the first column in the merge of the green and blue colors, figure 45. This localization is consistent with that of vDa26z which comprises amino acids 1-118 in a fusion with βgal. So E26 #1-2 (1-148) protein is unable to interact with the membrane due to missing domains or cysteine amino acids; or, the c-terminal T7 epitope alters folding and the exposure of the membrane association domain; or, E26 localized to the nucleosol and irreversibly bound to DNA making membrane association not possible once the cells were infected.

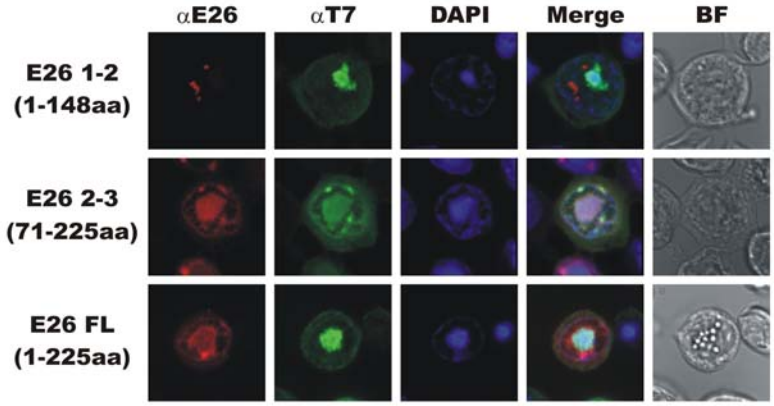


Figure 45. Immunoconfocal micrographs of transfected/infected *Sf* 9 cells expressing epitope tagged; n-terminal 1-148 aa, c-terminal 71-225, or full length E26. The first 148 aa direct the fusion to the virogenic stroma, whereas amino acids 71-225 direct the fusion to the virogenic stroma as well as membrane associations with intranuclear microvesicles. The full length protein appears to be directed only to the virogenic stroma.

The c-terminal region of E26 #2-3 (71-225) co-localized with the DAPI label identifying the virogenic stroma as well as discrete regions inside the nucleus, figure 45 middle row. This is shown the second column in the merge of the green and red colors. This localization is consistent with the localization of E26 in wild type infected cells

with one exception; these cells show increased labeling of the virogenic stroma. So E26 #2-3 (71-225) protein appears to be able to interact with the intranuclear membranes as well as DNA.

The full length construct, E26 FL (1-225), co-localized with the DAPI label identifying the virogenic stroma as seen in the third column in the merge of the green and blue colors. This localization is consistent with transfected E26 localization and vDa26z which is comprised of amino acids 1-118 in a fusion with βgal. So E26 FL (1-225) protein is unable to interact with the membrane due the c-terminal T7 epitope altering protein folding and the exposure of the membrane association domain or E26 efficiently and quickly localized to the nucleosol and bound to DNA making the membrane association not possible once the cells were infected.

This series of experiments demonstrates the importance of the temporal expression of E26 during the proper time points of infections. It adds support to the c-termini containing the membrane association domain or palmitoylated cysteine but with out further study complete conclusions can't be drawn, taken with the vDa26z data it merely allows educated speculation. Additional experiments were planed with the corresponding recombinant regional domain viruses; however these viruses did not produce detectable recombinant protein at the levels required for these experiments.

DISCUSSION

1. *The* da26 *Gene Produces Multiple Protein Products during Infection*

Table 19
Protein products of the *da26* gene. The 28 and 26 kDa forms have been identified during infection by multiple means, including mass spectrometry, epitope tagging with transient expression/infection studies, and with the recombinant virus polh E26-T7.

	Uninfected (kDa)	Infected (kDa)
M_r	28	34, 28, 26, 18

The proteins produced by the *da26* gene, reported in Benyia et al. 1998, include: 34, 28, 26, and 18 kDa forms, table 19. In infected cells, all of these proteins are detected by the E26 antiserum 7554 in a Western blot. The 7554 antiserum contains antibodies to an abundantly expressed E26 protein purified from cells infected with a polyhedrin expressed *da26* recombinant baculovirus. This antiserum has been extensively tested for cross-reactivity to the other known baculovirus proteins. A weak cross-reactivity was found to exist with ODV E25, which was removed by pre-absorption to bacterial expressed E25 which was bound to protein beads. After pre-absorption of the antiserum, detection of E26 by Western blot and confocal microscopy was consistent with the reported expression and localization patterns in Benyia et al., 1998. This study addresses two of these protein products, the 28 and 26 kDa forms.

In uninfected cells only the 28 kDa protein product is detected. The 28 kDa product appears to be the 28 kDa form detected at lower levels during infection, as visualized on the Western blot in figure 24 using the 7554 antiserum. This could be due to lower amounts of the 28 kDa protein product or that the 7554 antiserum exhibits a lower avidity for this form as compared to the 26 kDa form. Note the 26 kDa form is not detected in uninfected cells. MALDI-TOF identified molecular masses of peptide fragments that correspond to those predicted for the 28 and 26 kDa products from an 18 h p.i. cell extract which was separated in a SDS-PAGE, figure 25. In order to confirm the effects of infection on the production of the 26 kDa protein product by the *da26*

gene, uninfected cells which were transiently expressing *da26-T7* were infected and collected for Western blot analysis, figure 26. The T7 epitope was used for detection of the E26-T7 protein in order to differentiate it from the native E26 protein, produced by the virus infection. In the uninfected cells which were transiently expressing the *da26-T7* gene, a single protein product of approximately 28 kDa was detected by the T7 antiserum in a Western blot. When the cells were infected, multiple forms of the E26-T7 protein were detected by the T7 antiserum in a Western blot consisting of two dominate protein products of approximately 28 and 26 kDa and three minor protein products. Infection alters the protein products of the *da26* gene, resulting in the production of a 28 and 26 kDa forms as detected by 7554 antiserum, mass spectrometry of total cell extracts, and epitope tagged forms of the protein using antiserum to those epitopes.

In an attempt to produce a new rabbit polyclonal antiserum to the E26 protein the gene was expressed in bacteria and the protein purified. Rabbits were injected and the blood serum was collected. This serum, 12500, was used in Western blots for detection of E26. 12500, much like the serum produced by Imai et al., detects the 28 kDa form of the protein but not the 26 kDa form that is altered/produced by the baculovirus infection (figure 30). The 7554 antiserum detects both forms as both were present in the infected cell protein products presented to the rabbits for antiserum production. The results produced by Imai et al., 2004 and the 12500 antiserum can be explained because bacterial expression of the *da26* gene produces the 28 kDa form, thus the virally produced 26 kDa form is absent from the antigens presented to the animals for antibody production. If the 28 kDa protein has significantly different epitopes due to altered folding or other secondary modification, it is unlikely the two serums, Orf 8 and 12500, would identify the 26 kDa form.

In order to confirm that the 26 kDa form is derived from the *da26* gene during infection as suggested in the previously mentioned experiments, a polh E26-T7 recombinant virus was produced. In the polh E26-T7 virus the *polyhedrin* gene was replaced with the *da26-T7* gene fusion. Cells were infected with this virus and collected for analysis by Western blot. Total cell extracts from cells transiently expressing the

da26-T7 gene were also placed on this blot. As infection progressed detection of multiple protein products containing the T7 epitope were identified by Western blot, thus confirming that the *da26* gene produces multiple protein products during infection (figure 27). As a side note, it appears that detection of the E26-T7 protein by the 7554 antiserum demonstrates less affinity than detection of E26 protein by the 7554 antiserum. However, the transiently expressed *da26-T7* ~28 kDa form was detected as well as at 48 and 72 h p.i. by the 7554 antiserum, which corresponds to bands detected by the T7 antiserum. The inability to demonstrate an apparent strong antigen binding of the fusion protein could be due to an alteration in the protein folding, exposing different epitopes, or the fact that the 7554 serum has less affinity to the epitopes presented by the 28 kDa form of the protein.

There are no internal methionines present in the E26 protein to function as alternate start sites, ruling out an alternate start site to account for the 2 kDa alteration from 28 to 26 kDa. Splicing events are known to occur in *Ac*NPV, but are rare. Initial genome scans do not predict a splicing event. However, primer extension identifies as many as four promoters for the *da26* gene. Attempts to move two of these promoters with the *da26* gene to a non-coding region of the genome failed to produce any detectible recombinant protein supporting the hypothesis that trans-acting elements required for transcription are not present when the *da26* open reading frame is moved out of context (for this cloning strategy reference appendix B38). Because four promoters were identified for the *da26* gene and only two were used to express a second copy of the gene (due to the extreme distance from the start of *da26*), splicing is still a possibility. The other possibilities include cleavage of a signal sequence, an unidentified post translational modification of the protein, or a hydrophobic addition (lipid anchor) the effects of could allow for the binding of more SDS. This would add further charges to the protein resulting in a faster migration in an electric field. This would result in a decrease in the apparent molecular weight.

2. BV/ODV E26 Exists in Soluble and Peripheral Membrane Protein Forms

Table 20
Triton X-114 fractionation of E26 in uninfected and infected cells. The fractionation of the 26 kDa form is found in the detergent fractions of extracted wild type and polh E26-T7 virally infected cells. A soluble 26 kDa form is identified in the transfection/infection studies confirming that the membrane association is independent of the 2 kDa size modification.

	Uninfected	Infected	Transfection/Infection
Triton X-114 Fractionation	Insoluble & Aqueous (28)	Detergent (26)	Insoluble & Aqueous (28 & 26)

During the different stages of infection, E26 can be detected as either a soluble or membrane protein, table 20. Benyia et al., 1998, reported that E26 was a peripheral membrane protein; however, Imai et al., 2004 reported it to be a soluble protein. This confusion has led to a lot of difficulty in my studies. When triton X-114 extractions were performed on total cell extracts, the 26 kDa form is detected entirely in the detergent fraction by Western blots using antiserum 7554, categorizing it as a membrane protein (figure 30).

To further confirm the detergent fractionation or membrane association of E26, polh E26-T7 infected cells were triton X-114 extracted. The E26-T7 protein fractionated into both the insoluble (60%) and aqueous fractions (40%) by triton X-114 extraction at 18 h p.i. The timing of protein expression is consistent with the temporal patterns for the initiation of *polh* promoter, as this promoter is a late promoter of baculovirus. By 24 h p.i., E26-T7 was detected in the insoluble, detergent, and aqueous fractions, which implies that at least some population of the protein is now membrane associated (figure 31). The ability of the 26 kDa form to interact with membranes could be altered by two different factors; 1) the presence of the T7 epitope which could alter folding or 2) the time of expression of the recombinant E26-T7 protein. Because the recombinant protein is produced by the *polh* promoter, relative to the native E26 expression during normal infection the temporal expression of E26-T7 is altered, which relative to the putative roll of other viral proteins could affect modifications of the 26 kDa form to allow association with membranes.

To gain further insight for the membrane association of E26, its amino acid sequence was closely examined. A predicted trans-membrane sequence (TMS) could not be identified. In the absence of a clearly defined TMS other options were examined including amphipathic helices and lipid anchors. Detection of amphipathic helices traditionally uses circular dichroism spectroscopy, which is outside of my expertise; however a putative amphipathic helix was identified by sequence gazing and the use of a helical wheel between amino acids 94-111. As the E26 protein contains 5 cysteine amino acids, possible palmitoylation sites, palmitoylation experiments were performed to determine if E26 is associating with the membrane via a lipid anchor (Figure 32). E26, specifically the 26 kDa form, was found to be palmitoylated. When the E26 amino acid sequence is compared with the E26 homologues from other sequenced baculoviruses the three c-terminal cysteines are highly conserved, suggesting these as more probable palmitoylation sites. Specifically, the cysteine located at amino acid 129 is most likely the palmitoylated cysteine, as it is surrounded by charged amino acids. These charged amino acids have been shown to lower the pK_a thus increasing the likelihood of the palmitoylation event (Dietrich and Ungermann, 2004; Mossner, Iwai, and Glockshuber, 2000).

When cells transiently expressing the *da26* gene are extracted with triton X-114 and the proteins fractionated into insoluble, detergent, and aqueous fractions, the 28 kDa form was identified in the insoluble and aqueous fractions. These data support the hypothesis that the 28 kDa form is the only detectable product of *da26* in uninfected cells and it is a soluble protein, figure 35. To determine if the process of viral infection affects either the membrane association or change of 28 to 26 kDa, cells transiently expressing E26-T7 were infected and then extracted by triton X-114. The E26-T7 fusion allows one to distinguish the transiently expressed form from the native E26 produced from the virus. The insoluble fraction contained the 28 kDa form of the protein; however the aqueous fraction contained both the 28 and 26 kDa forms. These data imply that the process of infection some how alters the 28 kDa form to become 26 kDa M_r and that this allows for the membrane association. Due to the design of this

experiment, the production of the transiently expressed protein occurs before the cells are infected. Thus, if the 28 kDa protein functions as a DNA transcription binding factor, it likely traffics and binds to DNA before the alteration and change in M_r can occur. Therefore if the alteration and change in M_r still does occur, it would happen in the wrong compartment of the cell making the membrane association impossible. In addition, adding the T7 epitope to the c-terminal region of E26 could alter the ability of the E26 to be palmitoylated. This alteration could cause a change in the protein folding, masking the required cysteine amino acid.

Palmitoylation is one of several ways that E26 could interact with membranes. Another, known, membrane interaction of E26 is via a protein-protein interaction with ODV E66. In Braunagel et al., 2004 the authors were able to crosslink the ODV E66 INM-SM with E26 from infected nuclei and infected microsomes, which implies that these two proteins are in close proximity and may associate in the ER. This interaction could promote palmitoylation of E26 by one of two methods. Either by increasing the stability, thus increasing the accessibility of the sulphydryl group, or by lowering the pK_a of the cysteine, making the formation of the thiolate more likely (Dietrich and Ungermann, 2004; Duncan and Gilman, 1996; Veit, 2000).

Da26 was in vitro translated in the presence of microsomal membranes to determine if the encoded proteins have the ability to interact with the membrane in the absence of the soluble components of the cell. These in vitro translations were then salt extracted to determine the stringency of the membrane association. These translations were performed in the presence of canine pancreatic, uninfected insect, and infected insect microsomal membranes. The canine pancreatic and uninfected insect microsomal membranes should produce the 28 kDa form; whereas the infected insect microsomal membranes should produce both the 28 and 26 kDa forms unless soluble viral factors are required for the alteration and change in the M_r. In all three cases, the proteins produced by the *da26* gene showed minimal interactions with the membrane after all the soluble proteins were removed by centrifugation through a sucrose pad. This soluble protein accounted for 78.3 and 89.9% of the total radio labeled protein produced in the presence

of UIMM and IIMM, respectively as determined by a liquid scintillation counter. The infected insect microsomal membranes appeared to contain the most membrane associated protein produced by the in vitro translations (10.5% of the total radio labeled protein produced). But, the difference between the IIMM and the UIMM is minimal (~5% increase in the IIMM over the UIMM in the salt supernatant fraction). So, in the absence of the soluble components, the membrane association of E26 does not readily occur. Recall that in the E66 INM-SM/E26 crosslinking studies it was the INM-SM that was translated in vitro and that E26 is already associated with the infected microsomal membrane as it would have associated with these microsomes before they were extracted from infected cells.

3. BV/ODV E26 is a Soluble and Peripheral Membrane Protein of the Nucleus

E26 localization during infection has been superficially studied and reported by Benyia et al., 1998, and Imai et al., 2004. A comprehensive study is presented in this document containing a close examination of the early times post infection table 21. BV/ODV E26 localizes in discrete regions of the cytoplasm in the early phases of infection, 1-4 h p.i. By 6 h p.i. E26 localizes in the peripheral regions of the nucleus including electron dense regions inside the nucleus, confirmed by IEM presented in Beniya et al., 1998. E26 continues to accumulate in discrete nuclear regions between 8

Table 21
E26 localization by ICM in uninfected and infected cells. The localization of the E26 protein in uninfected cells is entirely nuclear and soluble. During infection with the modification of the proteins in various forms this localization pattern is more complex, early in infection, to 10 h p.i., the localization agrees with the data presented in both Benyia et al., 1998, and Imai et al., 2004. However, after the 12 h p.i. time point Imai et al., 2004 data varies from the data I have collected and that of Benyia et al., 1998. This is due to the manner in which the antiserum used was produced and its ability to detect the 26 kDa form of the protein.

	Uninfected	Infected – Early h p.i. (1-10 hpi)	Infected – Late h p.i. (12-72 hpi)
Localization	Nuclear (28)	Cytoplasmic and Nuclear regions void of DNA	Nuclear Membrane Associated

and 12 h p.i. and by 12 h p.i. starts to accumulate in nuclear regions absent of the DNA dye DAPI (figure 38). During the later phases of infection, E26 localizes to distinct peripheral regions of the newly forming and later mature virogenic stroma. These peripheral regions have been shown by electron microscopy to be foci of intra-nuclear microvesicles (figure 38 & 39). E26 is present in the nucleus as both a soluble and membrane associated protein depending on the time post infection, supported by this confocal data, triton X-114 extractions, and IEM (Benyia et al., 1998). Nuclear E26 is probably predominantly a soluble protein early in infection and a membrane associated protein late in infection; however quantitative amounts of soluble E26 versus membrane associated E26 are not possible at this time.

When cells transiently expressing the *da26* gene were examined by ICM, E26 was found to localize and accumulate in discrete regions of the nucleus (figure 39). Recall the 28 kDa form of the protein fractionates as both an insoluble (~65% of the total) and soluble protein (~35% of the total) as determined by Western blot of triton X-114 extractions and analysis using the spot densitometer of the Alpha Innotech ChemiImager 4000. The fractionation and localization results could complement each other as the insoluble form could be interacting with DNA (suggested in Imai et al., 2004), due to the fact that DNA fractionates into the insoluble fraction by triton X-114 extractions. The localization pattern of the 28 kDa form in uninfected cells resembles that of E26 at 10 h p.i. in wild type infected cells by the 7554 antiserum. However, as the transiently expressed protein is not detected in association with membranes nor does the uninfected cell produce intra-nuclear microvesicles, a membrane associated form of E26 is not detected in an uninfected cell. The nuclear localization of the 28 kDa form could occur by two different mechanisms: either free diffusion as it is below the size limit of the NPC; or NLS mediated as the N-terminal region of the protein contains two putative NLS, which could traffic this soluble form of the protein into the nucleus. By either mechanism the 28 kDa form of the protein localizes to electron dense regions of the nucleus and potentially interacts with DNA.

Unpublished data collected by a previous member of the Summers' lab from a yeast two-hybrid library screen (data not shown) predicts an interaction between E26 and IE-1. This interaction provides some evidence in support of the nuclear localization of the 28 kDa form and its association with DNA as the IE-1 protein is involved in viral transcription and therefore required in the nucleus. The E26 protein, as translated in yeast from the *da26* gene, was used as bait and the IE-1 protein was found from infected cell libraries. This putative interaction was confirmed in immunoprecipitations in the Summers' lab (unpublished data) and by Imai et al., 2004. As Imai et al., 2004 reported a DNA interaction with E26, and IE-1 is a transcriptional activator, the 28 kDa form likely plays a role in transcription as previously suggested in Guarino and Summers, 1998.

During infection the 26 kDa form is detected in association with membranes by triton X-114, palmitoylation, and cross links to INM-SM of E66 in microsomes. The 26 kDa form is incorporated into mature ODV envelopes and present in intra-nuclear microsomes (Beniya, Braunagel, and Summers, 1998; Braunagel et al., 2004). Protein palmitoylation predominantly occurs in, but is not limited to the ER and is reversible, allowing times of solubility and membrane association (Dietrich and Ungermann, 2004; Linder and Deschenes, 2003). The cross-linking studies identifying E26 used microsomes, which are predominantly composed of ER membranes. These data support, for a population of the E26 protein, the hypothesis that the membrane association of the 26 kDa form likely occurs in the ER and then traffics to the nucleus, similar to but clearly different from E66. This membrane associated form can be clearly localized inside the nucleus as early as 16 h p.i. by confocal and electron microscopy (Beniya, Braunagel, and Summers, 1998). The membrane association could be a result of the putative amphipathic helix or the palmitoylation event or both as a membrane association increases the likelihood of a lipid anchor modification via palmitoylation. As palmitoylation is the strongest membrane association identified for the protein, further examination of the cysteine amino acids is important. To examine the possible functions of 3 c-terminal cysteines, the virus vDa26z presented in O'Reilly et al. was

examined. This virus contains a truncation of the E26 protein at amino acid 118, removing the 3 c-terminal cysteine amino acids. However, recall that the authors could not purify this virus to homogeneity so antiserum to β-gal was used to study the fusion protein. This fusion protein without the c-terminal region fractionates into insoluble and aqueous fractions by triton X-114 extractions classifying it as a soluble protein. When its localization is examined by immuno-confocal microscopy it clearly co-localizes with the mature virogenic stroma. Together these data support a DNA interaction with the fusion protein and a NLS mediated trafficking event due to the extreme size of the fusion protein. This data lends support to the importance of the C-terminal region to the membrane association of E26. Further studies into this region specifically mutating each cysteine residue should be performed starting with the cysteine at amino acid 129, due to its surround charged amino acids.

Suraj Saksena, a fellow graduate student in the lab, studying INM protein integration at the translocon and sorting to the nucleus is the individual who confirmed the interaction of the INM-SM of E66 with E26. In the manuscript identifying this interaction, a postulated function for E26 was presented suggesting that it could act as a sorting factor for E66 (Braunagel et al., 2004; Saksena et al., 2004). Suraj identified two cellular proteins termed SMAP 10 and SMAP 25 that crosslinked with in vitro translated INM-SM protein in uninfected and canine pancreatic cells. Reviewing Suraj's data, I suggested that the SMAP proteins could be cellular homologs of E26 as the virus induces an amplified pulse of INM proteins to the nucleus. Thus it stands to reason that the infected cell would require additional sorting factors beyond those supplied by the cell. Knowing that the 12499 and 12500 antisera identified cellular proteins of similar size before pre-absorption, I suggested that these sera be used in an attempt to identify the SMAPs. Cross reactivity between the SMAPs and the 12499 and 12500 antisera was successful in uninfected *Sf*9 and canine pancreatic cells. A probable function of the 26 kDa membrane associated form is disclosed by this cross reactivity. In Suraj's experiments using the 12499 and 12500 antisera the SMAP 10 gene was identified from a cDNA *Sf*9 library and the translated amino acid sequence was found to have a high

degree of similarity with importin-α. Recently membrane association of importin-α has been identified, which suggests it likely has other roles beyond importing proteins through the nuclear pore via NLS sequences (Hachet et al., 2004). Suraj went on to find that antiserum directed against importin-α, specifically an antiserum to the region of similarity between importin-α and SMAP 10 cross reacted with SMAP 10. Thus the current hypothesis is that SMAP 10 is a translocon associated protein, an early sorting factor for INM proteins, and likely an importin-α isoform. Thus the 26 kDa membrane associated form by similarity likely shares these functions as antiserum directed against E26 initially identified these findings (Personal communication and collaboration with Suraj Saksena).

SUMMARY

1. *The Model: BV/ODV E26 Functions as Both a Soluble and a Membrane Protein during Infection*

E26 has at least two roles during infection which correspond to the stage of infection, summary figure 46. Early during infection the protein has one role as a soluble protein and late in infection a population of E26 becomes a membrane associated protein a second role occurs. That is not to say that the two roles are exclusive of each other, they do appear to overlap in the time course of infection. The membrane association of E26 is likely mediated and regulated by other viral proteins or maturation events, as E26 only associates with membranes during infection.

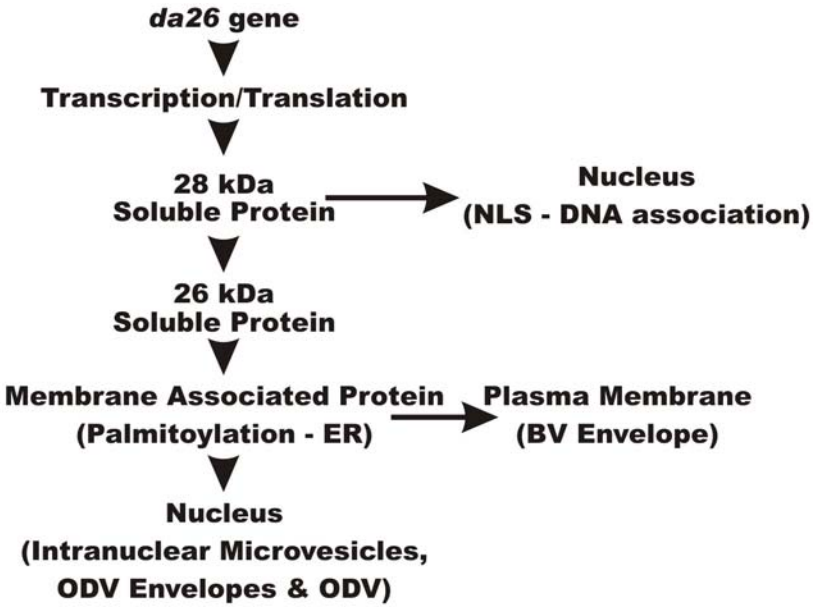


Figure 46. E26 production, modification, and localization during infection. The gene is transcribed and translated producing the 28 kDa form, which is either or both, efficiently modified into the 26 kDa form or shuttled to the nucleus (NLS) to interact with DNA. The 26 kDa form then associates with membranes, likely the ER by lipid anchor (palmitoylation). From the ER the 26 kDa form traffics to the nucleus where it is incorporated into ODV. As a side note the 26 kDa form is also incorporated in the BV envelope at the plasma membrane.

Early in infection, 1-10 h p.i., E26 is produced from the promoters identified previously at -38 amino acids from the methionine (Guarino and Summers, 1986; O'Reilly et al., 1990). The 28 kDa protein contains putative NLS, which traffic it to the nucleus where it probably interacts with both IE-1 and DNA. This is supported by Imai et al., the transient expression of the gene in uninfected cells, and the polh E26-T7 recombinant virus data I've presented. Specifically, that 28 kDa form is a soluble protein localizing to discrete nuclear regions similar to transcription sites. The IE-1 and DNA interactions could occur in the electron dense regions detected in immuno-electron microscopy at 6 h p.i. (Beniya, Braunagel, and Summers, 1998). Immuno-confocal microscopy using both 7554 and Orf 8 antisera detected similar localization patterns for the forms E26 that these antiserum detect through 10 h p.i., which could indicate the switch from the early soluble role to the late membrane associated role.

By 6 h p.i. the 26 kDa form is readily detected on Western blots using the 7554 antiserum. The post-translational modification of the 28 kDa form to the 26 kDa form occurs independently from the 26 kDa forms membrane association as demonstrated by the cells transiently expressing the *da26-T7* gene followed by viral infection. The size change occurs followed by the membrane association only if the protein expression and change in size corresponds to the proper temporal time point, implying that other viral factors are involved. The timing of this change in size and the membrane association of E26 is supported by 6 h p.i. immuno-electron microscopy which detects E26 at the plasma membrane and cytoplasmic vesicles (Beniya, Braunagel, and Summers, 1998).

By 12 h p.i., E26 is membrane associated, however the soluble form is present through out infection at very low levels as indicated by Western blot analysis. This is supported by immuno-confocal microscopy which identifies E26 in the peripheral edges of the virogenic stroma, a region enriched in DNA and the presence of the 28 kDa form on Western blots of late and very late total cell extracts. The localization to the peripheral regions of the virogenic stroma corresponds to the localization detected by Orf 8 antiserum. E26 begins to accumulate in discrete nuclear regions where there is no detectable DNA as determined by the DNA dye DAPI at 12 h p.i. By 16 hpi these

regions can be co-localized to regions containing microvesicles by IEM (Beniya, Braunagel, and Summers, 1998). E26 is found in microvesicles, ODV envelopes, nuclear envelope, the periphery of the virogenic stroma, and at very low levels in the cytoplasm at 18 – 72 h p.i. (Beniya, Braunagel, and Summers, 1998). The 26 kDa form is detected as early as 4 h p.i. and was confirmed present by Maldi-TOF in 18 h p.i. total cell extracts. Palmitoylated E26 was detected at plus 34 h p.i. (34 h p.i. infection with a 1.5 hour starvation period and an 8 hour labeling period), correlates to a time when E26 is found to be membrane associated. Also, when the ODV E66 INM-SM was in vitro translated in the presence of these membranes, it was found to crosslink to E26. These crosslinking studies were performed using 33 hour infected nuclei or microsomes, also correlating to a time when E26 is membrane associated. The interaction between E26 and E66 INM-SM along with the charges surrounding the cysteine at amino acid 129, make it a likely location for a lipid addition via palmitoylation. The membrane association occurring later in infection corresponds to the data collected from cells infected with polh E26-T7 as detected in triton X-114 extractions. Recall the recombinant protein is initially detected in the insoluble and aqueous fractions at 18 h p.i., however by 24 h p.i. E26-T7 is detected in the insoluble, detergent, and aqueous fractions.

Therefore the *da26* gene produces at least 2 protein products with different functions, which correlate with localization, solubility, membrane association, and temporal implications. The 28 kDa form is likely a soluble protein that interacts with transcriptional activators and DNA in the nucleus in the early stages of infection. Where as, a part of the 26 kDa population is a membrane bound form that interacts with an integral membrane protein in the ER, likely functions as a sorting factor directing INM proteins from the translocon to the INM, and found in the inner nuclear membrane, intra-nuclear microvesicles, ODV envelopes, and ODV in the nucleus.

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APPENDIX A

PRELIMINARY OR INCOMPLETE DATA

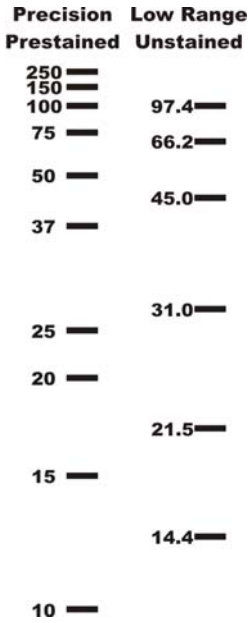


Figure A1. Molecular weight standards for SDS-PAGE. E26 protein sizes were originally determined by unstained standards, most experiments in this dissertation utilize prestained standards, and as such molecular weights for the various forms were established and kept for consistency. Note the variation in the 31 to 14 kDa region of the two standards.

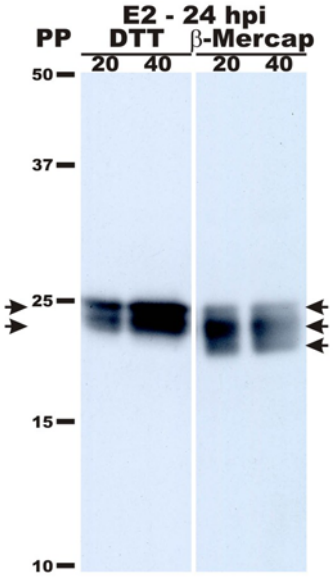


Figure A2. DTT vs β-Mercaptoethanol as a protein denaturant. β-Mercaptoethanol does not denature the 26 kDa forms of E26 as effectively as 100 mM DTT. When DTT is used the 3 band smear identified in Benyia et al., 1998 resolves to two bands. Samples above are 24 h p.i. wild type infected cell extracts loaded at 20 and 40 μg of total protein per lane as determined by Bradford's assay.

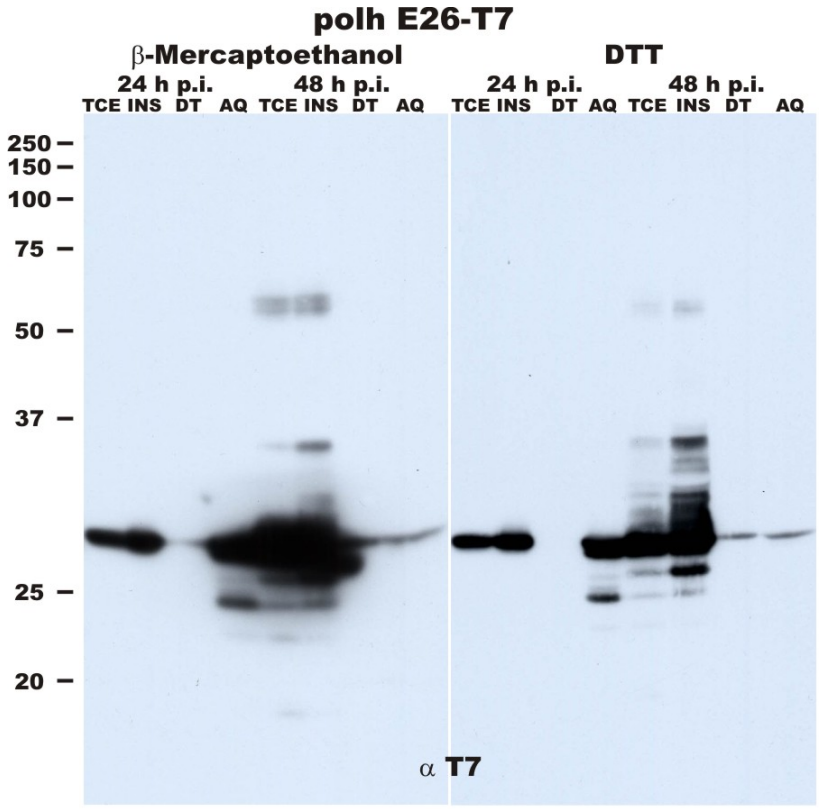


Figure A3. DTT vs β-Mercaptoethanol as a protein denaturant. β-Mercaptoethanol does not denature the protein produced by the *da26-T7* gene as effectively as 125 mM DTT. When DTT is used smearing results, however when DTT is used the proteins are more fully denatured resulting in greater resolution. Samples above in the 24 and 48 h p.i. polh E26-T7 infected total cell extracts (TCE) lanes are loaded at 25 μg of total protein per lane as determined by Bradford's assay and the triton X-114 extractions were loaded by equal volume. Protein is detected by the T7 antiserum (1:8,000). INS = Insoluble fraction, DT = Detergent fraction, and AQ = Aqueous fraction.

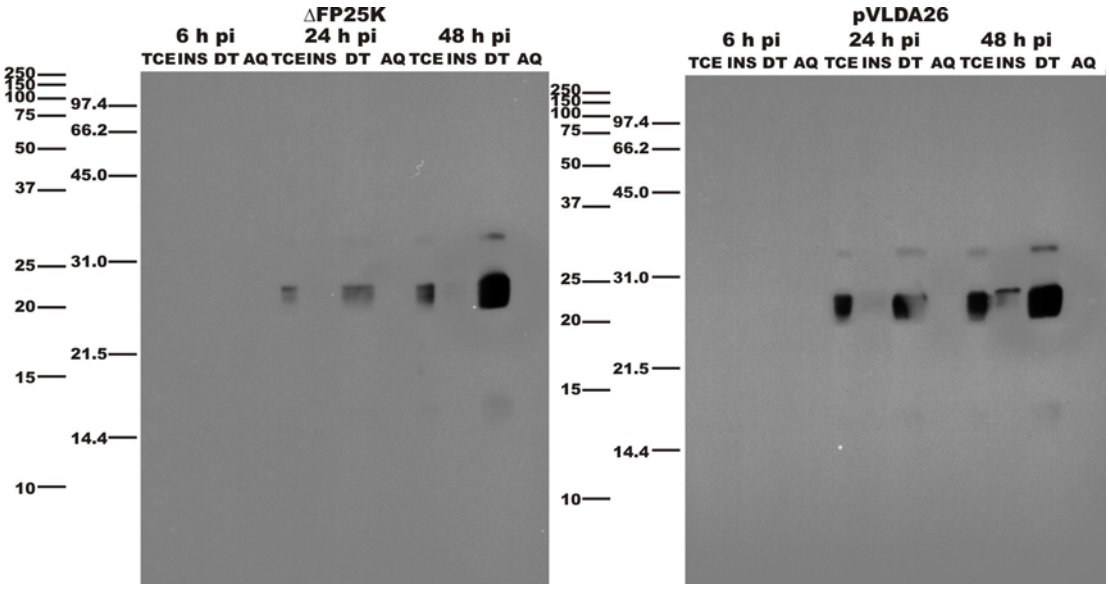


Figure A4. Triton X-114 extraction of E26 from two different recombinant viruses. Cells infected with ΔFP25K and pVLDA26 viruses were collected and extracted by triton X-114 at various time points post infection. Note that E26, including the 34, 28, 26, and 18 kDa forms are detected in the detergent fractions at 48 h p.i. in both recombinant viruses. Specifically note that a ~28 kDa band is detected in the insoluble fraction of the pVLDA26 virus extraction. ΔFP25K virus has had the *fp25k* gene removed, which alters E66 localization. The pVLDA26 virus is a recombinant virus where a second copy of the *da26* gene was placed under the control of the polh promoter, resulting in higher than normal expression of E26. Total protein loaded in the TCE lanes was 10 μg as determined by Braford's assay and the triton X-114 extractions were loaded by equal volume. Protein is detected by the 7554 antiserum (1:20,000). INS = Insoluble fraction, DT = Detergent fraction, and AQ = Aqueous fraction.

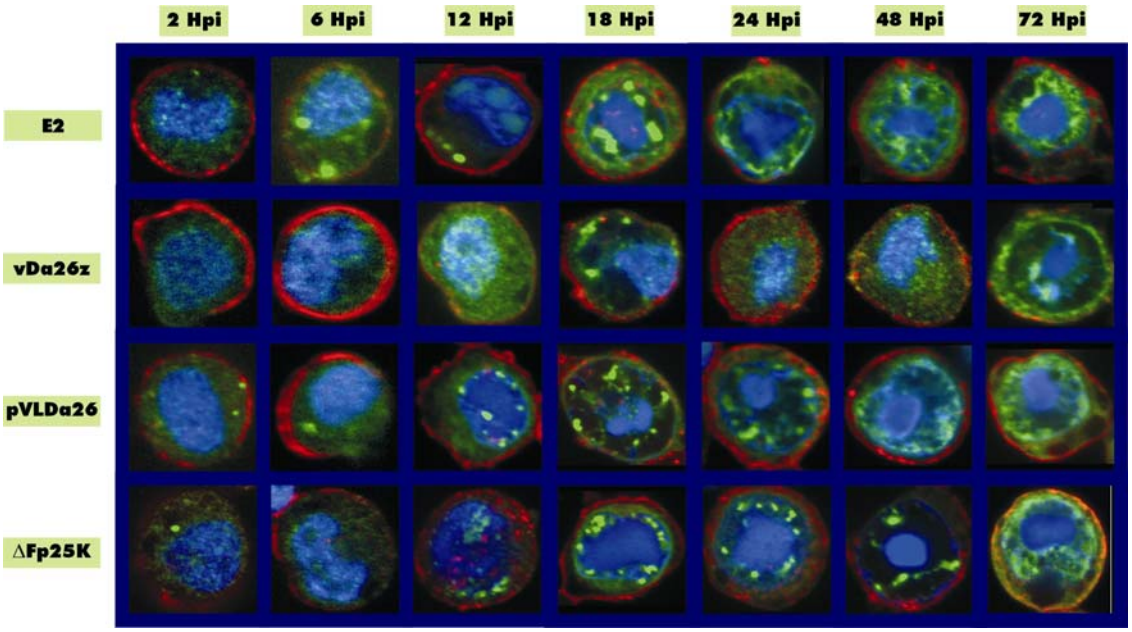


Figure A5. Immuno-confocal micrographs of E26 localization throughout infection from several viruses. E26 localization as detected by the 7554 antiserum (1:1000) in wild type (E2), vDa26z, pVLDa26, and ΔFP25K. Recall that the vDa26z virus is the virus made in error and presented in O'Reilly et al., 1990, the 7554 antiserum would be detecting the E26 protein produced by the intact gene in this virus as such its' localization is not distinguishable from wild type throughout infection. The pVLDa26 virus contains a second copy of the *da26* gene under the control of the polh promoter. IEM detects a hyper-condensation of the virogenic stroma and while this structure does appear smaller in these micrographs correlation is not possible in these ICMs. ΔFP25K virus has had the *fp25k* gene removed, which alters E66 localization. E26 localization in this virus is not distinguishable from wild type throughout infection. The red color denotes cellular actin as detected by αactin mAb C6 (Sigma)(1:500) the green denotes E26 as detected by 7554, and the blue denotes DNA as labeled by the dye DAPI.

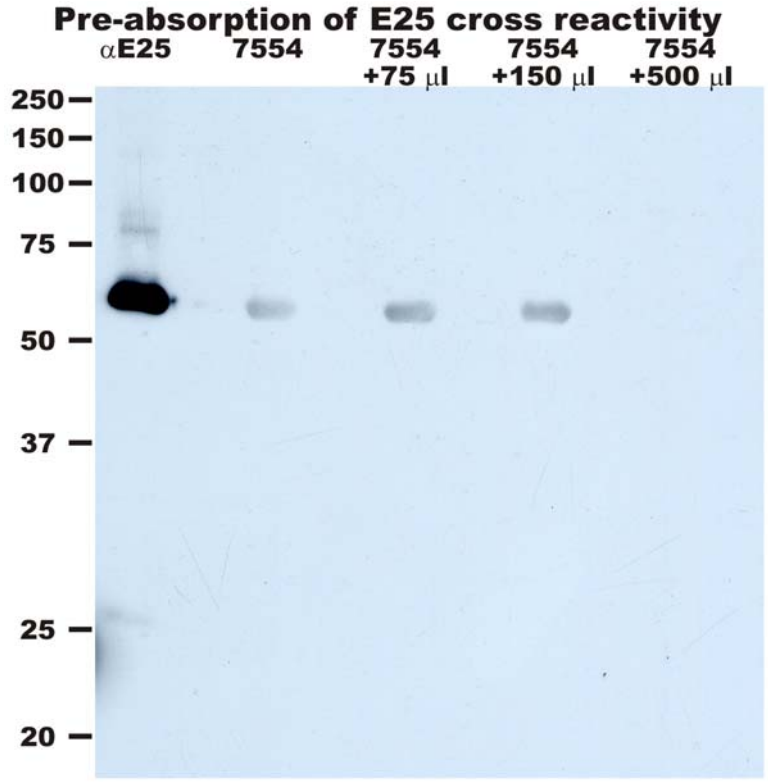
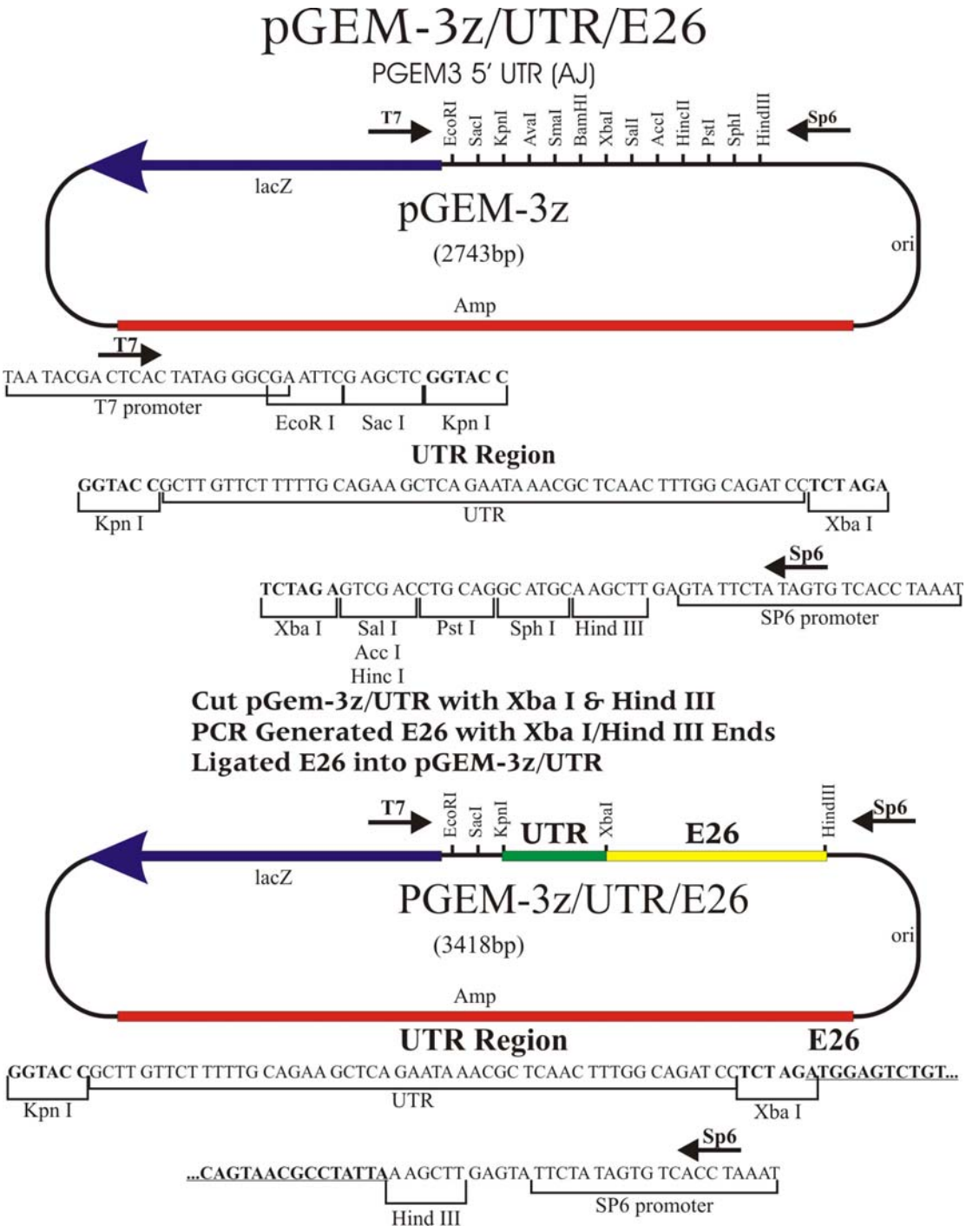


Figure A6. Pre-absorption of the 7554 antiserum to remove cross-reactivity with E25. Bacterial expressed E25 fusion protein (10 µl of the induced soluble fraction) was separated on a SDS-PAGE gel and exposed to various antisera including treated 7554 antiserum. The antiserums used were E25 (1:10,000), 7554 (1:40,000), 7554 + 75 µl of protein beads with bacterial expressed E25 bound (1:34,783), 7554 + 150 µl of protein beads with bacterial expressed E25 bound (1:30,769), and 7554 + 500 µl of protein beads with bacterial expressed E25 bound (1:20,000). The 7554 antiserums various dilutions account for the additional diluting volumes. Exposure time on this Western blot is 5 minutes, which is extreme, displaying the minimal cross-reactivity that exists and the removal in the + 500 µl sample. This pre-absorbed antiserum (7554 + 500 µl of protein beads) was used in all experiments using the 7554 antiserum in this dissertation.

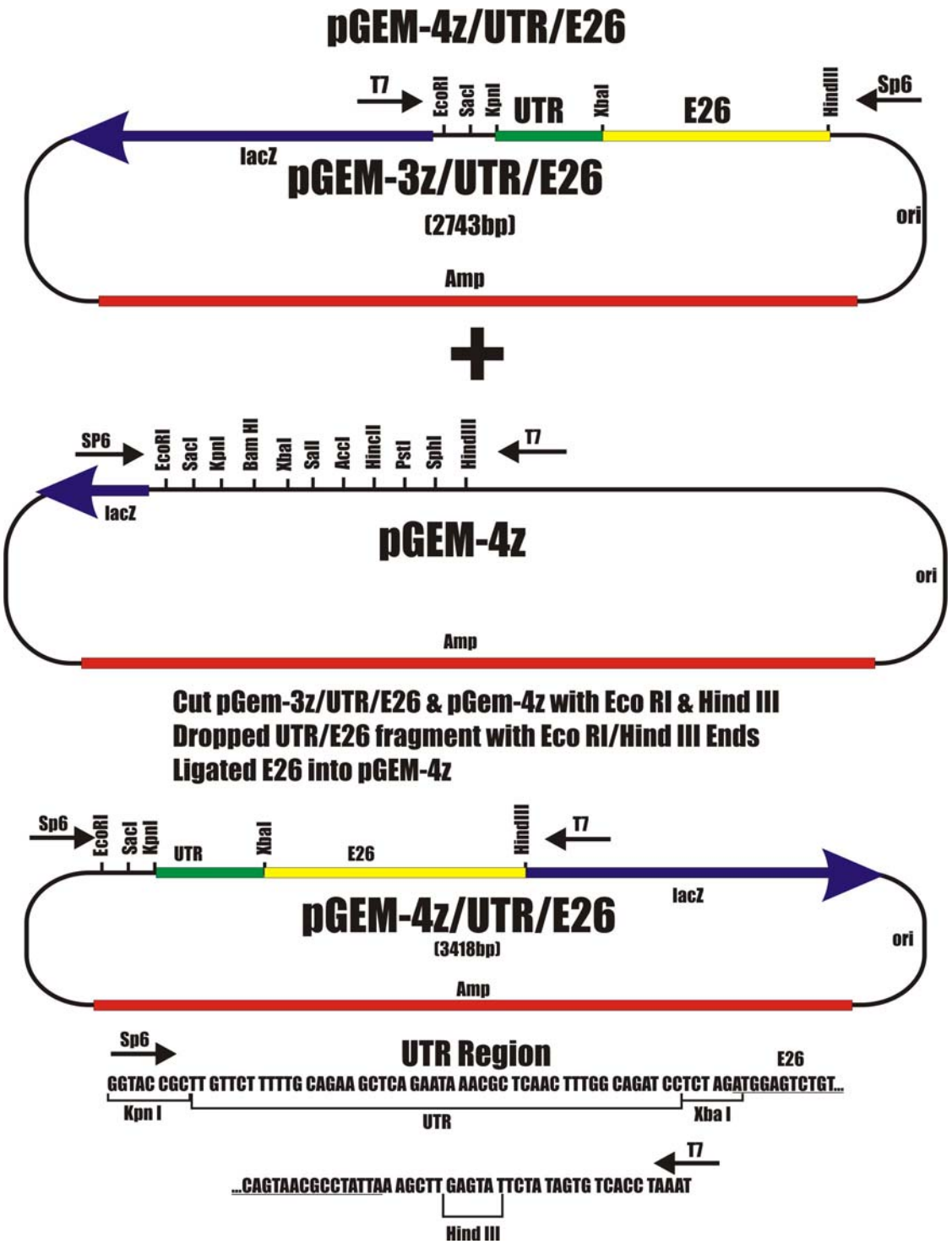
APPENDIX B

CLONE MAPS

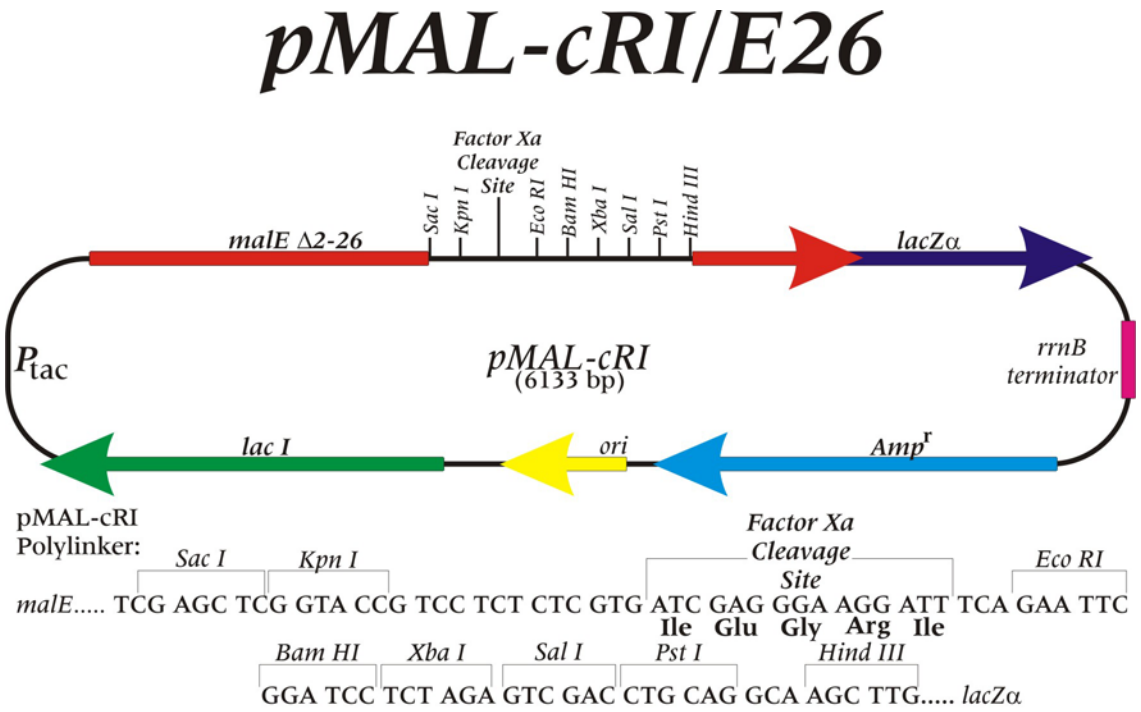
B1 : pGEM3z/UTR/E26



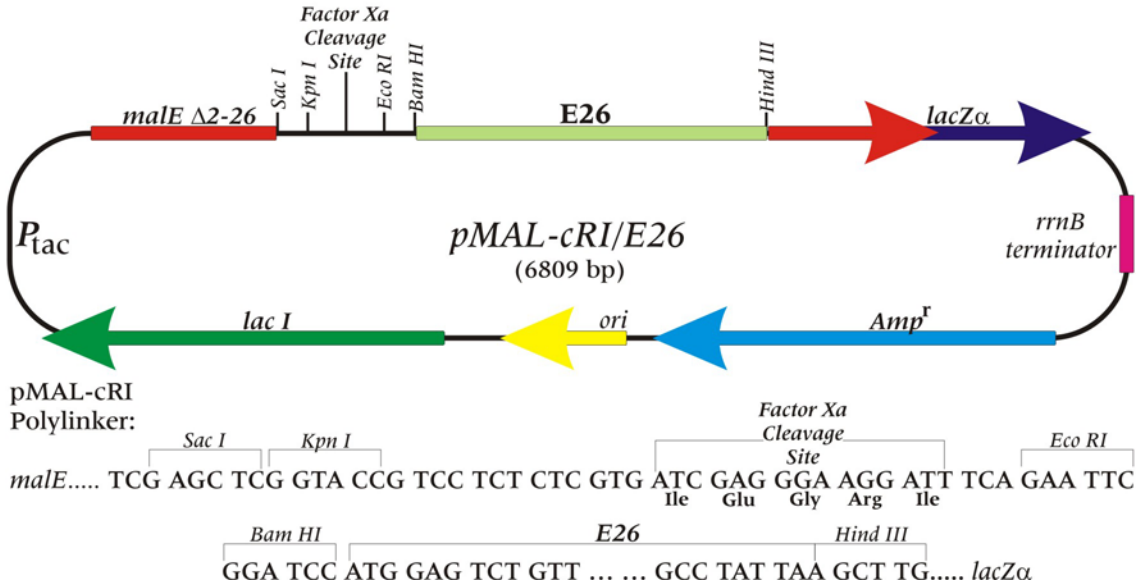
B2 : pGEM4z/UTR/E26



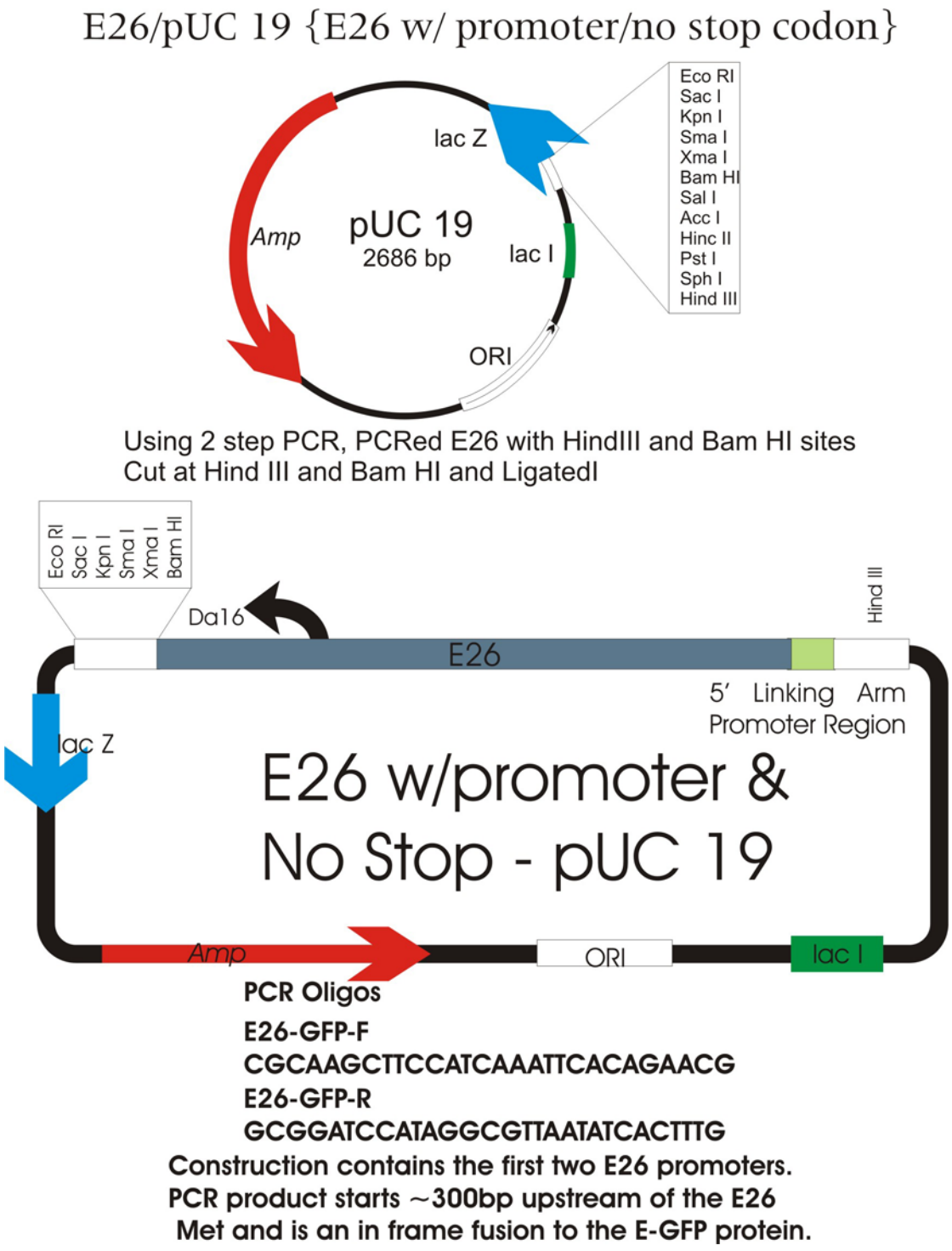
B3 : pMAL-cRI/E26



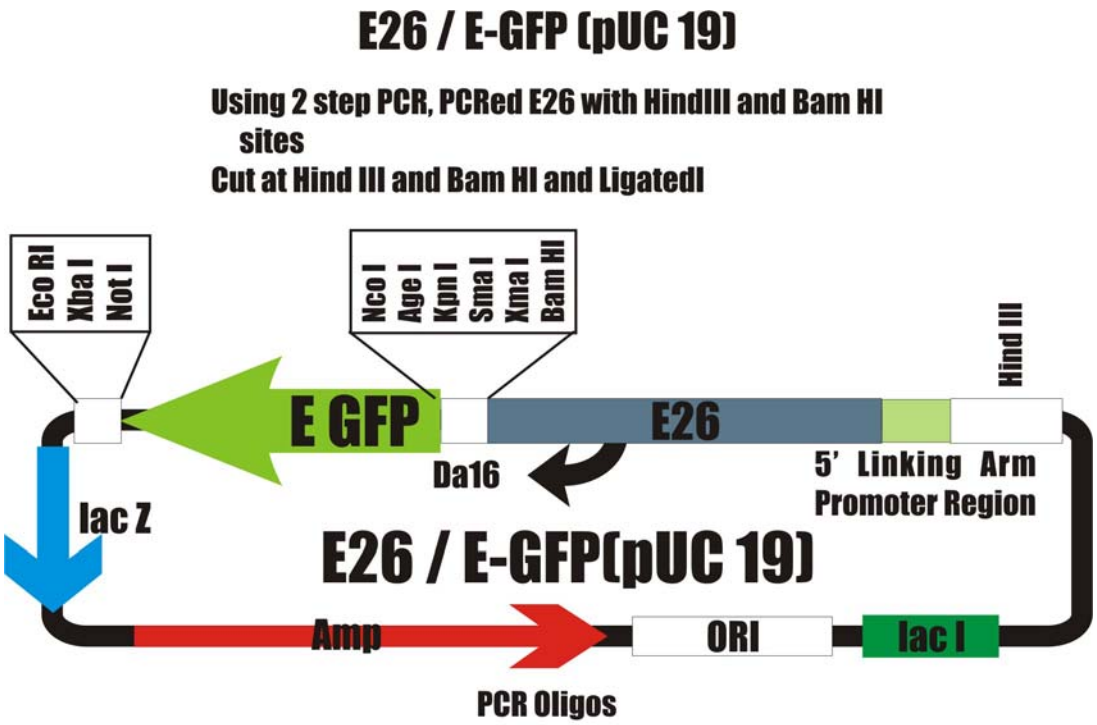
Cut pMAL-CRI with Bam HI and Hind III
PCR generated E26 with Bam HI and Hind III ends
Ligated E26 into pMAL-CRI



B4 : pUC19/E26 (no stop condon)

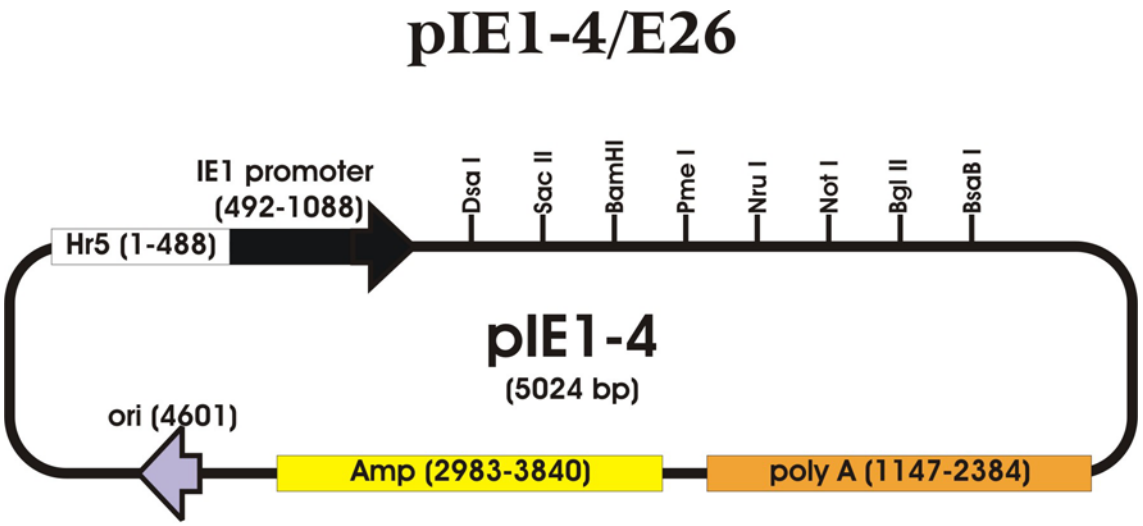


B5 : pEGFP/E26

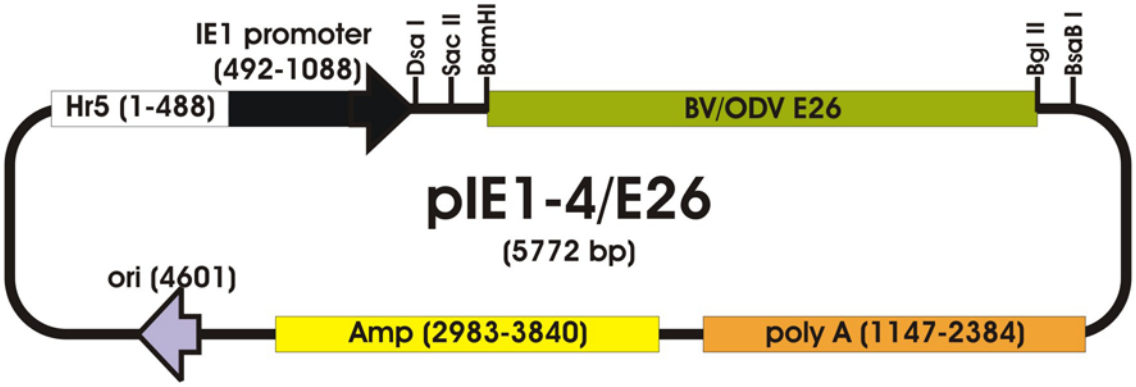


Construction contains the first two E26 promoters. PCR product starts ~300bp upstream of the E26 Met and is an in frame fusion to the E-GFP protein.

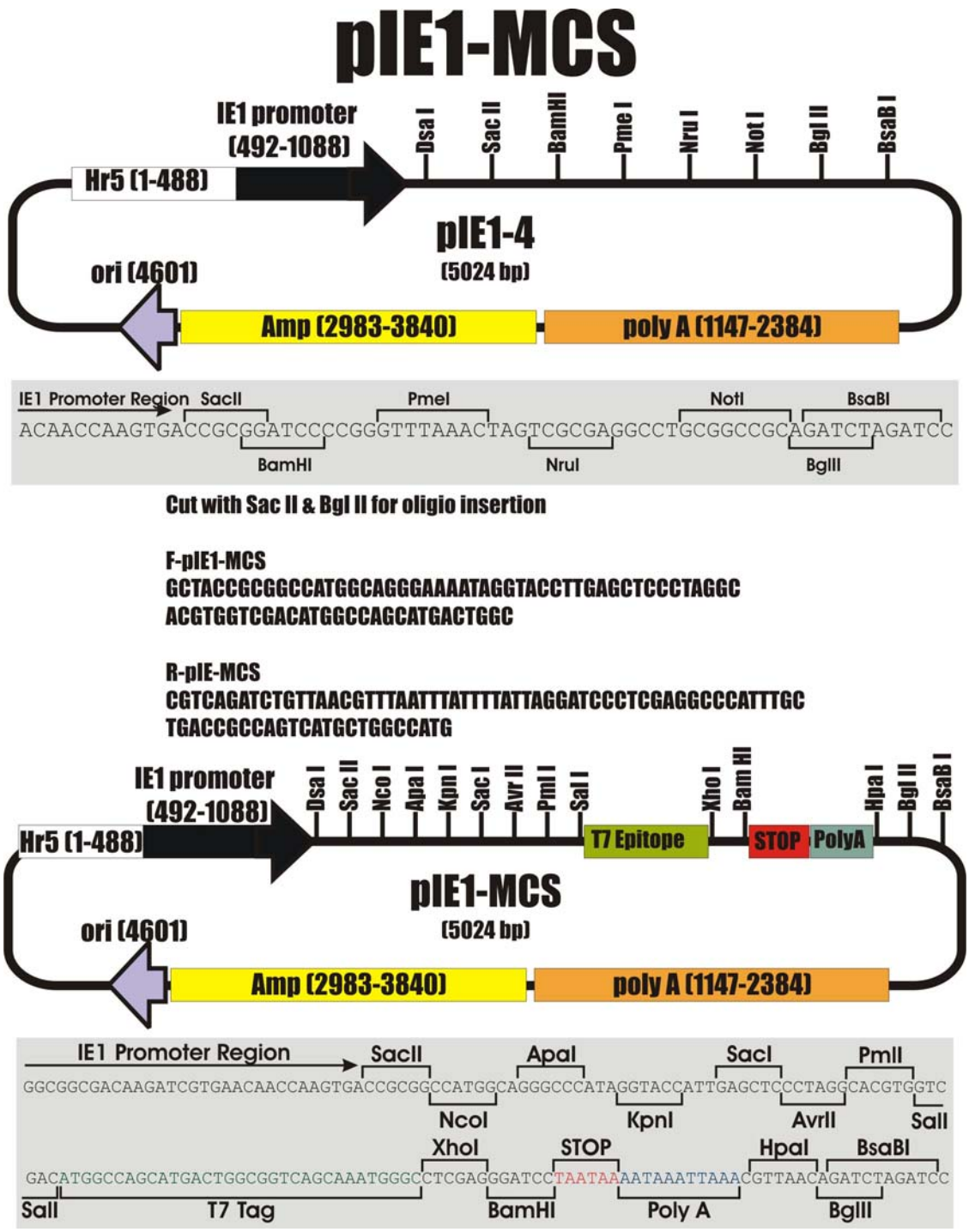
B6 : pIE1-4/E26



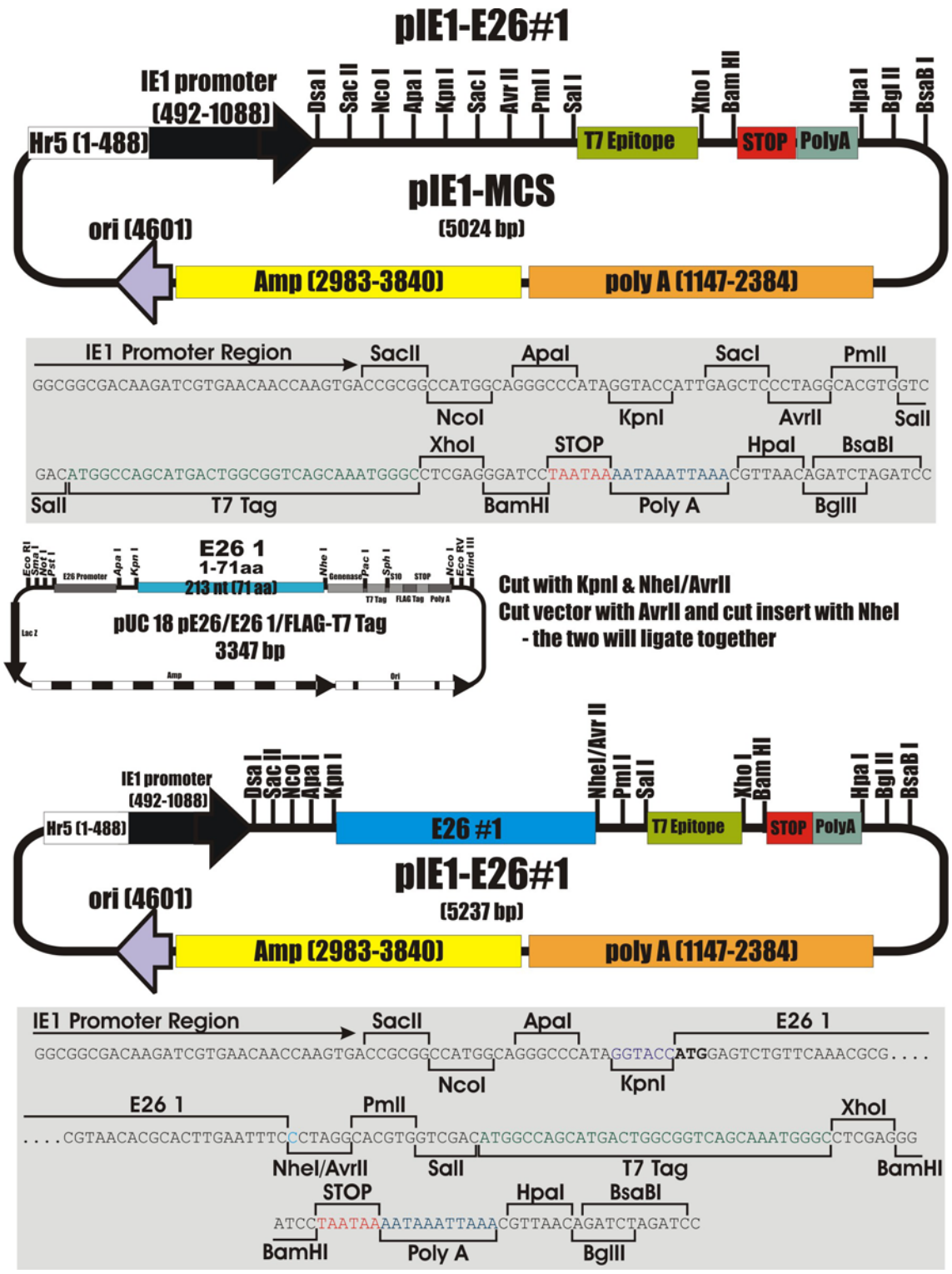
PCR generated E26 with BamH I and Bgl II sites and cut pIE1and inserted gene



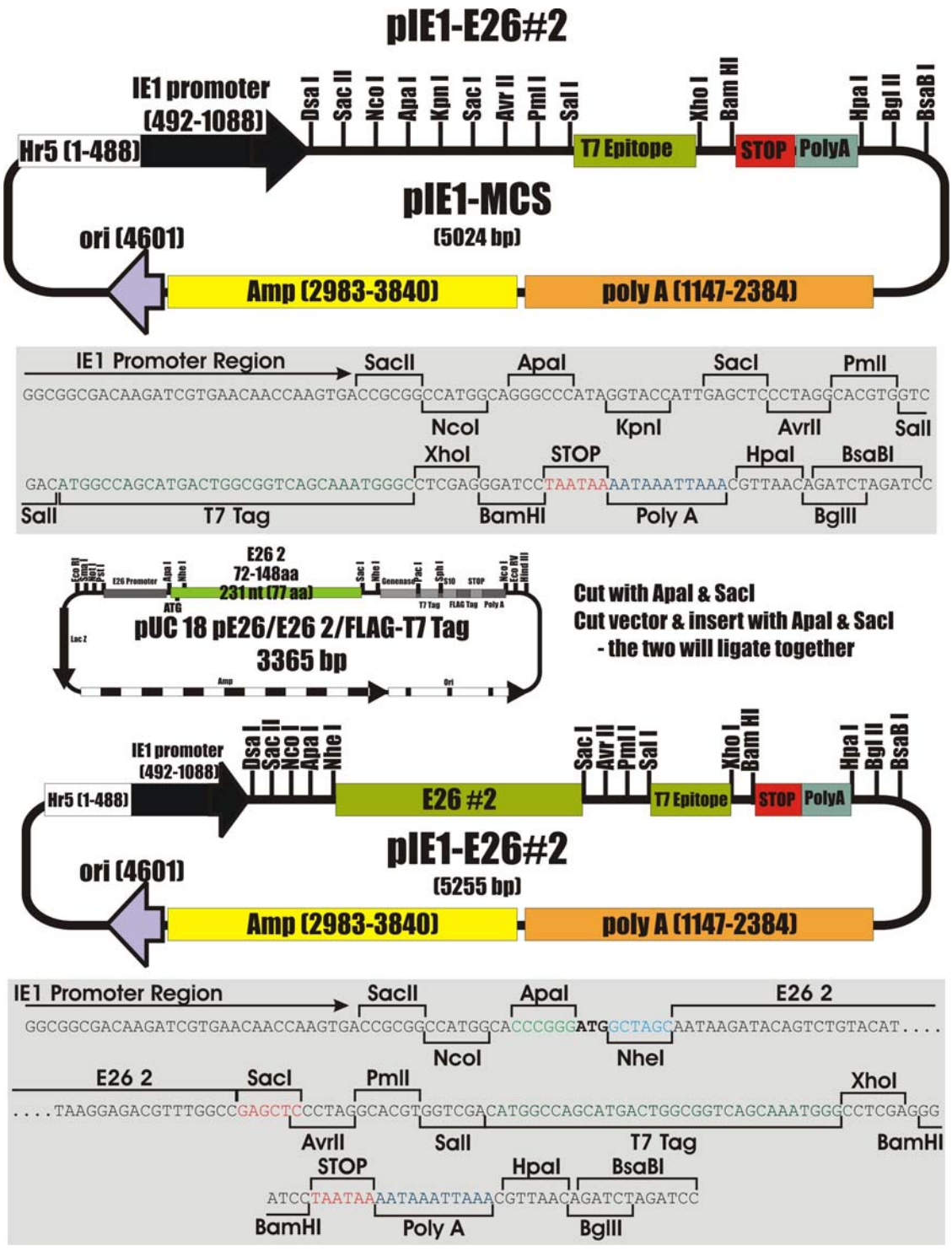
B7 : pIE1-MCS


pIE1-MCS
IE1 promoter
(492-1088)
Hr5 (1-488)
Dsa I
Sac II
BamHI
Pme I
Nru I
Not I
Bgl II
BsaB I
pIE1-4
(5024 bp)
ori (4601)
Amp (2983-3840)
poly A (1147-2384)
IE1 Promoter Region
SacII
PmeI
NotI
BsaBI
ACAACCAAGTGACCGCGGATCCCCGGGTTTAAACTAGTCGCGAGGCCTGCGGCCGCAGATCTAGATCC
BamHI
NruI
BglII
Cut with Sac II & Bgl II for oligio insertion
F-pIE1-MCS
GCTACCGCGGCCATGGCAGGGAAAATAGGTACCTTGAGCTCCCTAGGC
ACGTGGTCGACATGGCCAGCATGACTGGC
R-pIE-MCS
CGTCAGATCTGTTAACGTTTAATTTATTTTATTAGGATCCCTCGAGGCCCATTTGC
TGACCGCCAGTCATGCTGGCCATG
IE1 promoter
(492-1088)
Hr5 (1-488)
Dsa I
Sac II
Nco I
Apa I
Kpn I
Sac I
Avr II
Pml I
Sal I
T7 Epitope
Xho I
Bam HI
STOP
PolyA
Hpa I
Bgl II
BsaB I
pIE1-MCS
(5024 bp)
ori (4601)
Amp (2983-3840)
poly A (1147-2384)
IE1 Promoter Region
SacII
ApaI
SacI
PmlI
GGCGGCGACAAGATCGTGAACAACCAAGTGACCGCGGCCATGGCAGGGCCCATAGGTACCATTGAGCTCCCTAGGCACGTGGTC
NcoI
KpnI
AvrII
SalI
XhoI
STOP
HpaI
BsaBI
GACATGGCCAGCATGACTGGCGGTCAGCAAATGGGCCTCGAGGGATCCTAATAAAATAAATTAAACGTTAACAGATCTAGATCC
SalI
T7 Tag
BamHI
Poly A
BglII

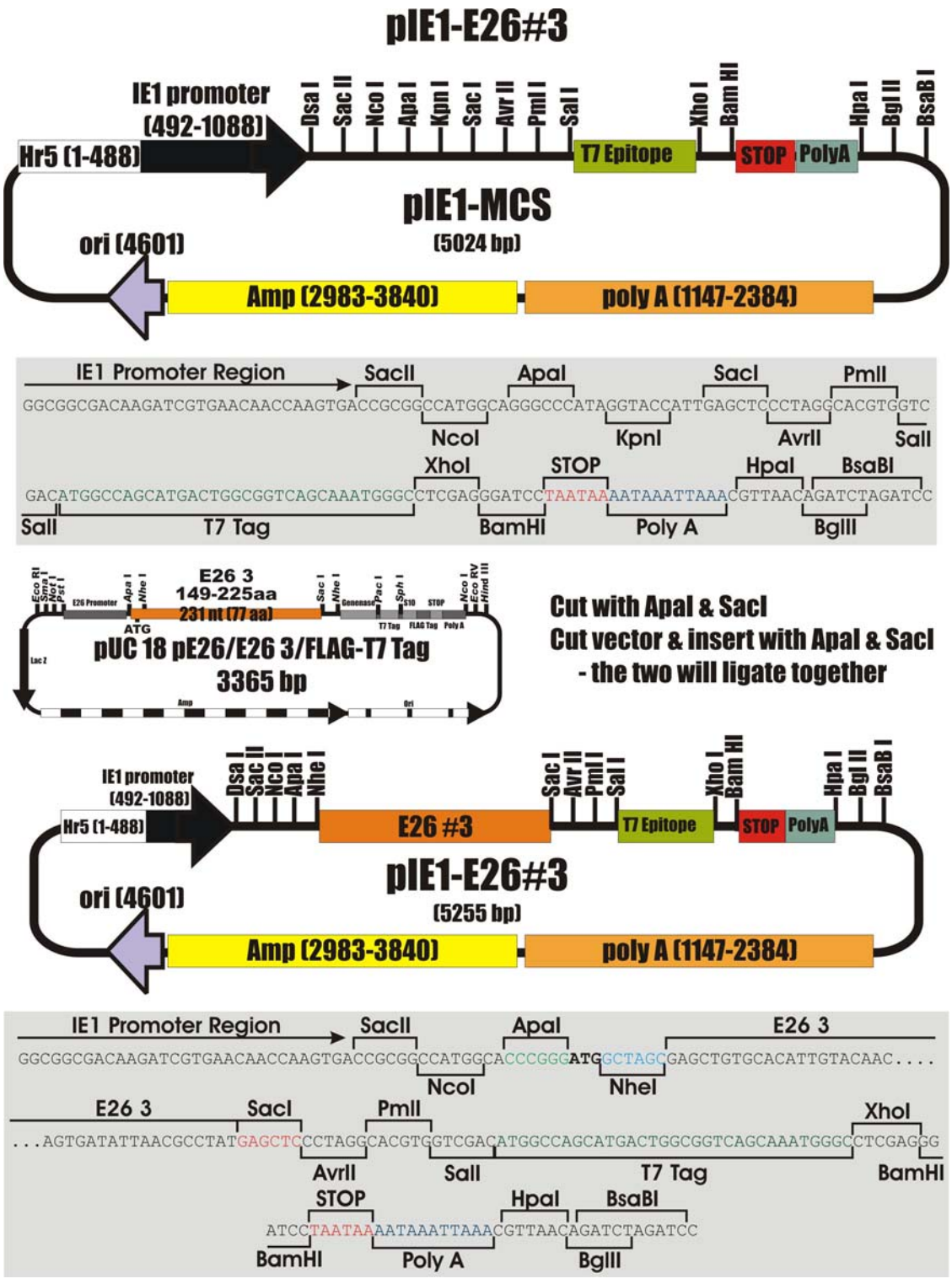
B8 : pIE1-E26#1


pIE1-E26#1
IE1 promoter
(492-1088)
Hr5 (1-488)
Dsa I
Sac II
Nco I
Apa I
Kpn I
Sac I
Avr II
Pml I
Sal I
Xho I
Bam HI
Hpa I
Bgl II
BsaB I
T7 Epitope
STOP
PolyA
pIE1-MCS
(5024 bp)
ori (4601)
Amp (2983-3840)
poly A (1147-2384)
IE1 Promoter Region
SacII
ApaI
SacI
PmlI
GGCGGCGACAAGATCGTGAACAACCAAGTGACCGCGGCCATGGCAGGGCCCATAGGTACCATTGAGCTCCCTAGGCACGTGGTC
NcoI
KpnI
AvrII
SalI
XhoI
STOP
HpaI
BsaBI
GACATGGCCAGCATGACTGGCGGTCAGCAAATGGGCCTCGAGGGATCCTAATAAAATAAATTAAACGTTAACAGATCTAGATCC
SalI
T7 Tag
BamHI
Poly A
BglII
E26 1
1-71aa
213 nt (71 aa)
pUC 18 pE26/E26 1/FLAG-T7 Tag
3347 bp
Cut with KpnI & NheI/AvrII
Cut vector with AvrII and cut insert with NheI
- the two will ligate together
IE1 promoter
(492-1088)
Hr5 (1-488)
E26 #1
NheI/Avr II
pIE1-E26#1
(5237 bp)
ori (4601)
Amp (2983-3840)
poly A (1147-2384)
IE1 Promoter Region
SacII
ApaI
E26 1
GGCGGCGACAAGATCGTGAACAACCAAGTGACCGCGGCCATGGCAGGGCCCATAGGTACCATGGAGTCTGTTCAAACGCG....
NcoI
KpnI
E26 1
PmlI
XhoI
....CGTAACACGCACTTGAATTTCCCTAGGCACGTGGTCGACATGGCCAGCATGACTGGCGGTCAGCAAATGGGCCTCGAGGG
NheI/AvrII
SalI
T7 Tag
BamHI
STOP
HpaI
BsaBI
ATCCTAATAAAATAAATTAAACGTTAACAGATCTAGATCC
BamHI
Poly A
BglII

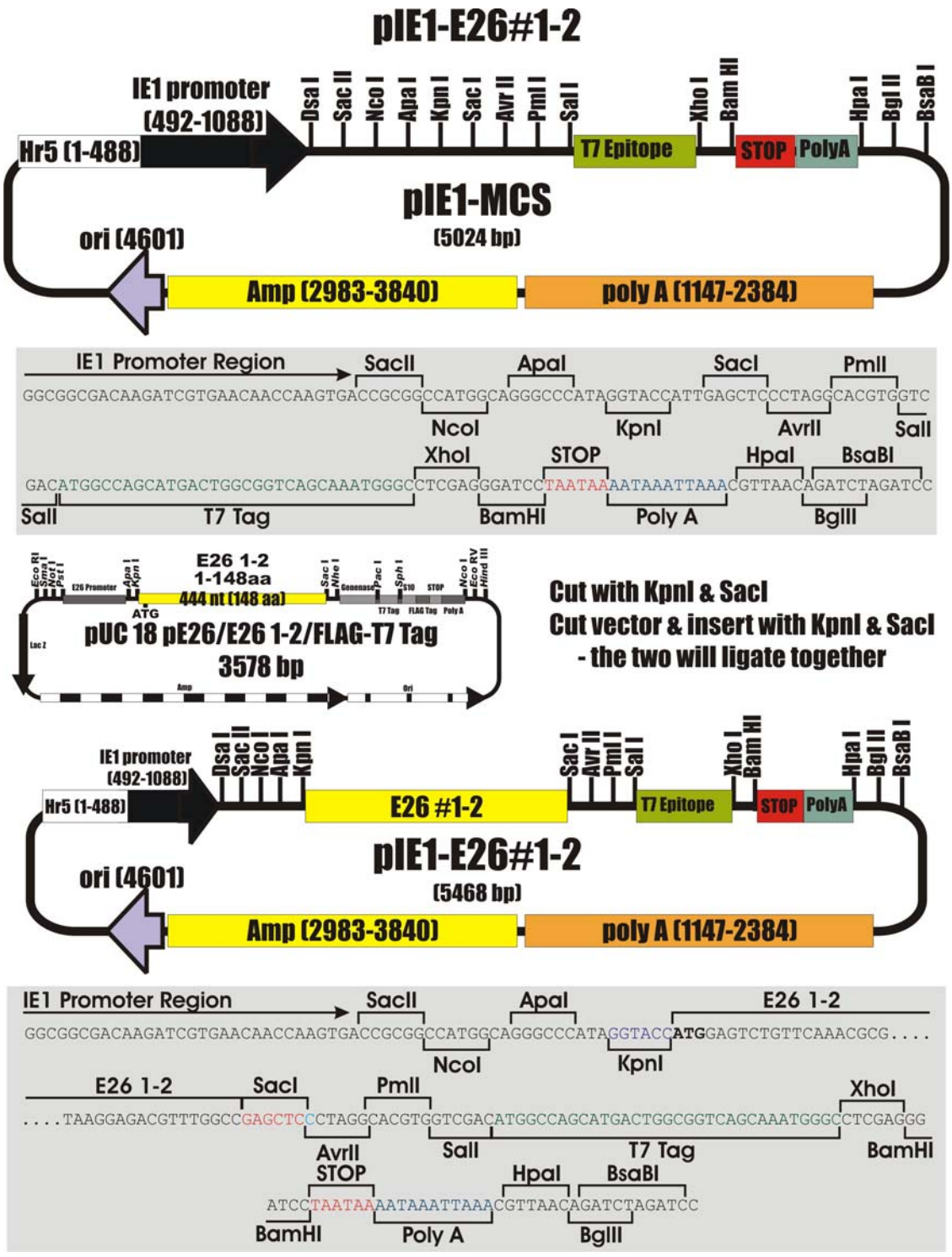
B9 : pIE1-E26#2


pIE1-E26#2
IE1 promoter
(492-1088)
Hr5 (1-488)
T7 Epitope
STOP
PolyA
pIE1-MCS
(5024 bp)
ori (4601)
Amp (2983-3840)
poly A (1147-2384)
IE1 Promoter Region
SacII
ApaI
SacI
PmlI
NcoI
KpnI
AvrII
SalI
XhoI
STOP
HpaI
BsaBI
BamHI
Poly A
BglII
T7 Tag
E26 2
72-148aa
231 nt (77 aa)
pUC 18 pE26/E26 2/FLAG-T7 Tag
3365 bp
Cut with ApaI & SacI
Cut vector & insert with ApaI & SacI
- the two will ligate together
E26 #2
pIE1-E26#2
(5255 bp)

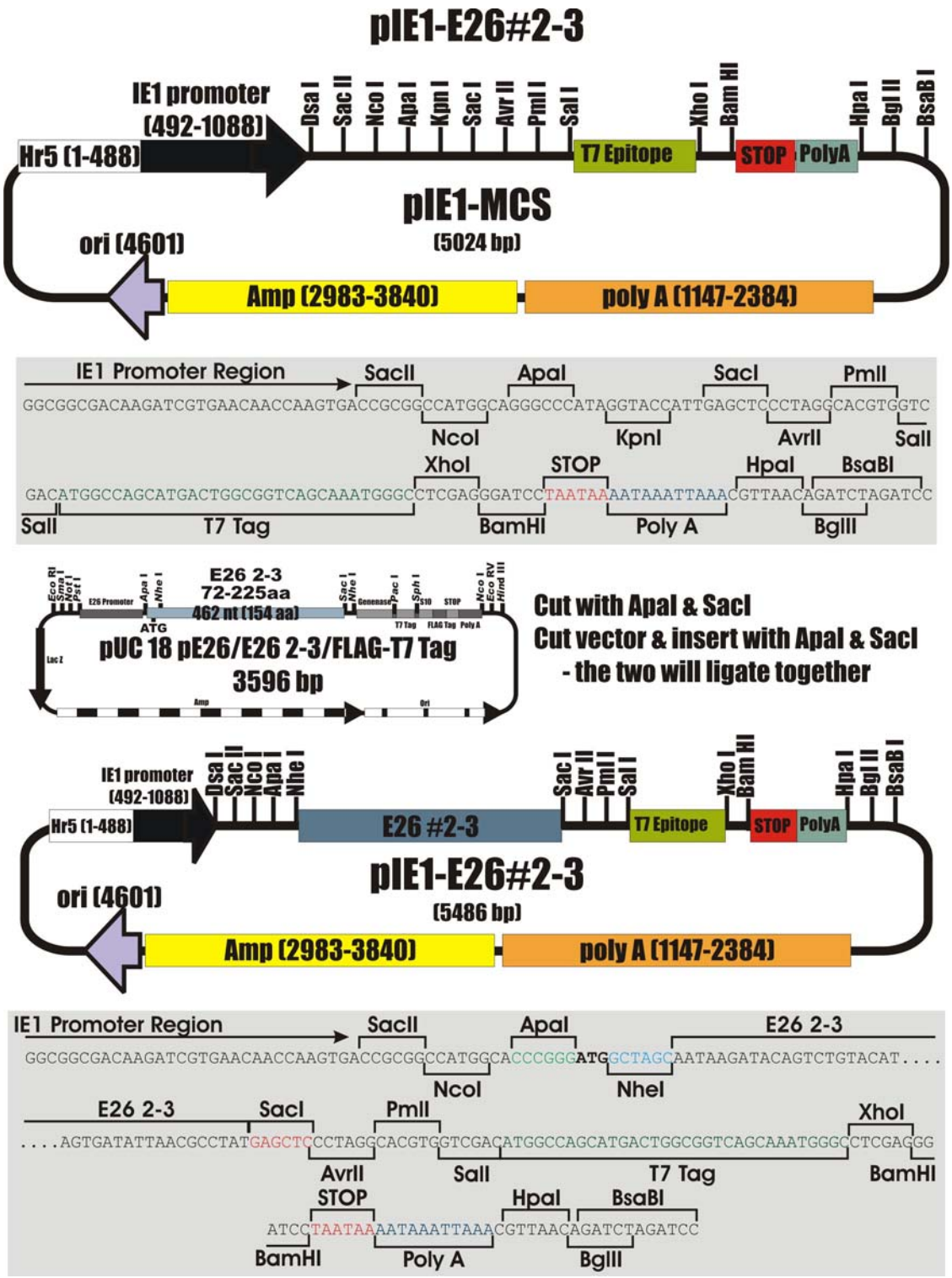
B10 : pIE1-E26#3



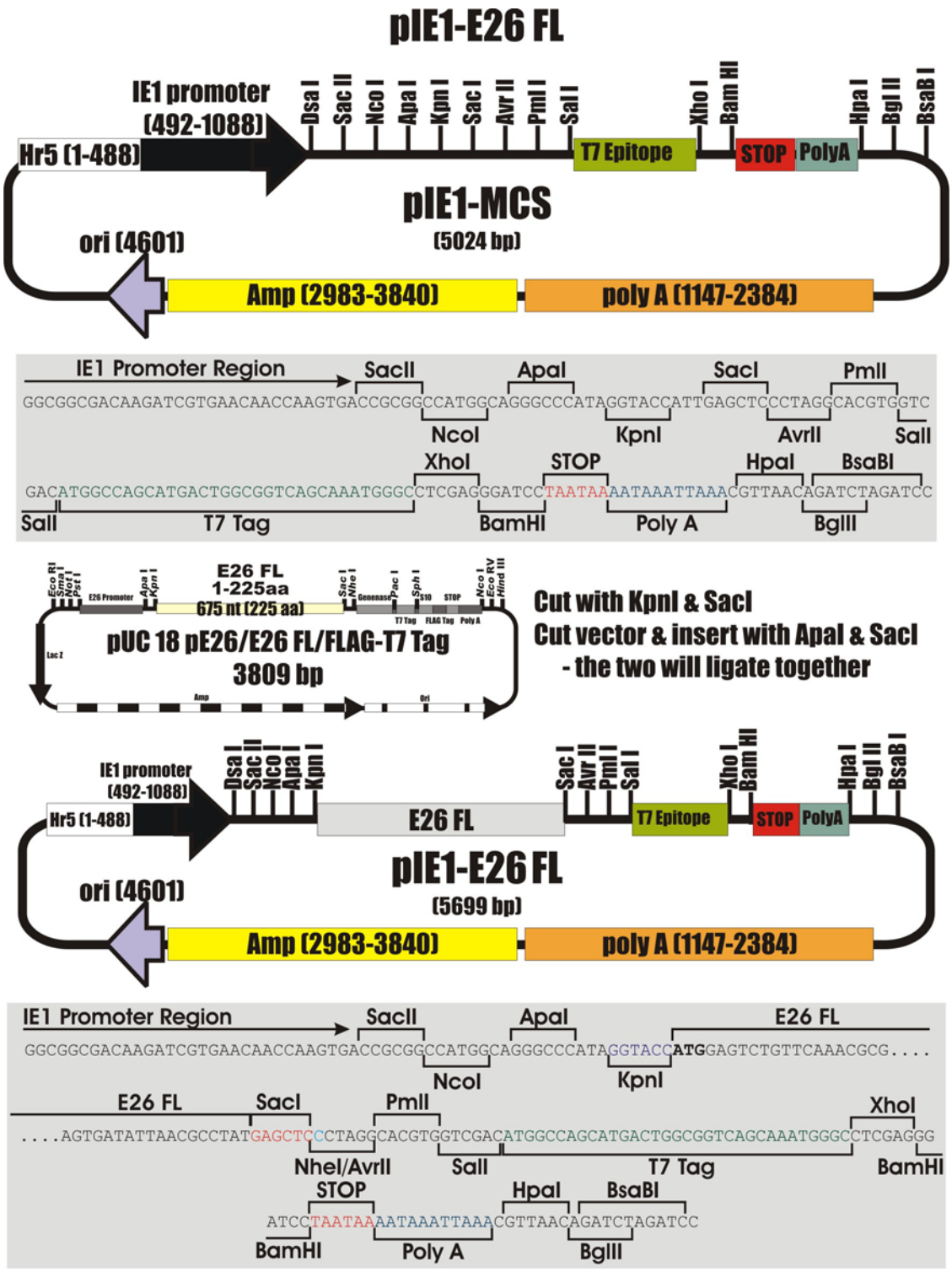
B 11： pIE1-E26#1-2



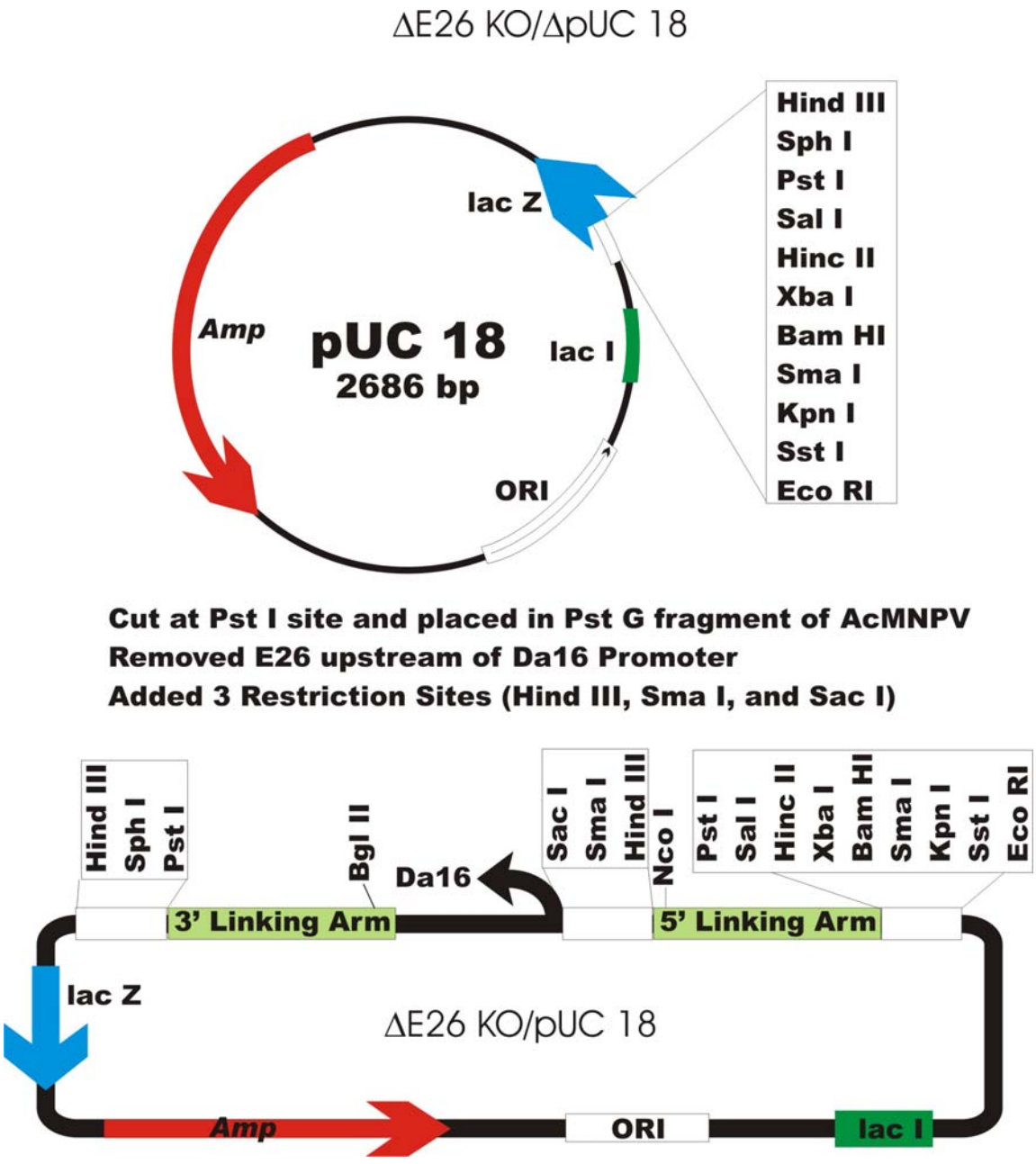
B12 : pIE1-E26#2-3



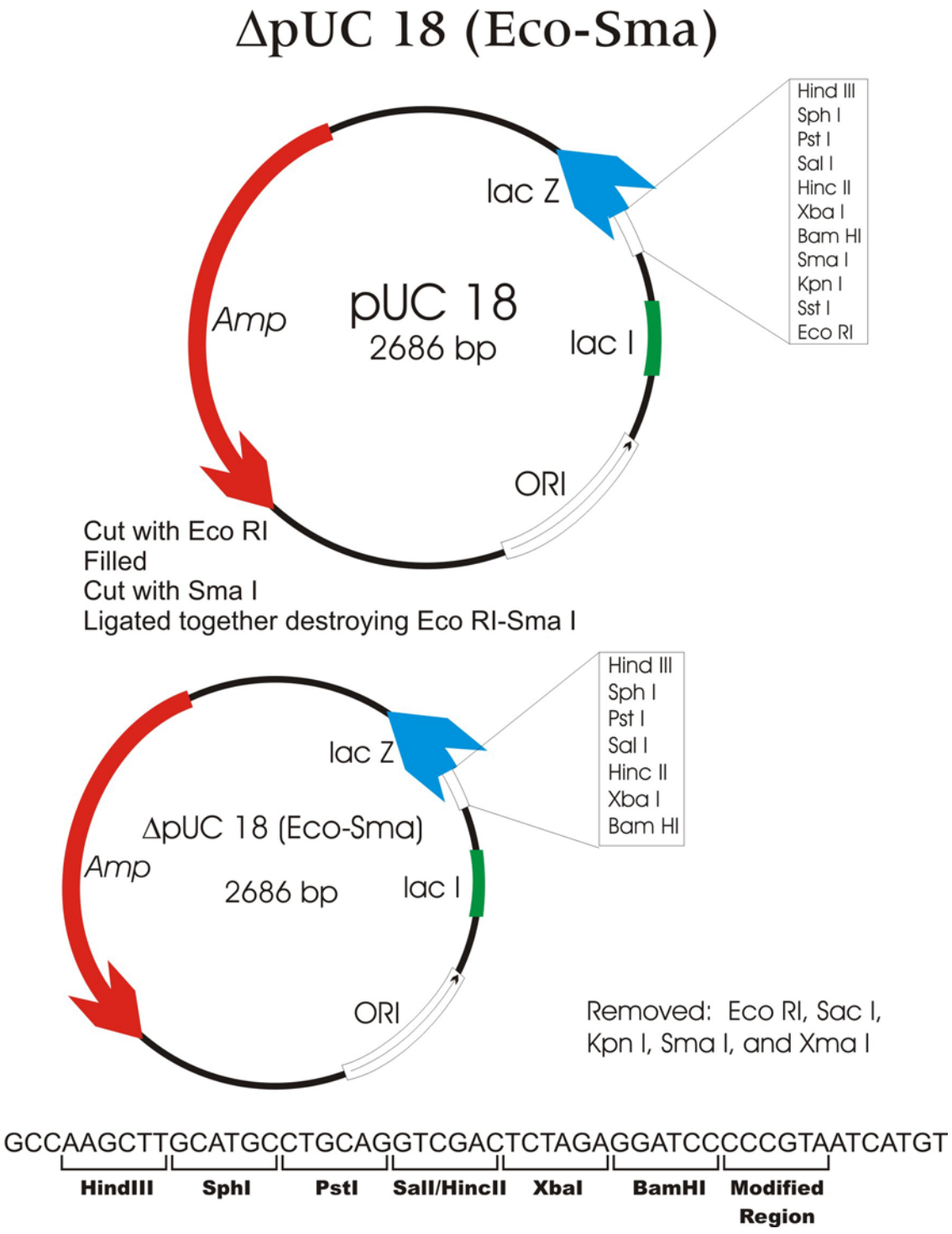
B13 : pIE1-E26 FL



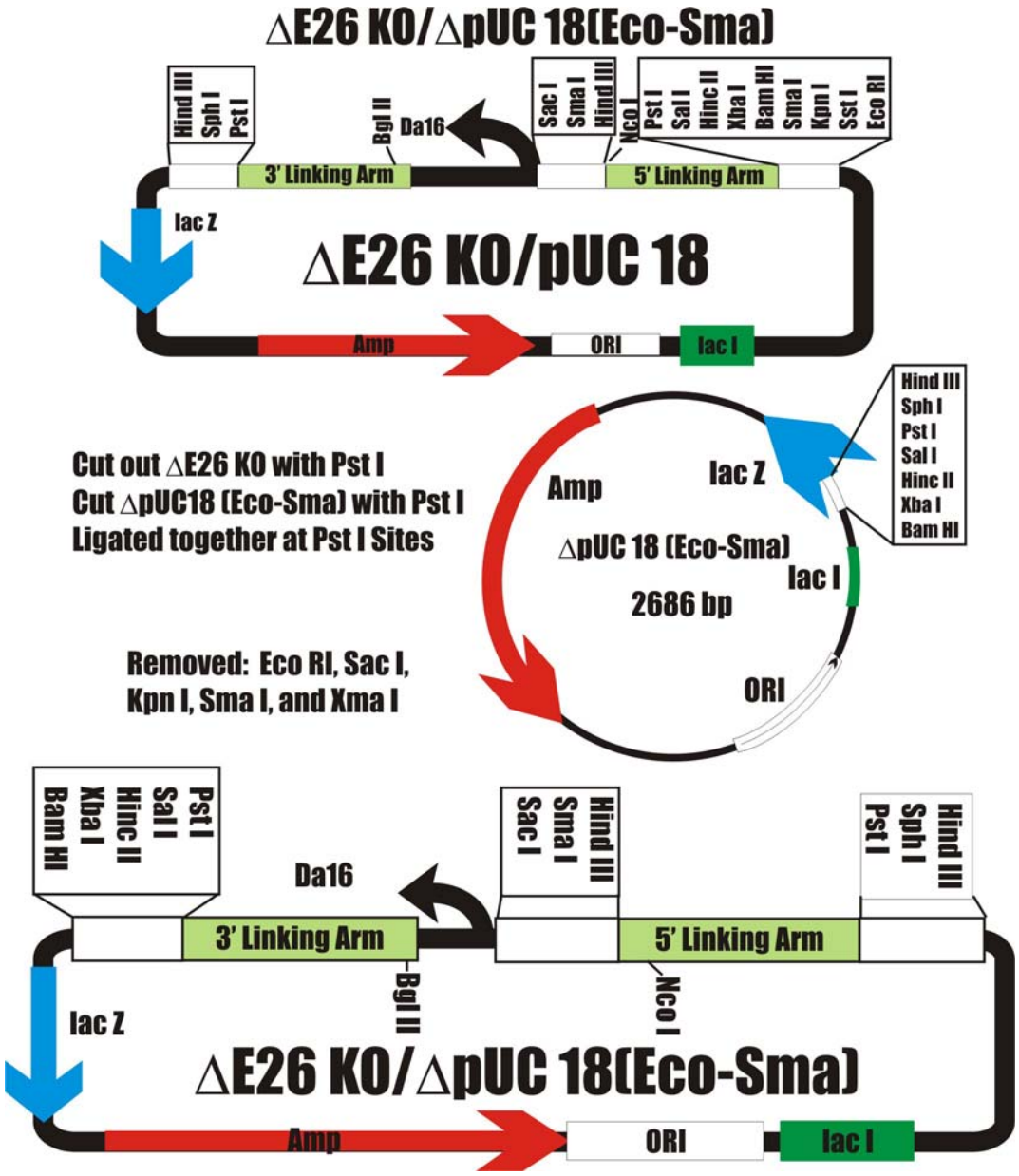
B14 : pUC18/ΔE26 KO



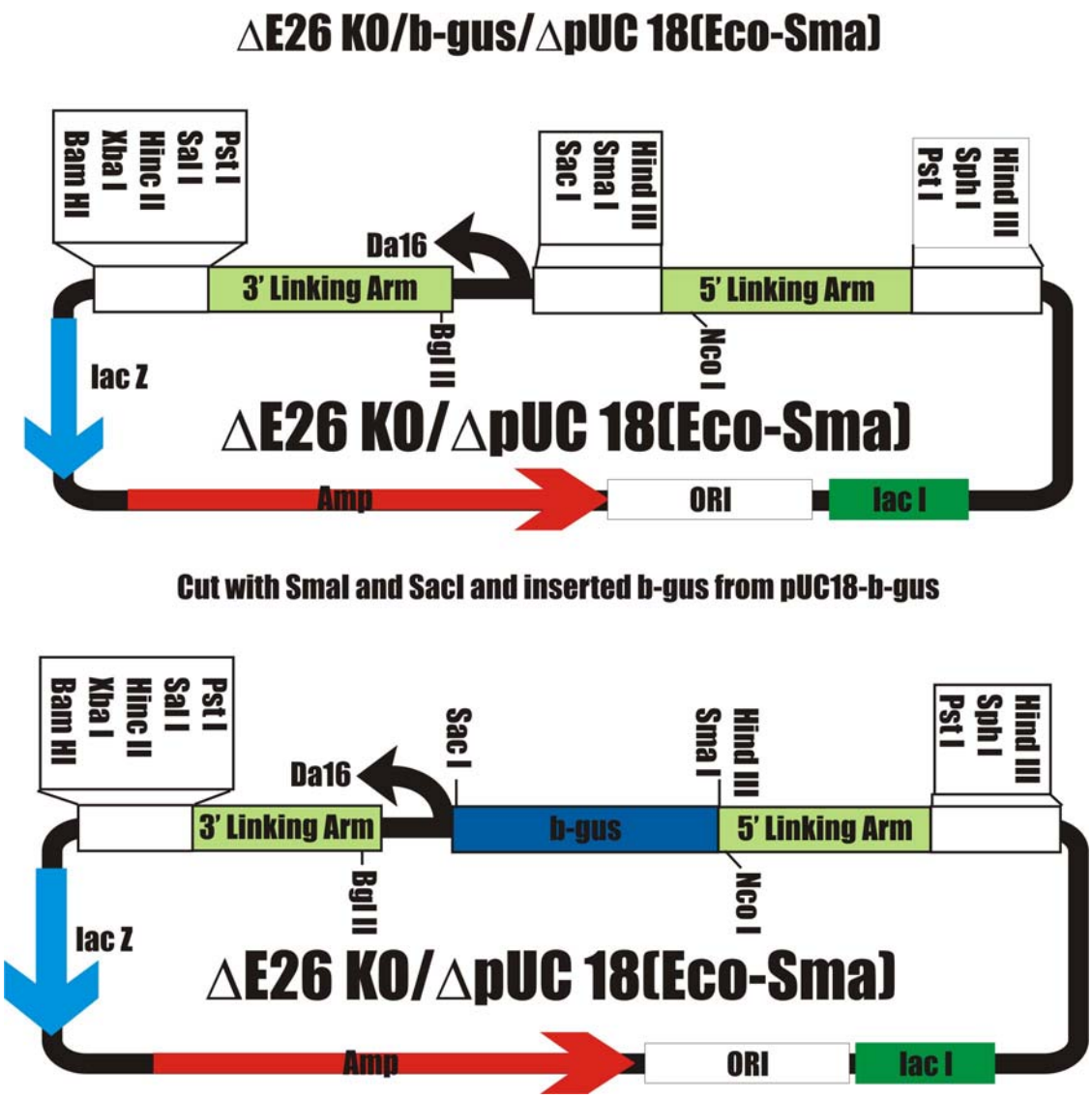
B15 : ΔpUC18 (EcoRI-SmaI)



B16 : ΔpUC18 (EcoRI/SmaI)/ΔE26 KO

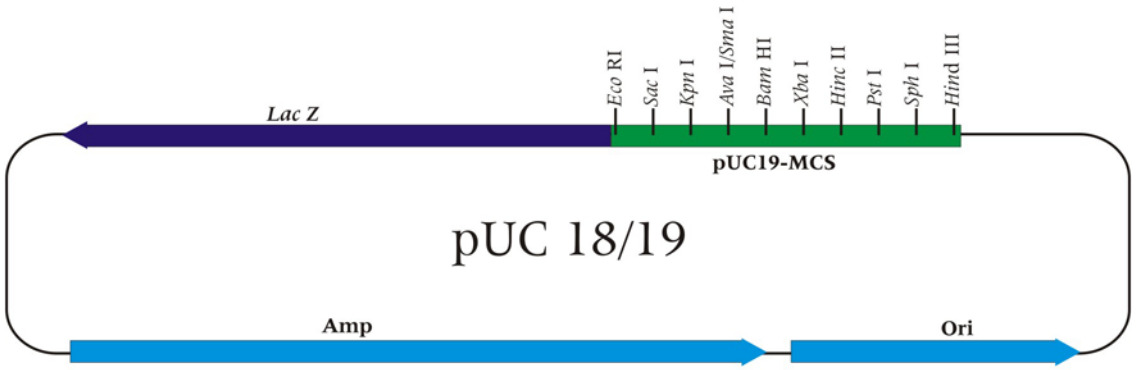


B17 : ΔpUC18 (EcoRI/SmaI)/ΔE26 KO/β-Gus



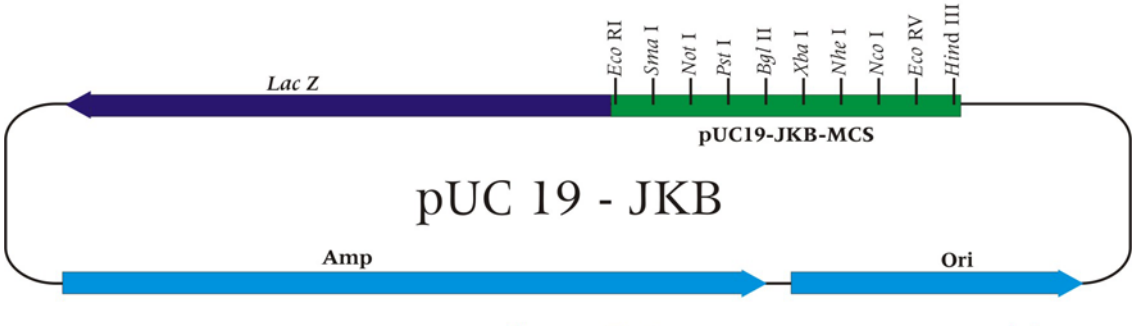
B18 : pUC18/19 – JKB

pUC18/19 - JKB



Generation of pUC-JKB
MCS Insertion into pUC with oligos

Eco RI *Not* I *Bgl* II *Xba* I *Nco* I *Hind* III
AATTCCCCGGGCGGCCGCTGCAGATCTGCTCTAGATGCTAGCAACCATGGATATCA
GGGGCCCGCCGGCGACGTCTAGACGAGATCTACGATCGTTGGTACCTATAGTTCGA
Sma I *Pst* I *Nhe* I *Eco* RV

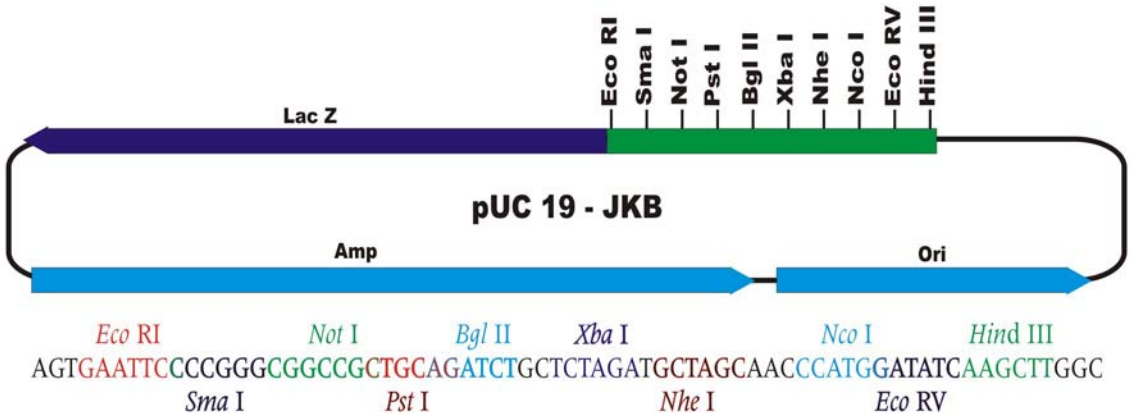


Eco RI *Not* I *Bgl* II *Xba* I *Nco* I *Hind* III
GCCAGTGAATTCCCCGGGCGGCCGCTGCAGATCTGCTCTAGATGCTAGCAACCCATGGATATCAAGCTTGG
Sma I *Pst* I *Nhe* I *Eco* RV

pUC 18 - JKB has a reversed MCS to the one above.
*** No blue/white selection due to accidental insertion of stop codon**

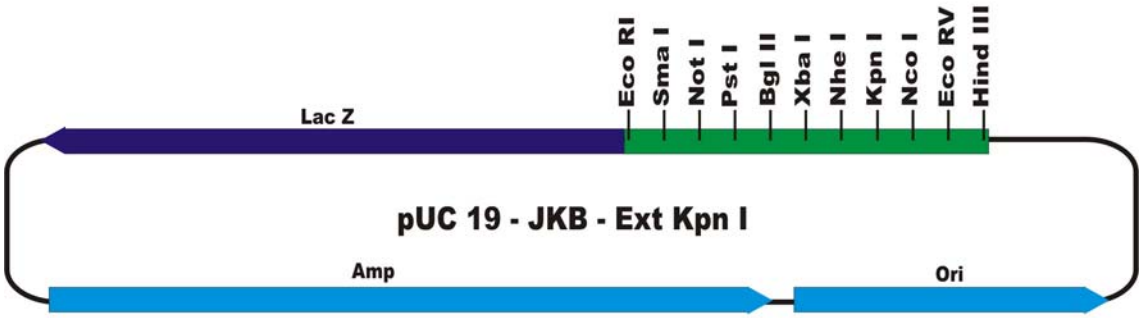
B19 : pUC18/19 – JKB-Ext KpnI

pUC18/19 - JKB - Extended Kpn I



Eco RI *Not* I *Bgl* II *Xba* I *Nco* I *Hind* III
AGTGAATTCCCCGGGCGGCCGCTGCAGATCTGCTCTAGATGCTAGCAACCCATGGATATCAAGCTTGGC
Sma I *Pst* I *Nhe* I *Eco* RV

Cut pUC18/19 - JKB with Nhe I & Hind III
Oligo inserted new region that includes Kpn I Site
Ligated with oligos

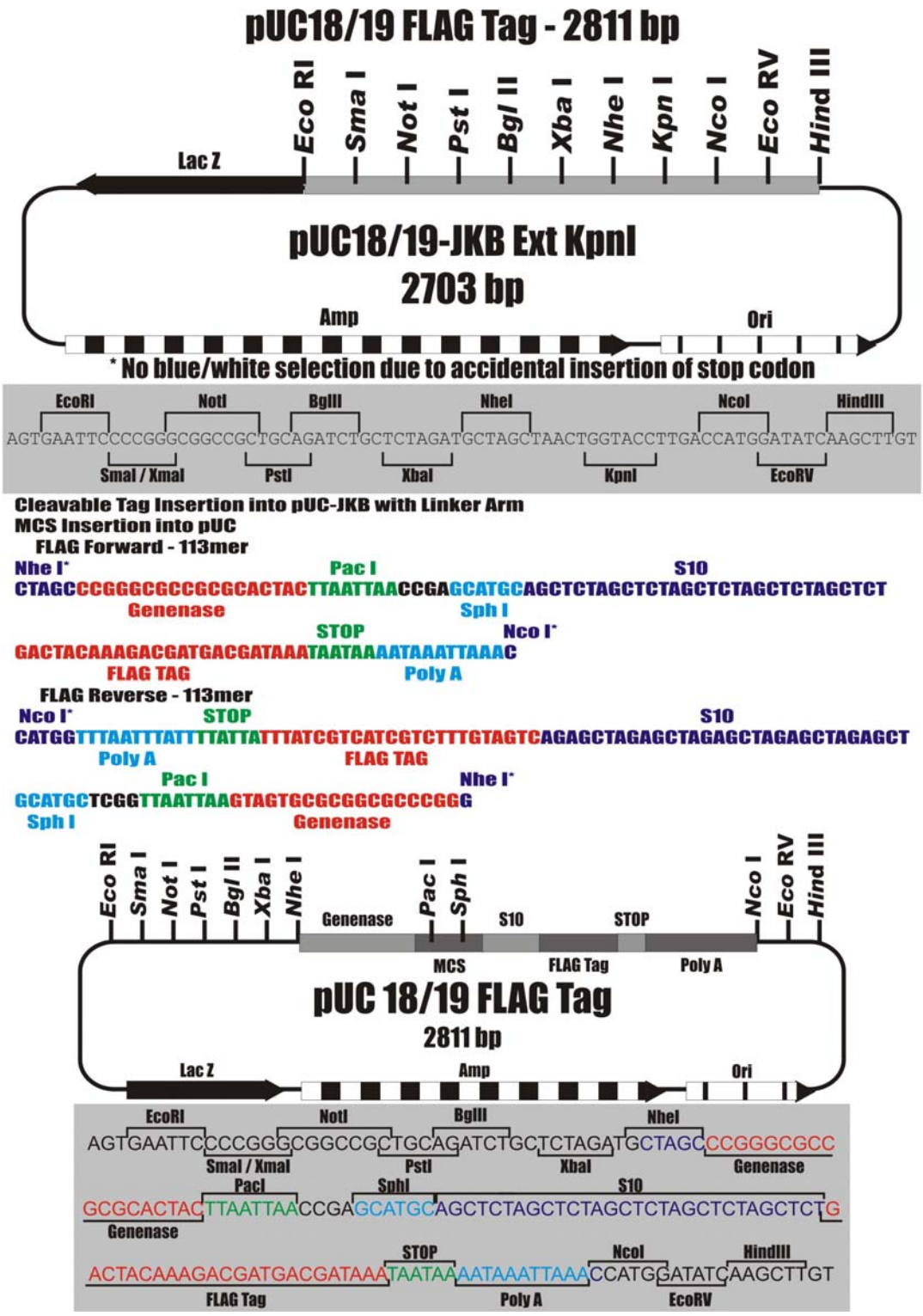


Eco RI *Not* I *Bgl* II *Xba* I
CGGCCAGTGAATTCCCCGGGCGGCCGCTGCAGATCTGCTCTAGATGCTAGCT
Sma I *Pst* I *Nhe* I

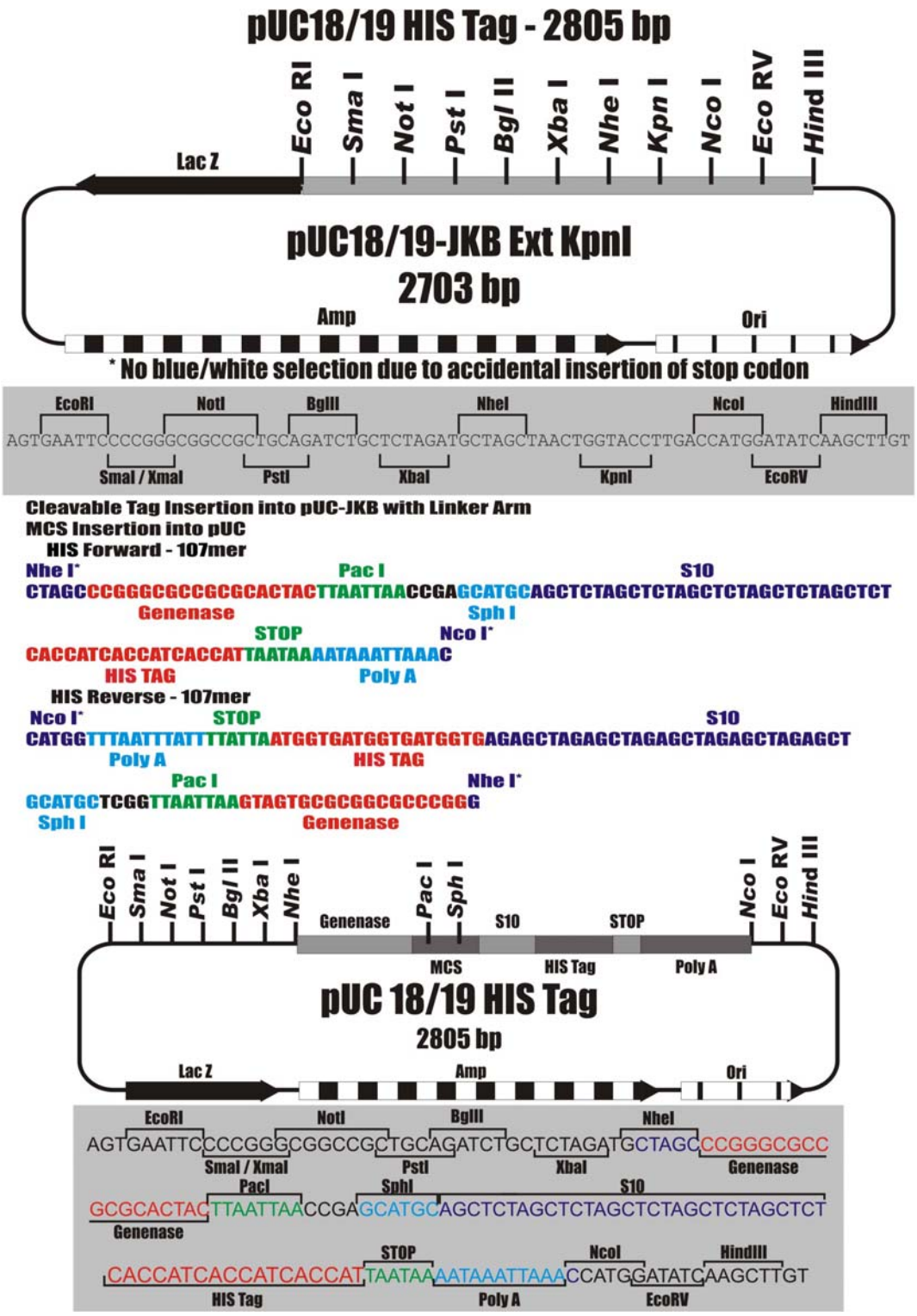
Nco I *Hind* III
AACTGGTACCTTGACCATGGATATCAAGCTTGGCGTAAT
Kpn I *Eco* RV

pUC 18 - JKB - Extended Kpn I has a reversed MCS to the one above.
*** No blue/white selection due to accidental insertion of stop codon**

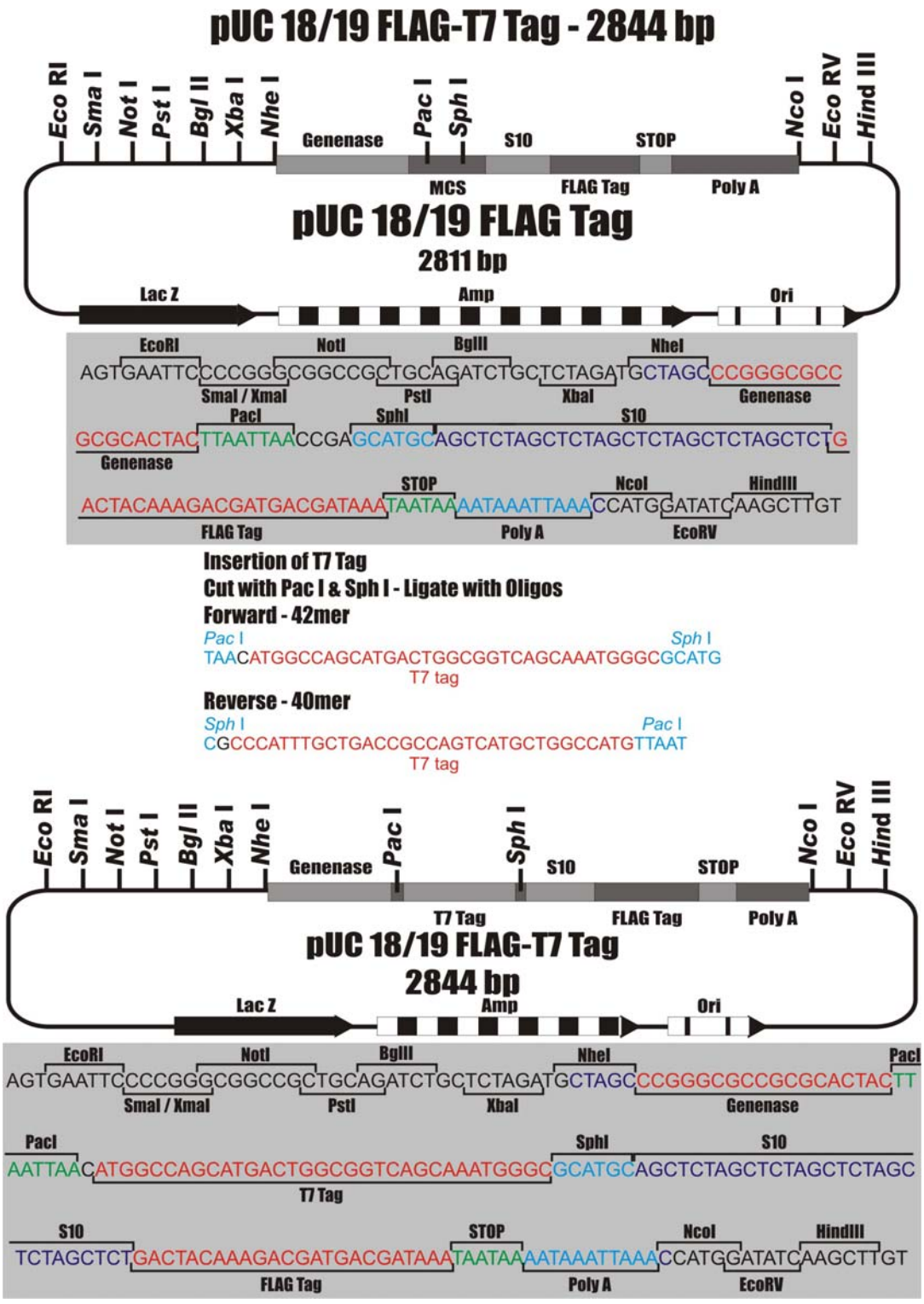
B20 : pUC18/19 FLAG Tag



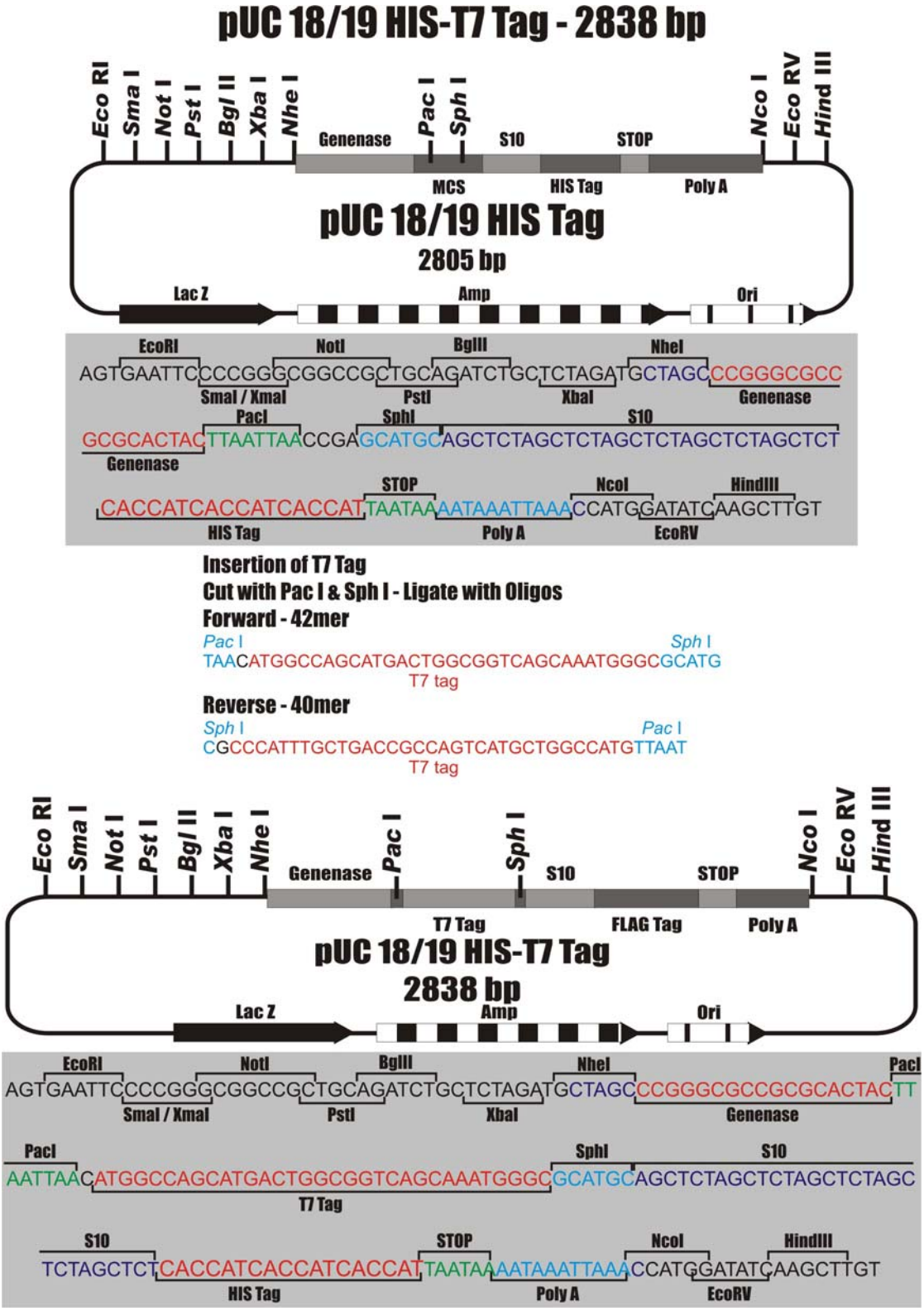
B21 : pUC18/19 HIS Tag



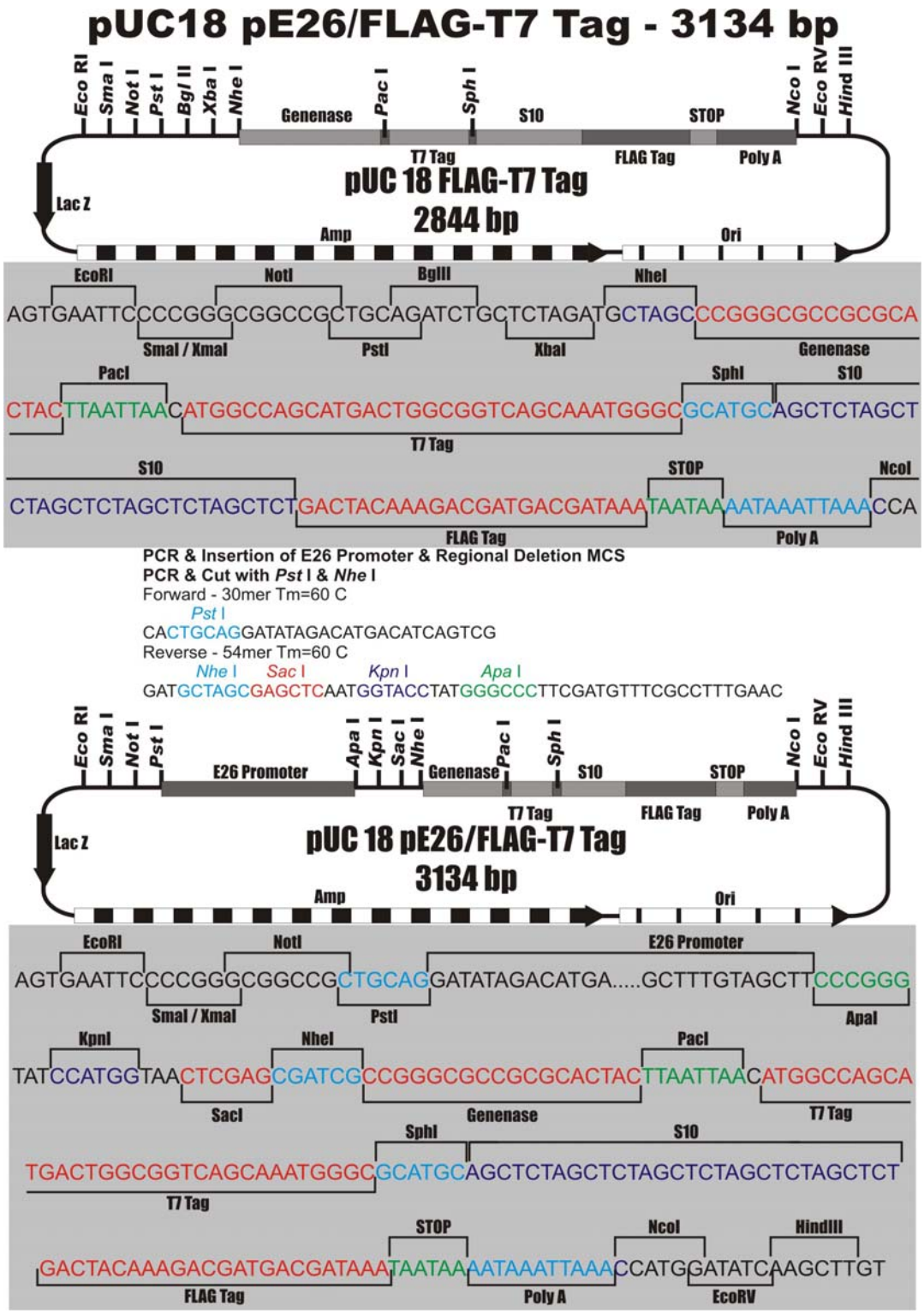
B22 : pUC18/19 FLAG-T7 Tag



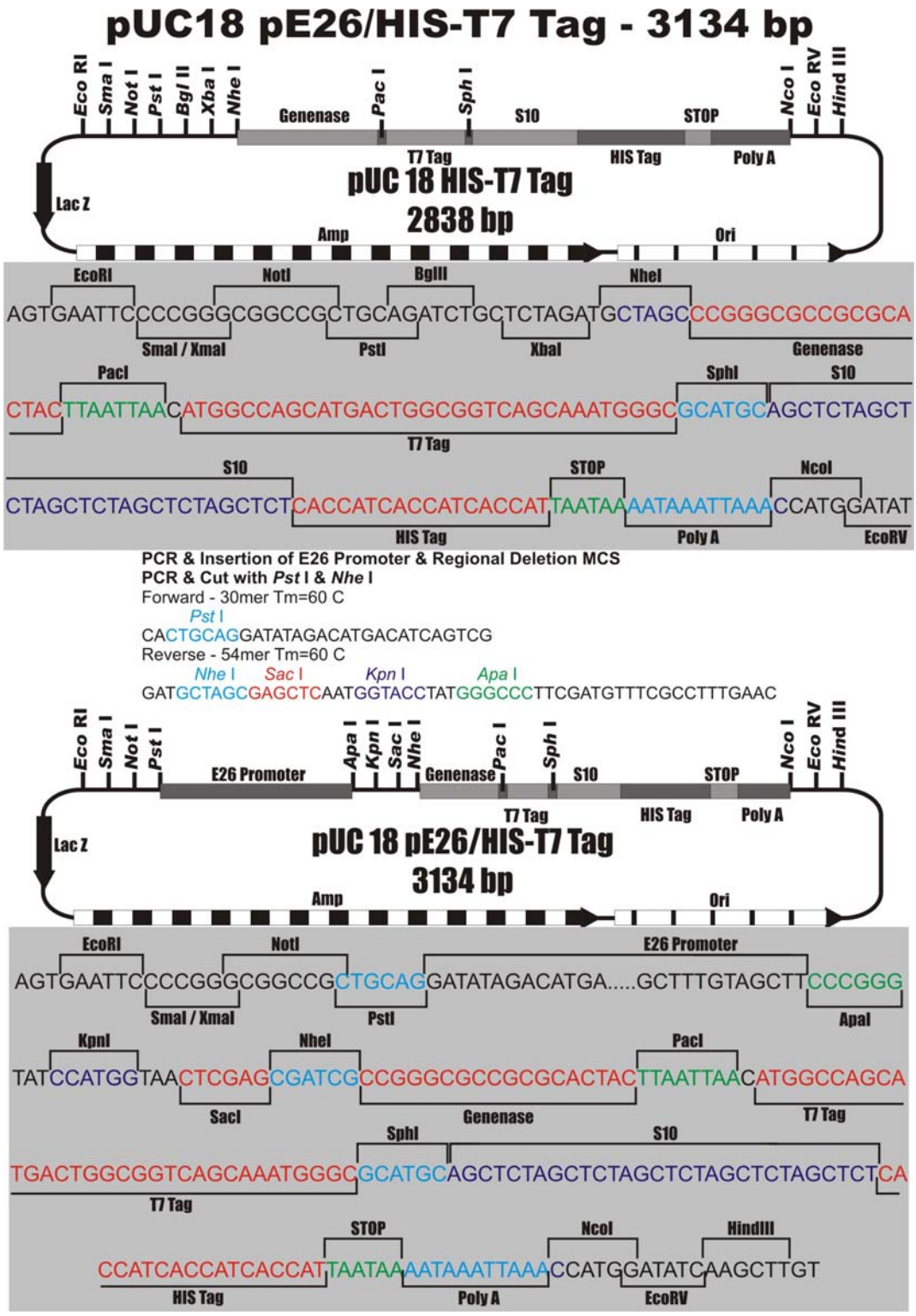
B23 : pUC18/19 HIS-T7 Tag



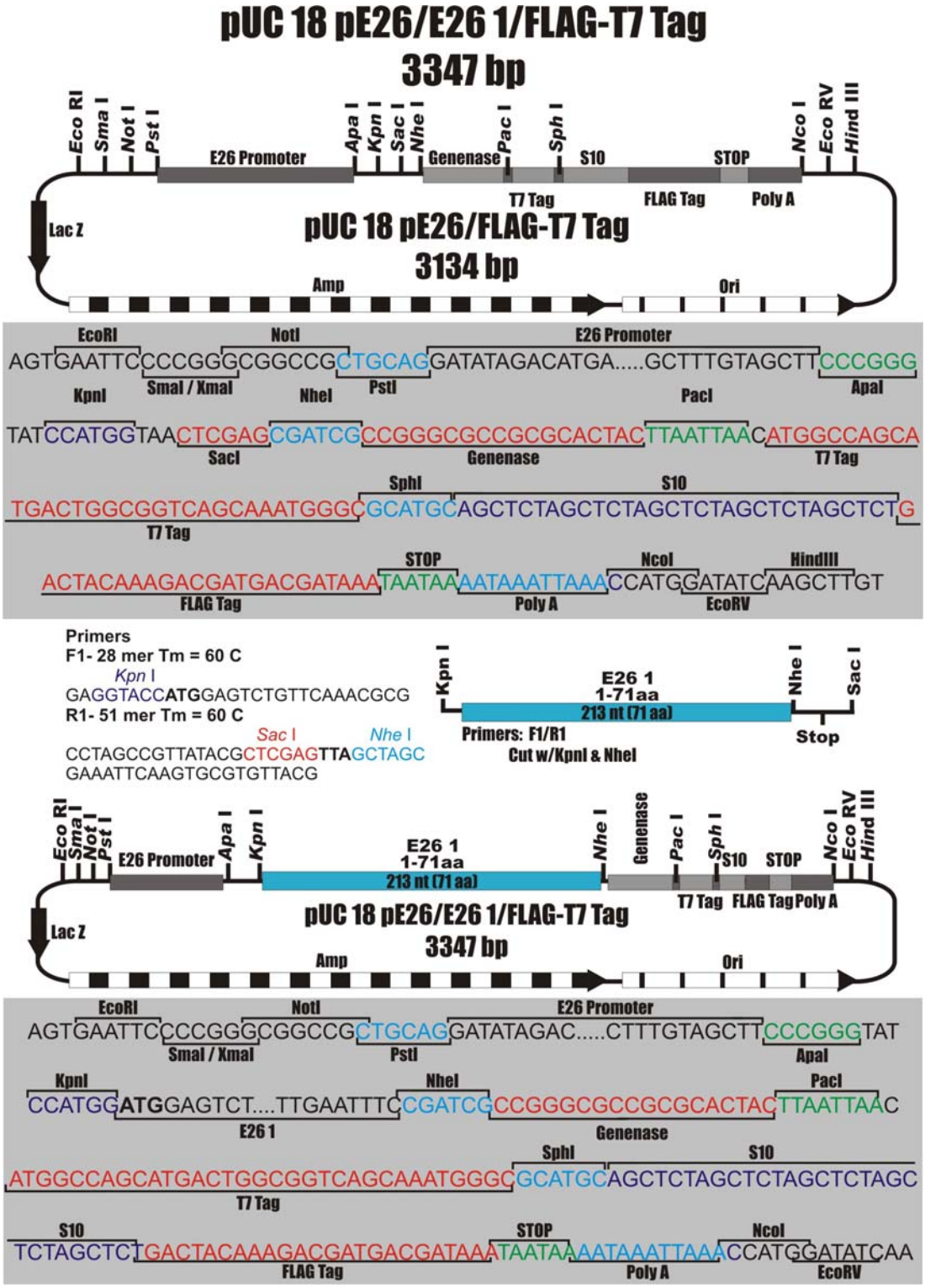
B24 : pUC18/pE26/FLAG-T7 Tag



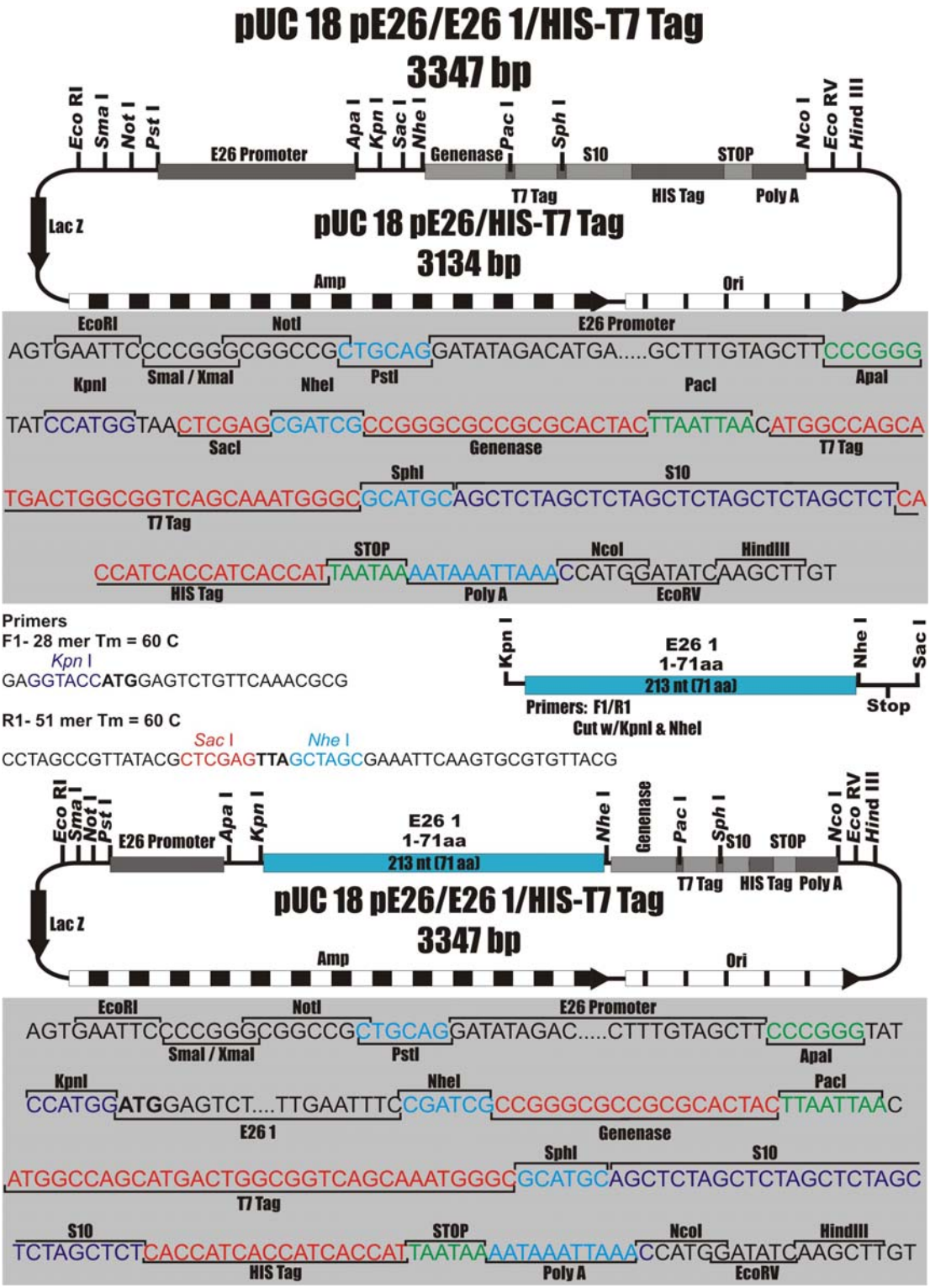
B25 : pUC18/pE26/HIS-T7 Tag



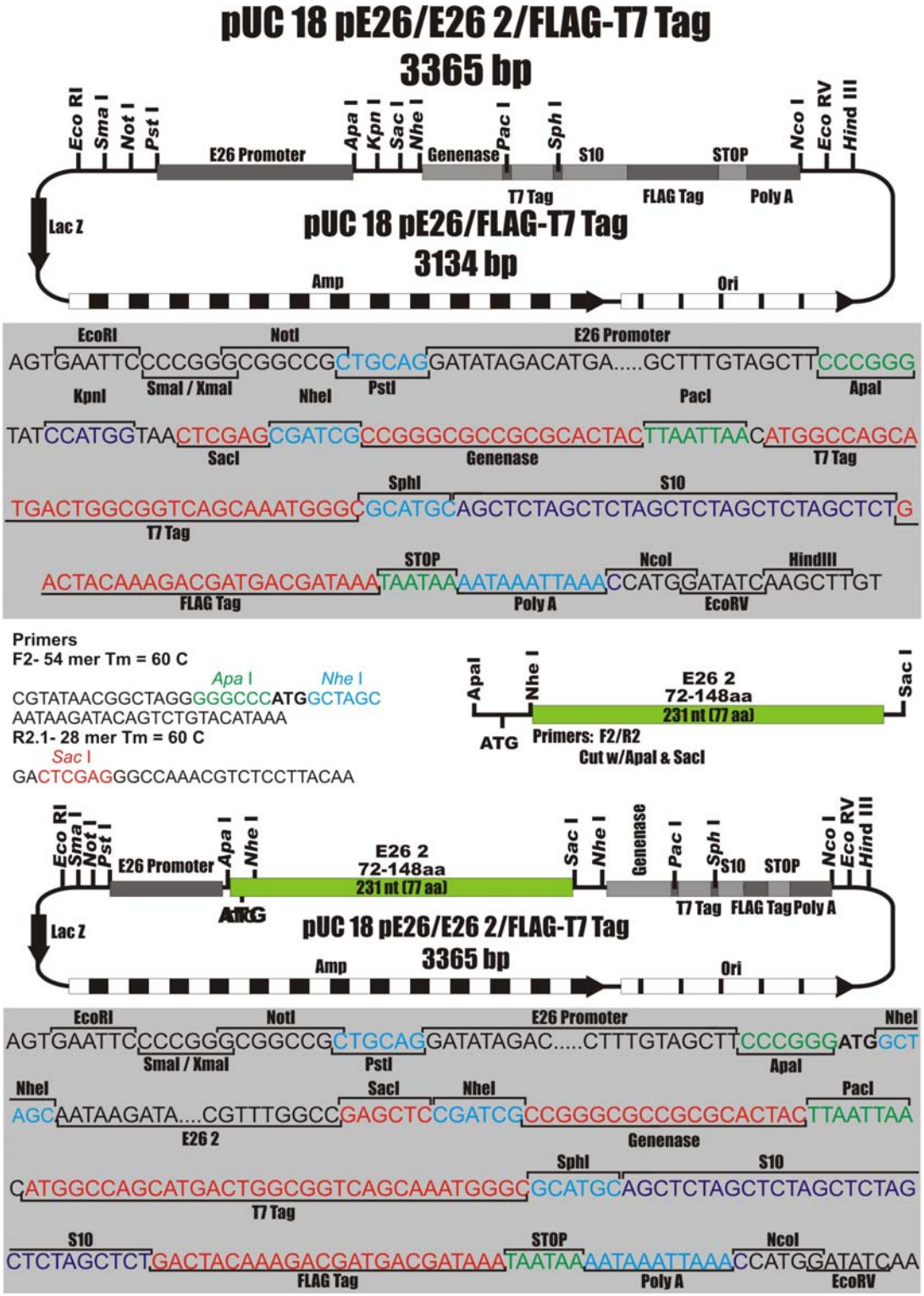
B26 : pUC18/pE26/E261/FLAG-T7



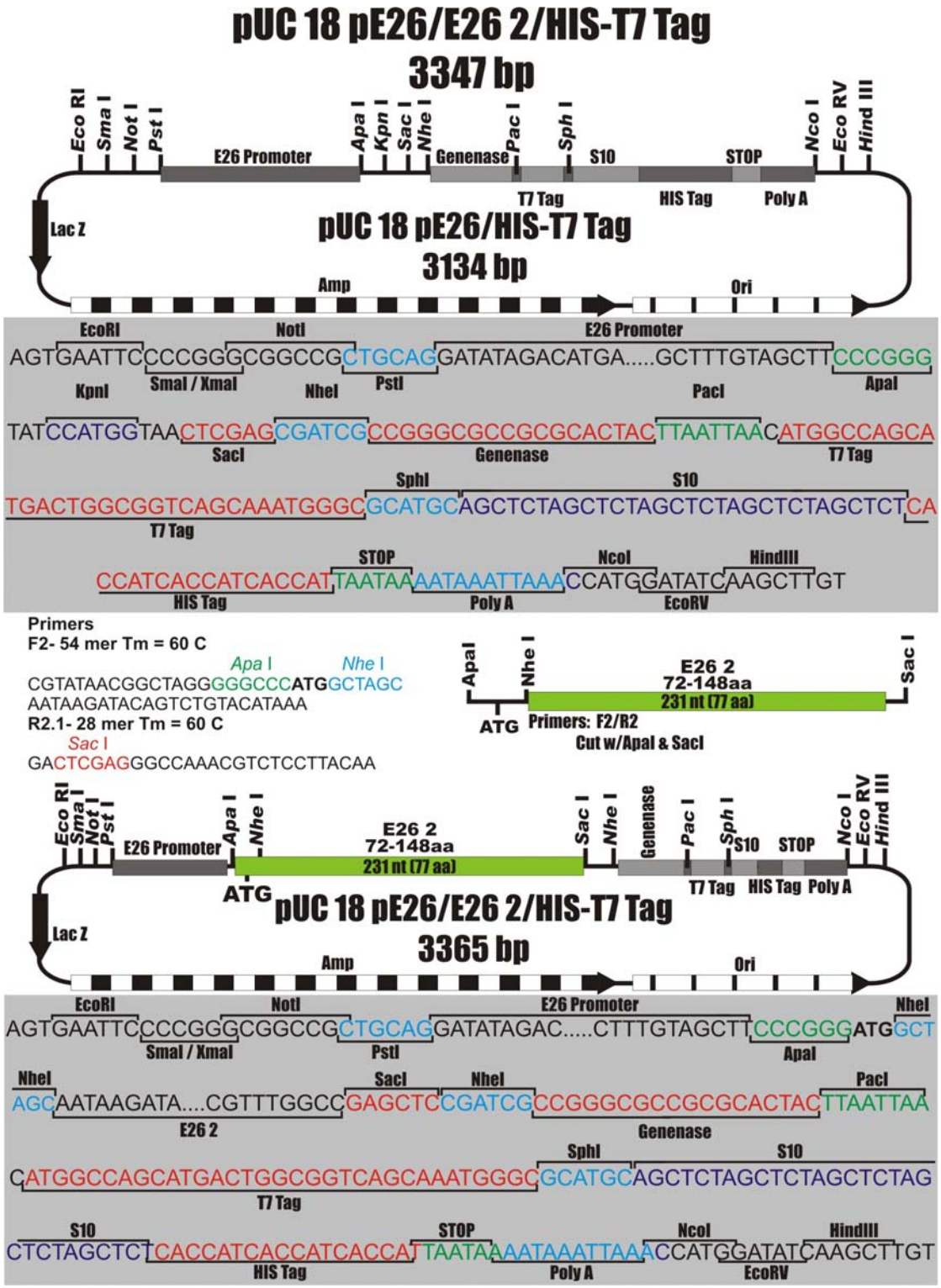
B27 : pUC18/pE26/E261/HIS-T7



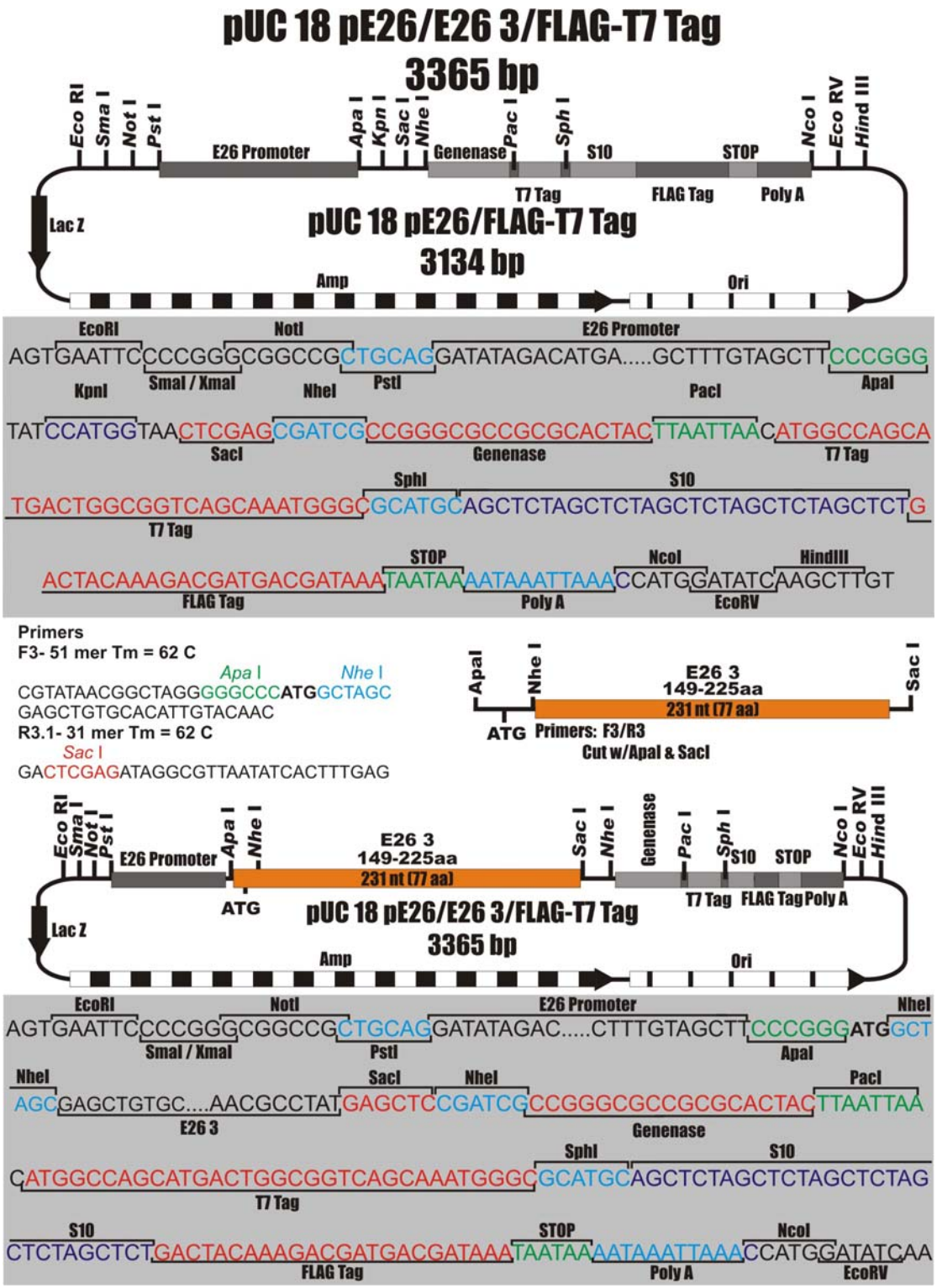
B28 : pUC18/ pE26/E26 2/FLAG-T7



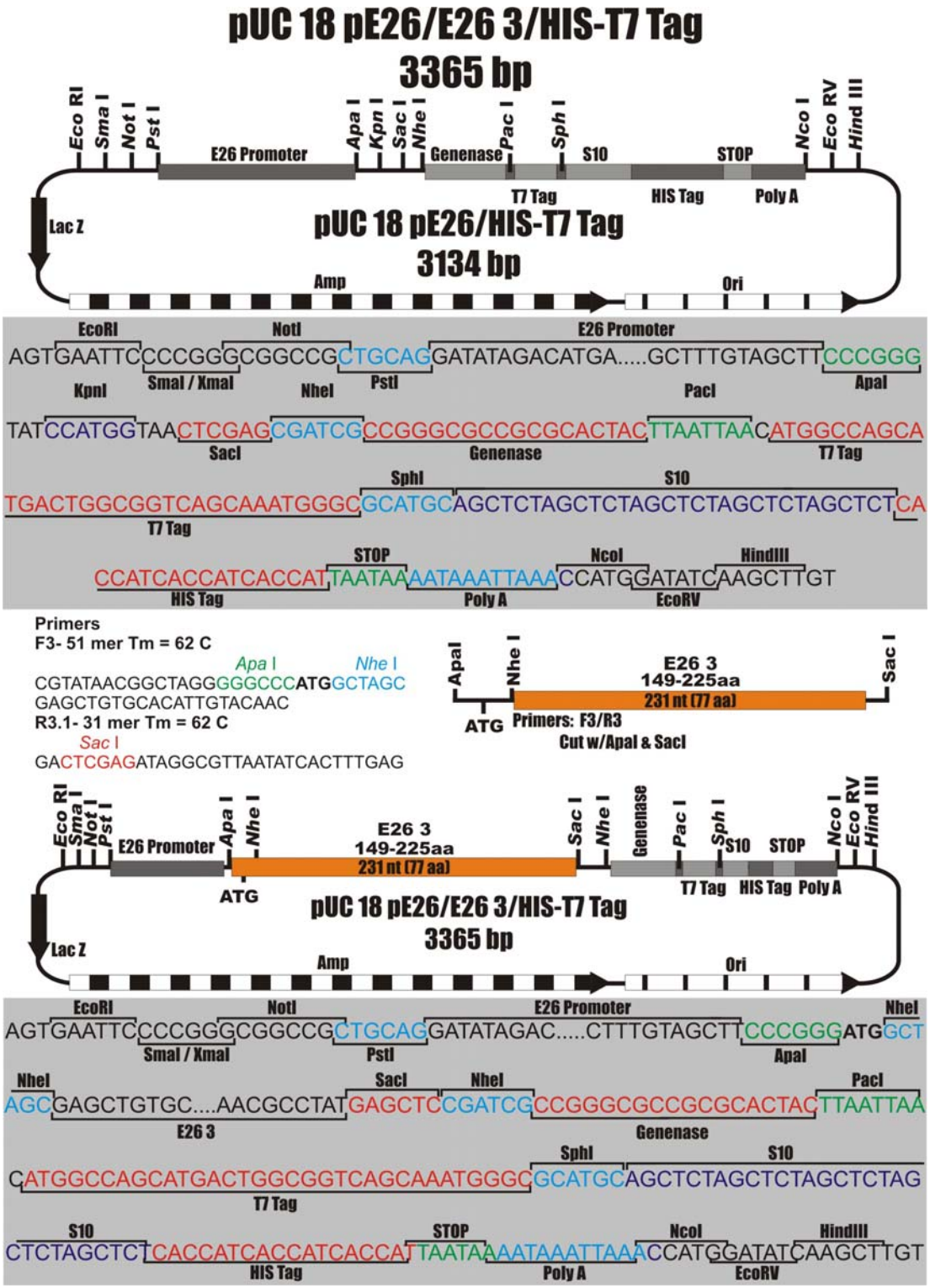
B29 : pUC18/pE26/E26



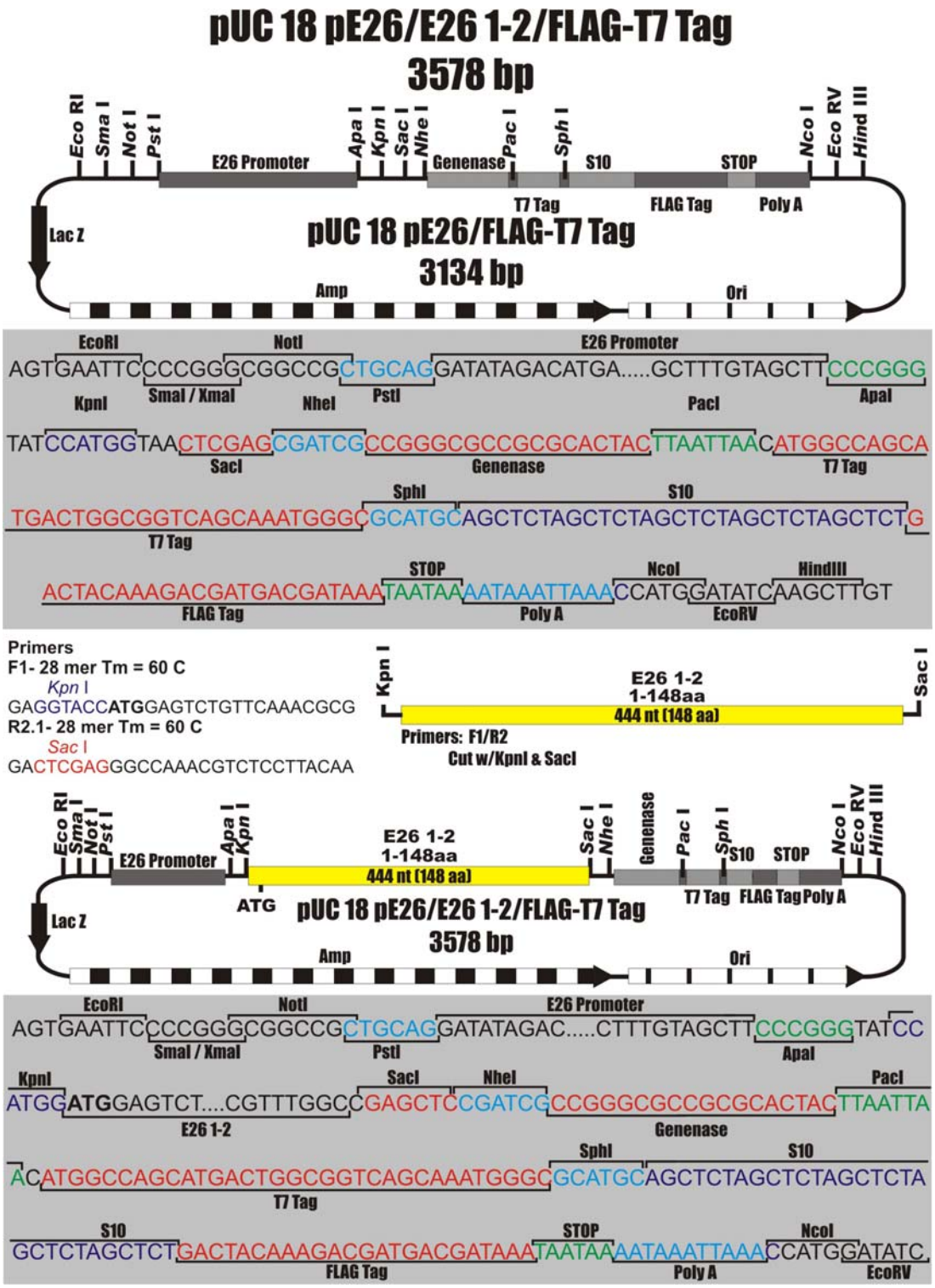
B30 : pUC18/pE26/E263/FLAG-T7



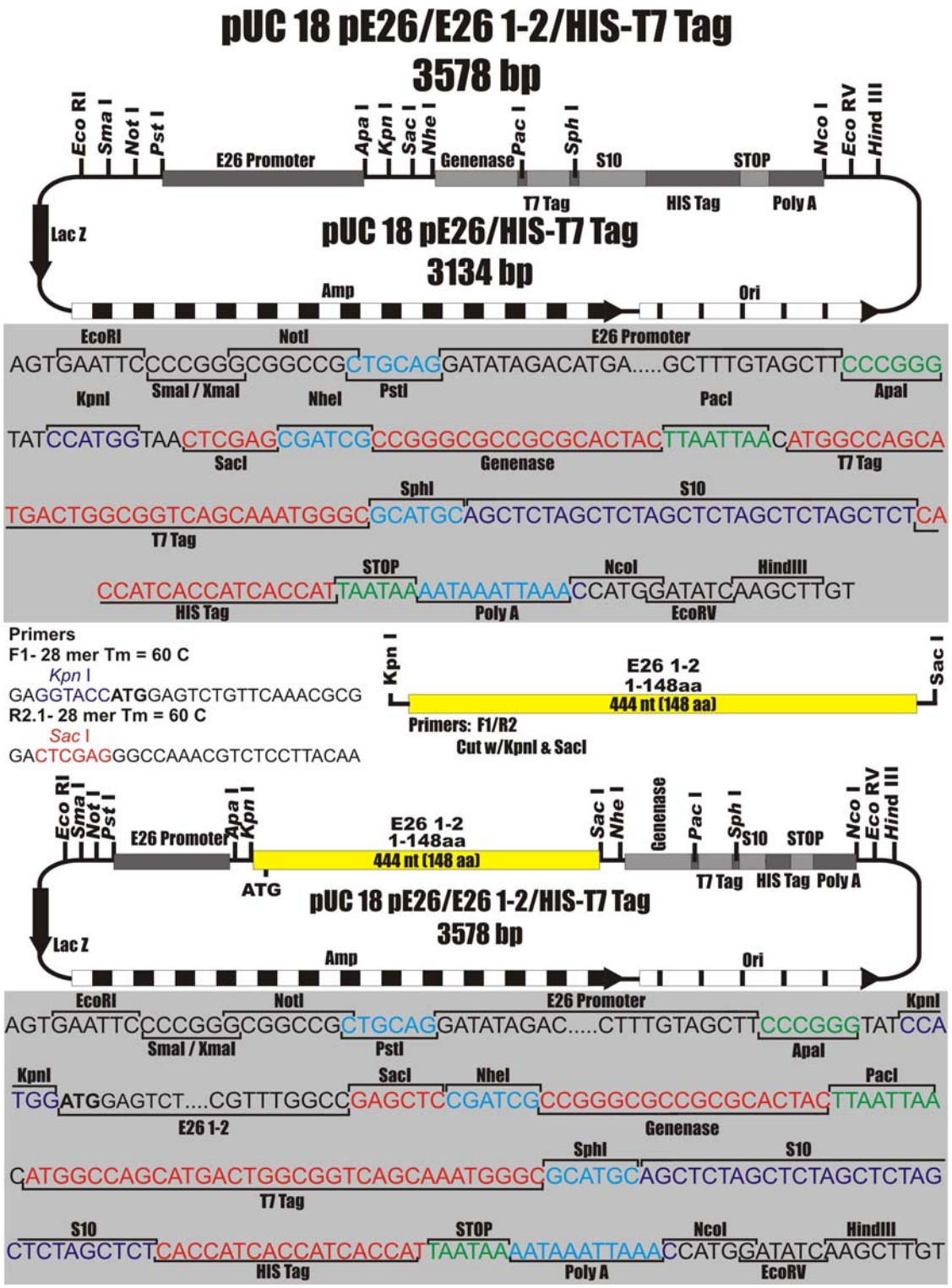
B31 : pUC18/pE26/E263/FLAG-T7



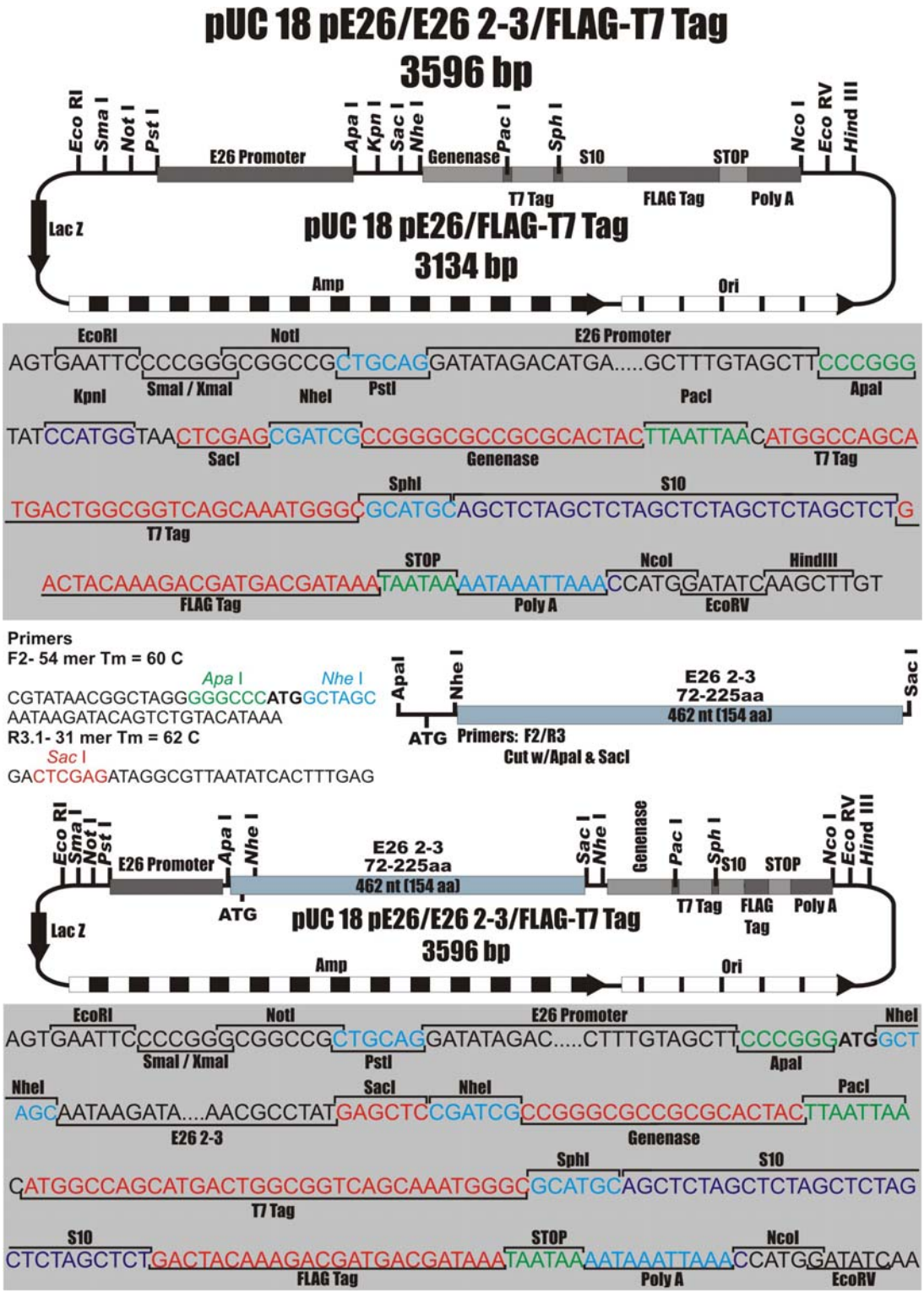
B32 : pUC18/pE26/E26 1-2/FLAG-T7



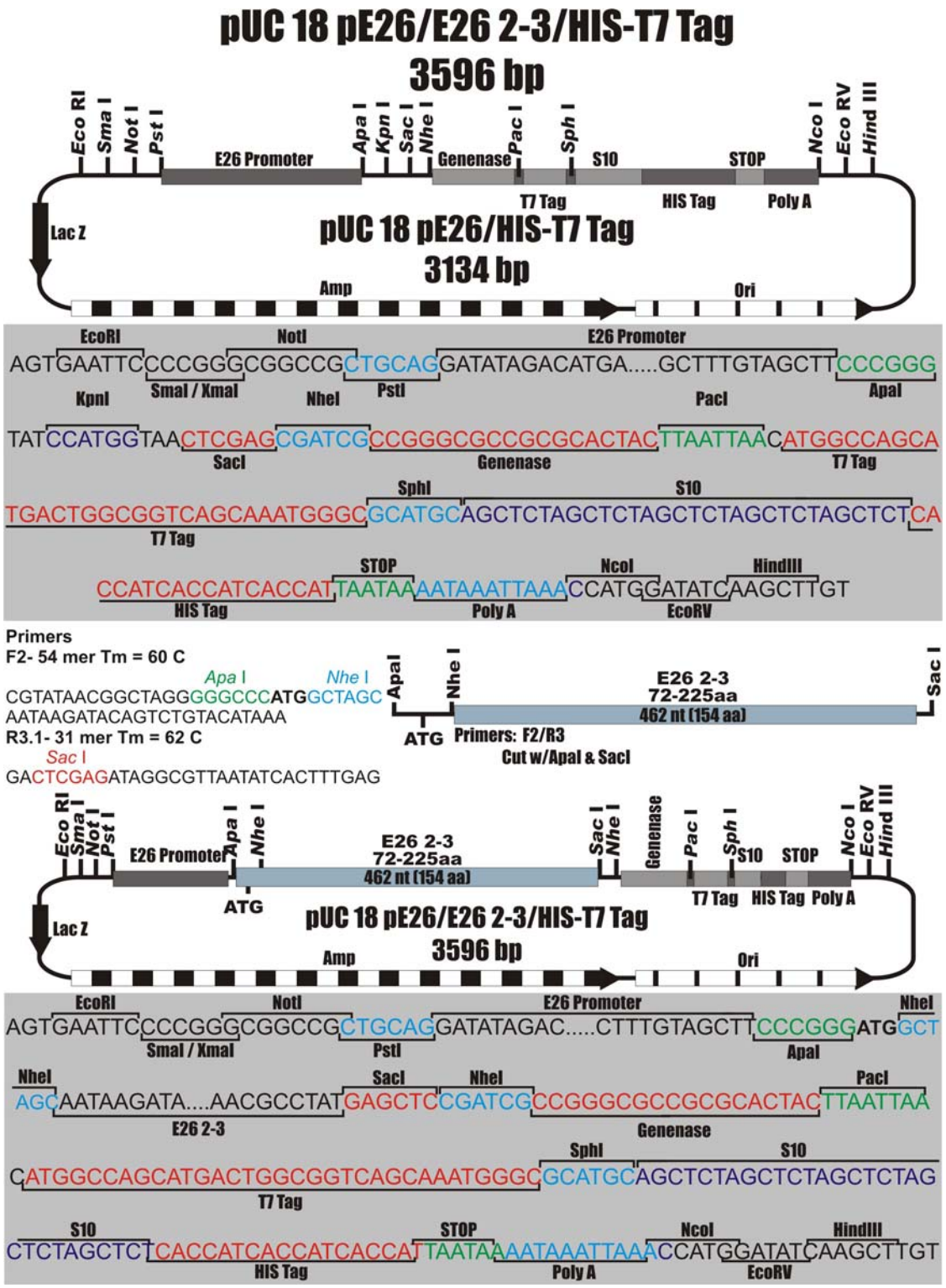
B33 : pUC18/pE26/E26 1-2/HIS T7



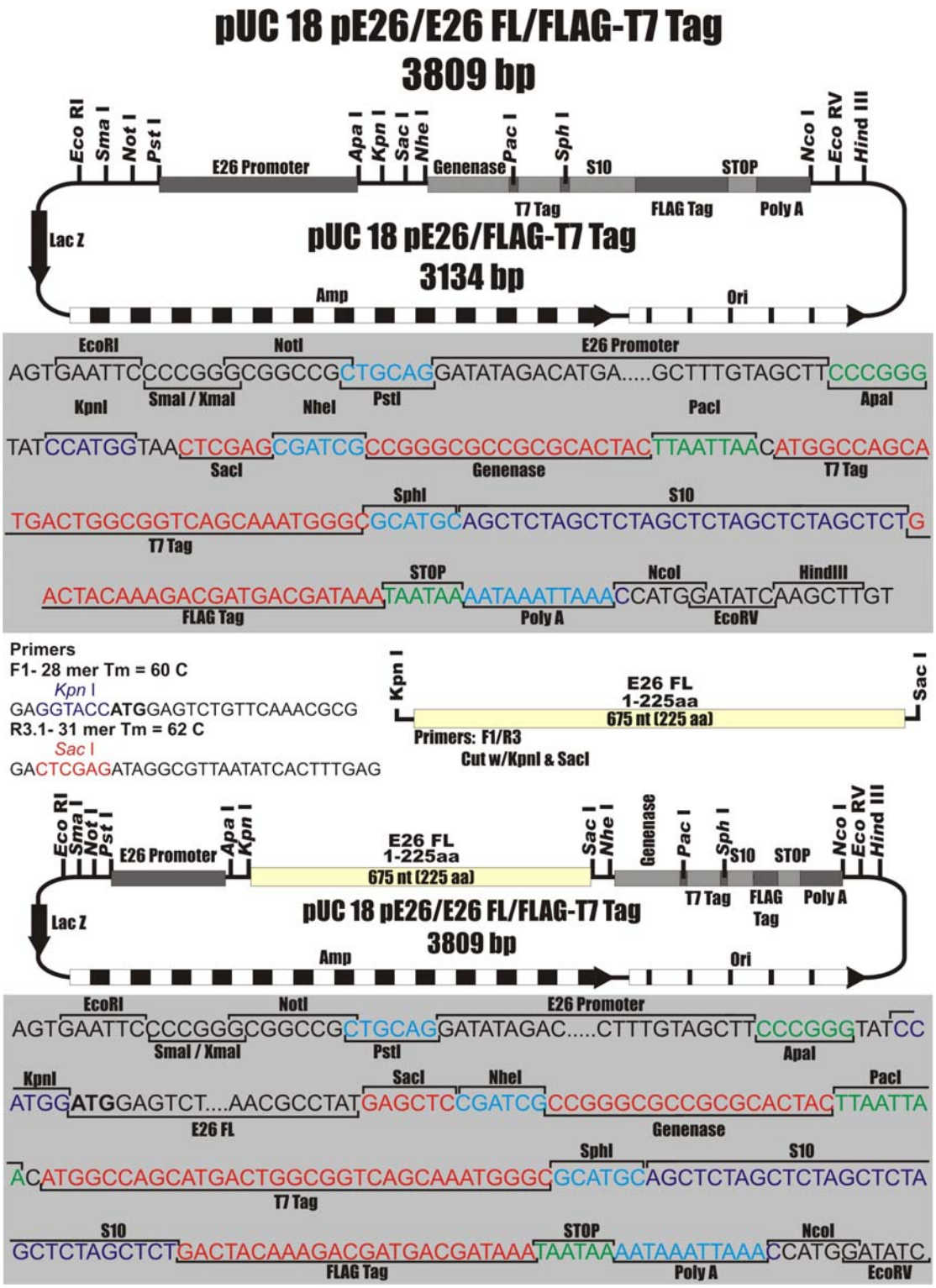
B34 : pUC18/pE26/E26 2-3/FLAG-T7



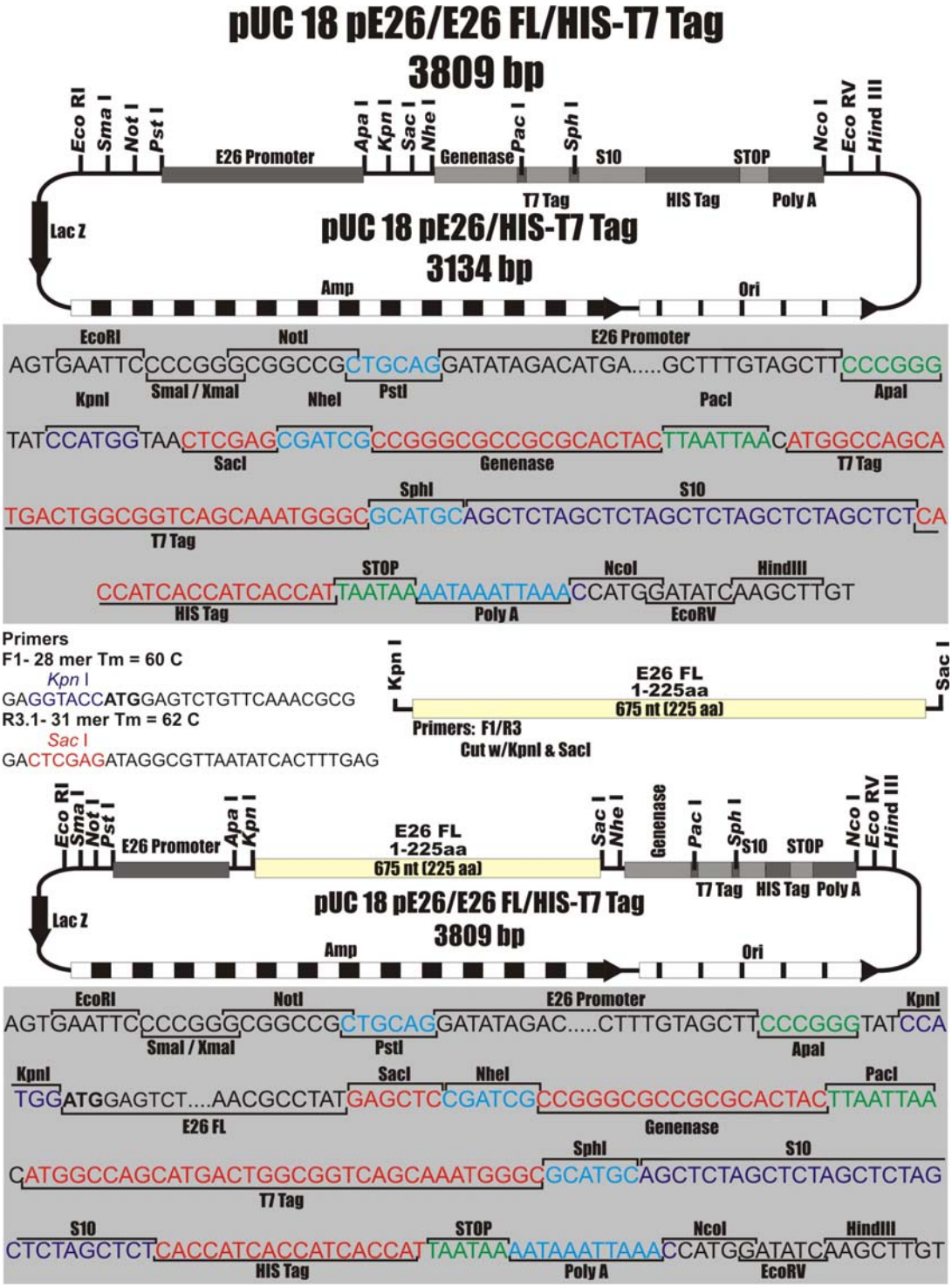
B35 : pUC18/pE26/E26 2-3/HIS-T7



B36 : pUC18/pE26/E26 FL/FLAG-T7



B37 : pUC18/pE26/E26 FL/HIS-T7

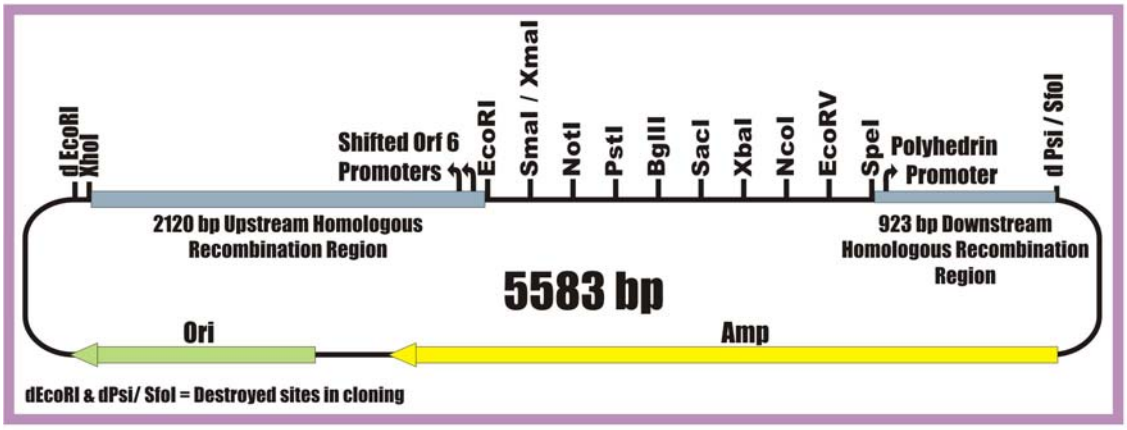


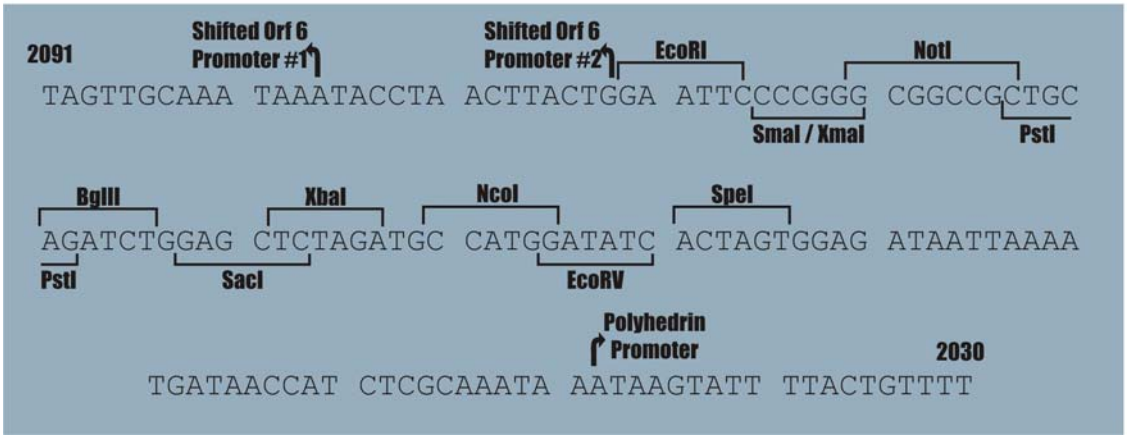
B38 : pEcoRV-MCS

EcoRV-MCS Recombinant Virus Clone

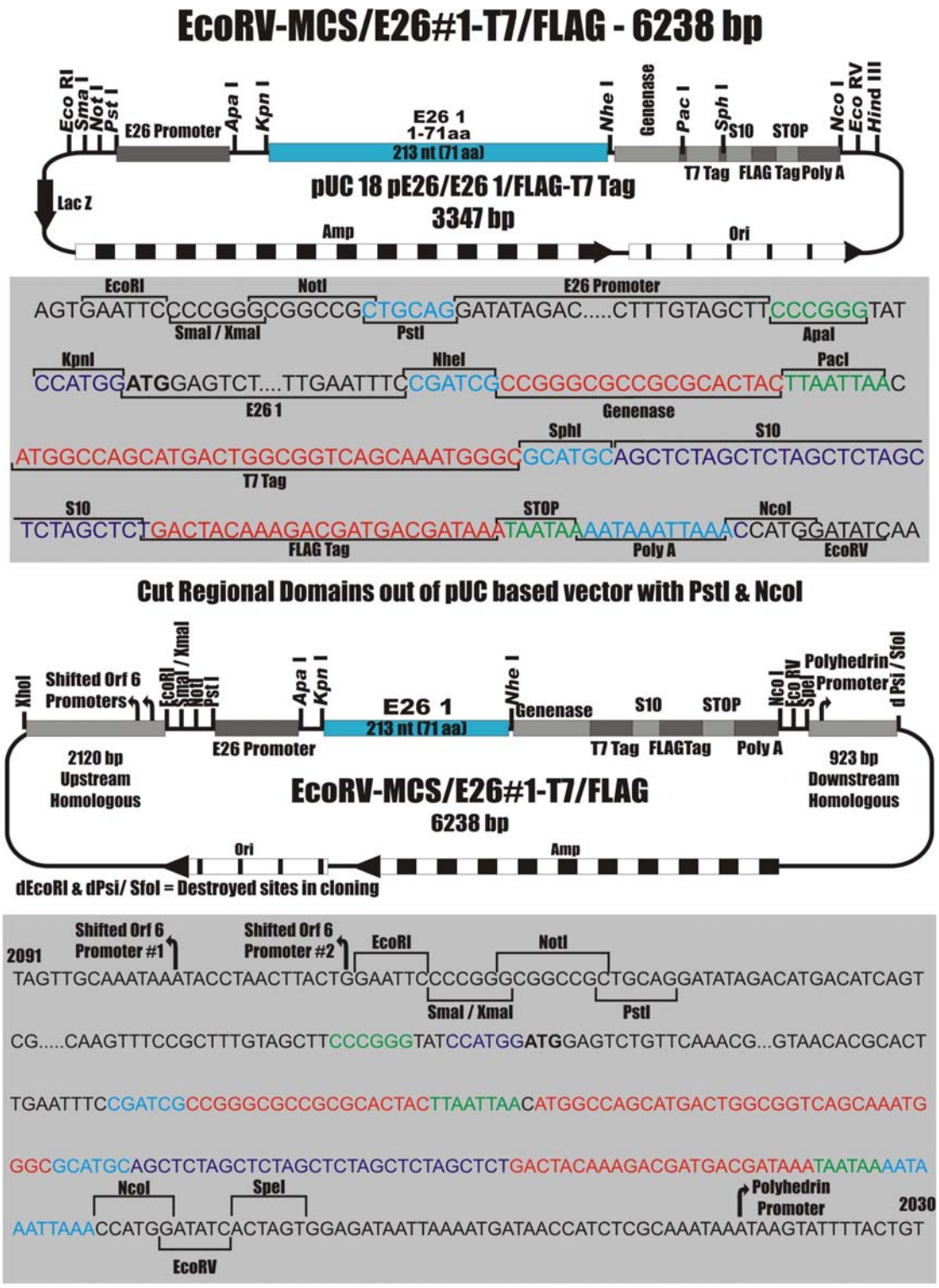
EcoRV-MCS recombinant virus clone allows for insertion of your gene and promoter of interest. This vector is based on a modified pUC18. The modified pUC 18 is missing the majority of the *lac Z* gene and has had the EcoRI site destroyed as noted below. The map also shows the relative positions of the coding sequences and the origin of replication. The exact position are: ampicillin 3441-4401; multiple cloning site 2120-2176; ColE1 compatibility group origin of replication 5277.

The homologous recombination regions are 2120 and 923 bp respectively. The insertion of the MCS required shifting the Orf 6 promoters upstream of the MCS. The polyhedrin promoter is located downstream of the MCS. This region of the AcMNPV genome has been cloned into a modified pUC18 between XhoI and SfoI. The AcMNPV region was created by PCR, cut using XhoI and PsiI, and ligated into the XhoI/SfoI of the modified pUC18. The blunt SfoI site was ligated to the blunt PsiI site destroying both as noted below.

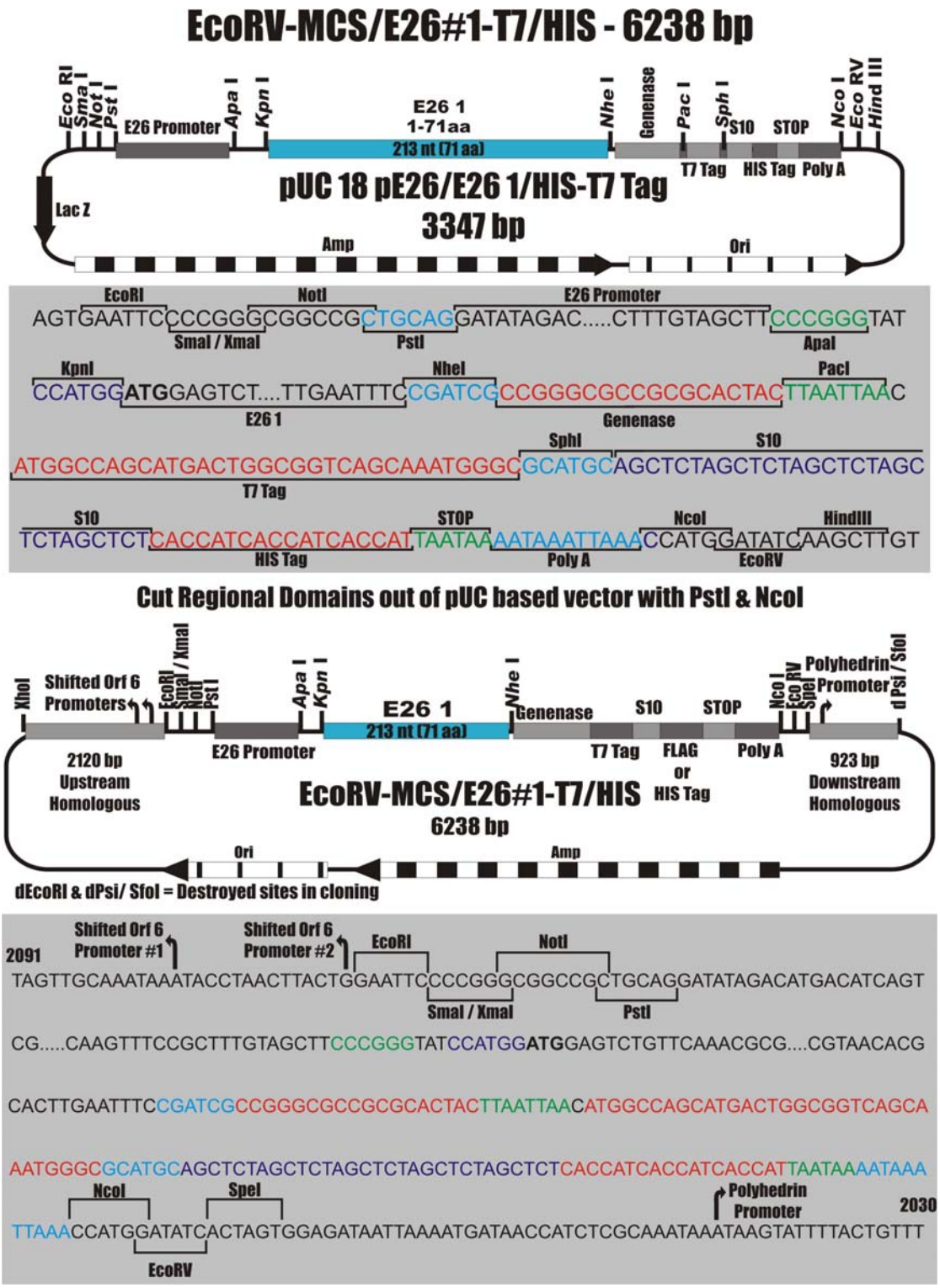




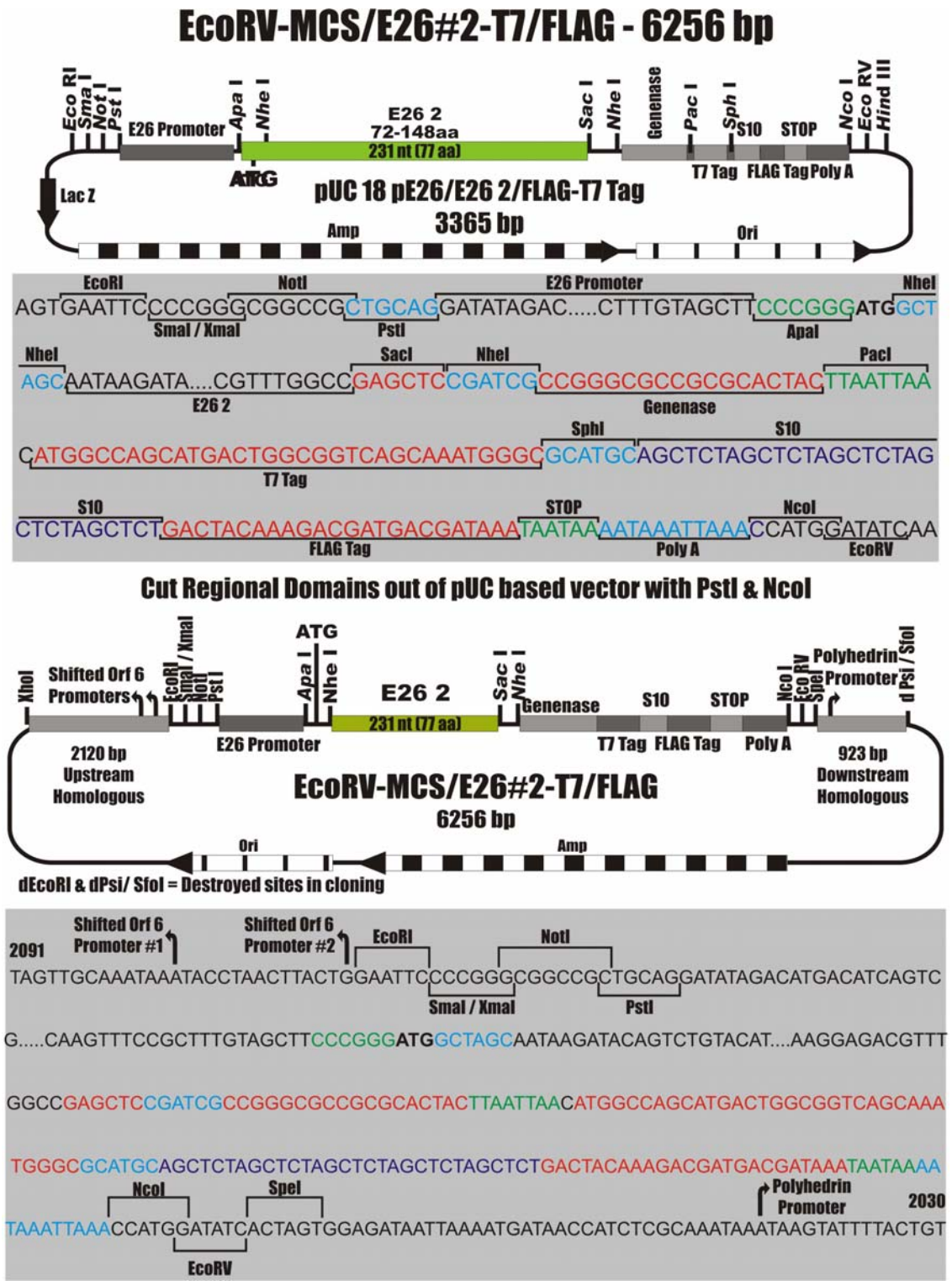
B39 : pEcoRV-MCS/E26 1/FLAG-T7



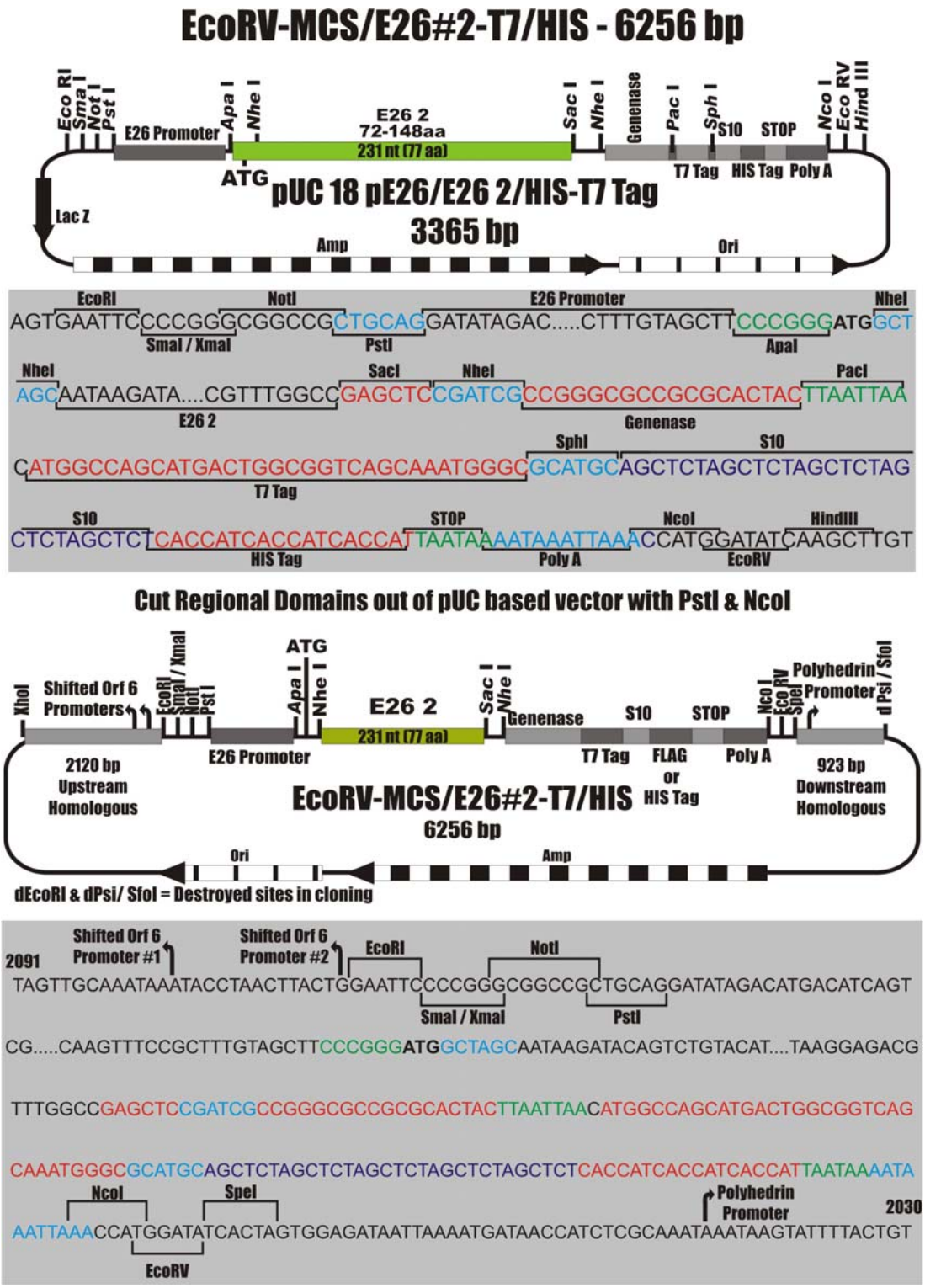
B40 : pEcoRV-MCS//E26 1/HIS-T7



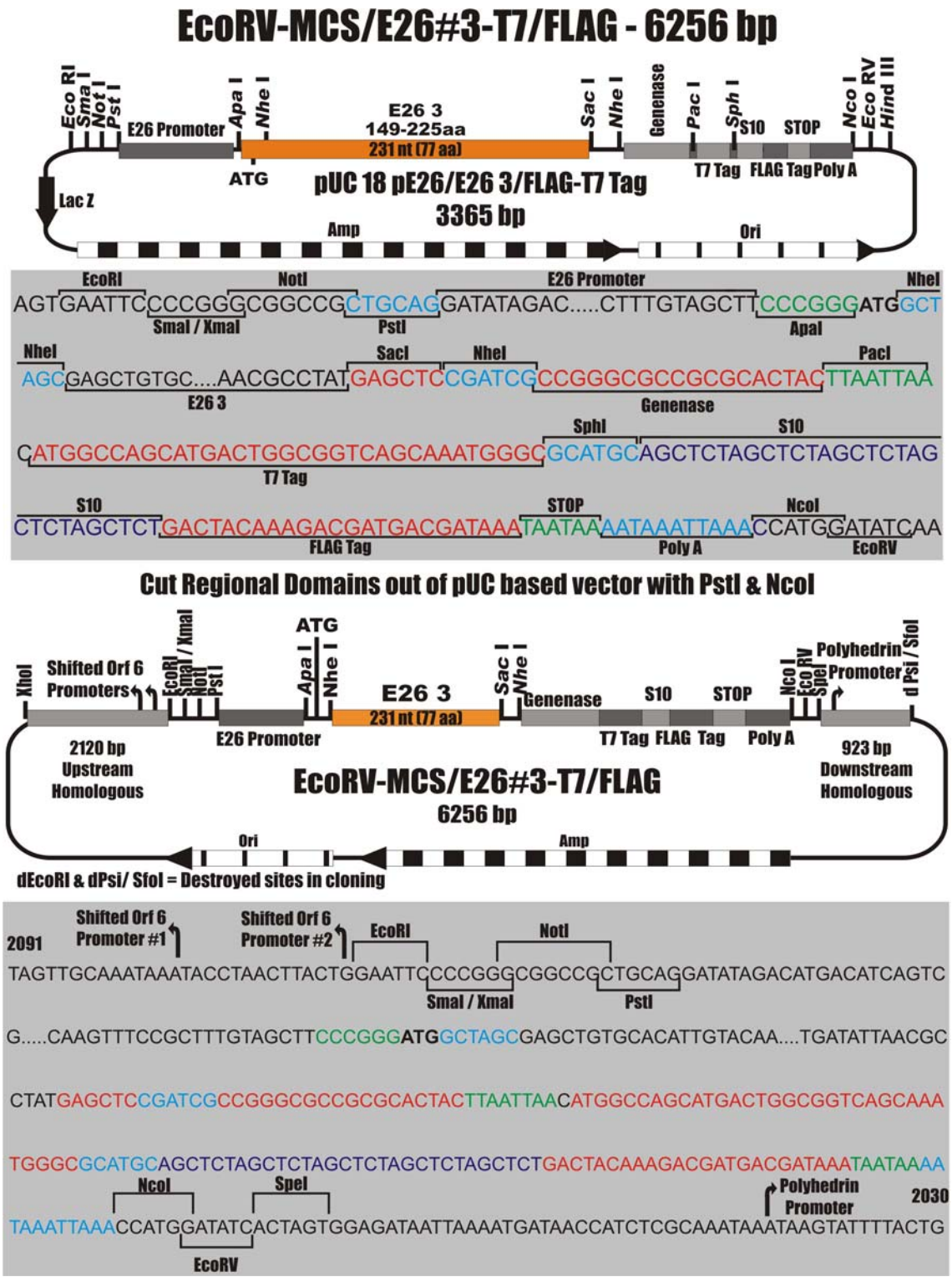
B41 : pEcoRV-MCS/E26 2/FLAG-T7



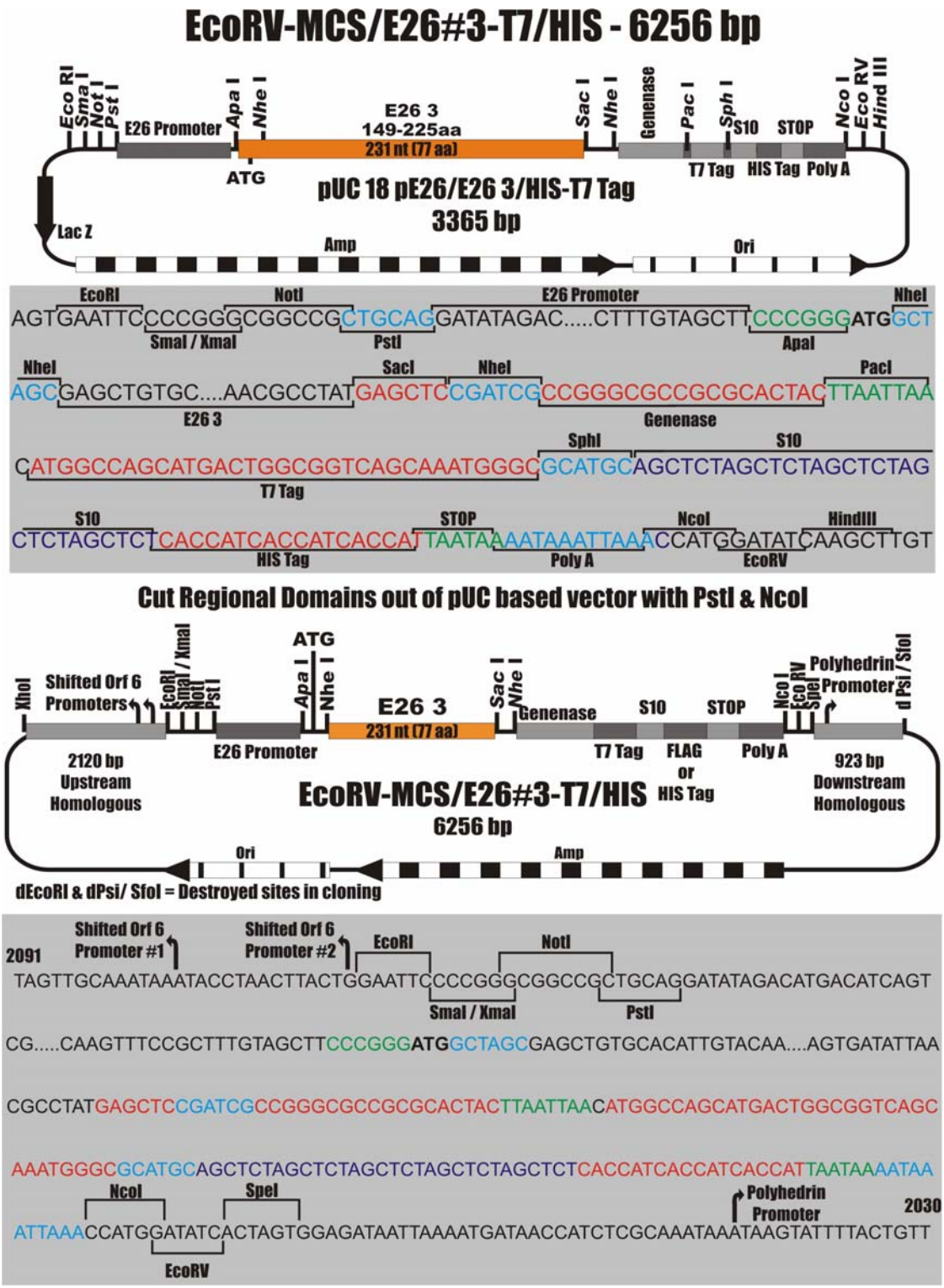
B42 : pEcoRV-MCS/E26 2/HIS-T7



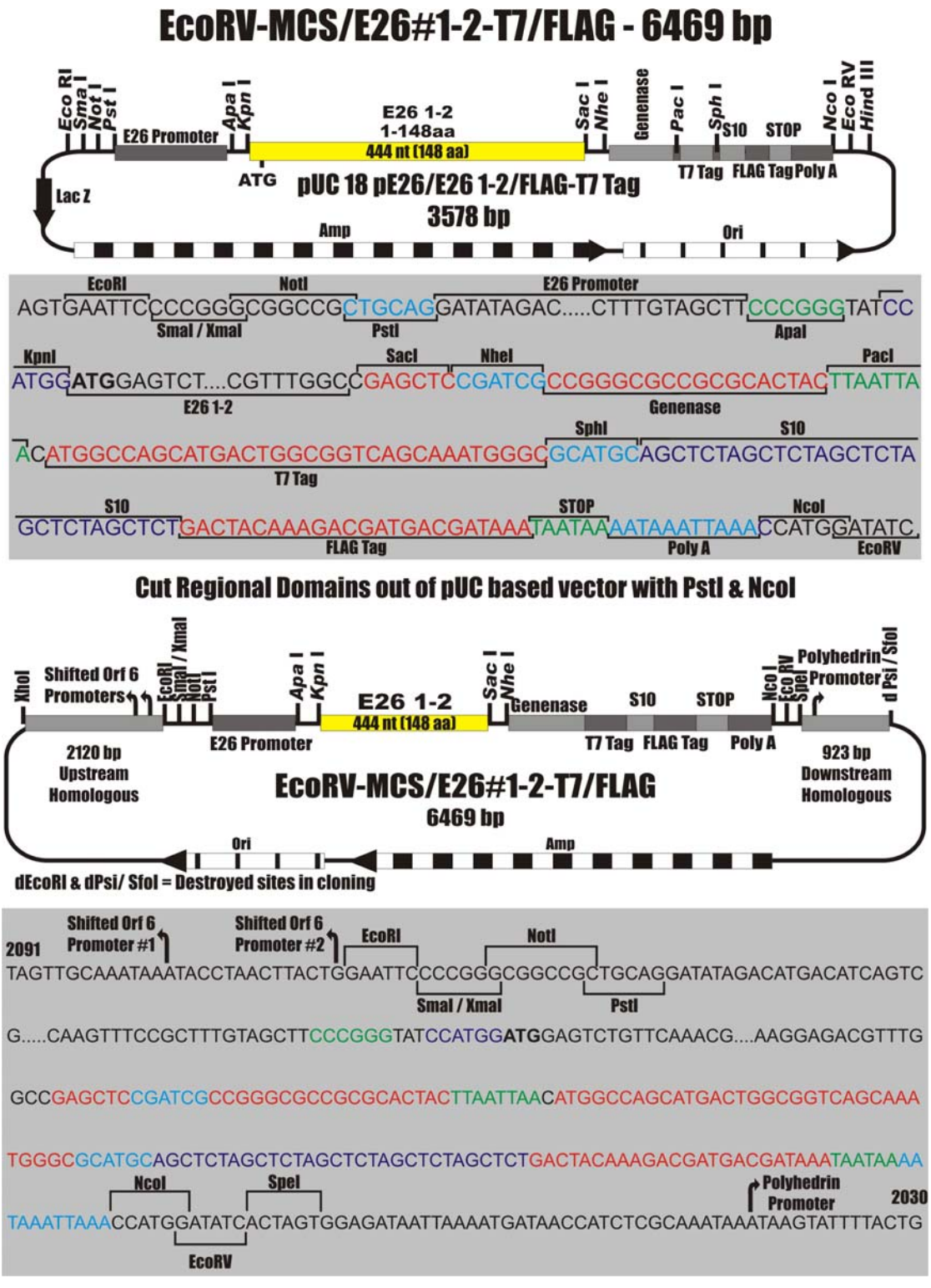
B43 : pEcoRV-MCS/E26 3/FLAG-T7



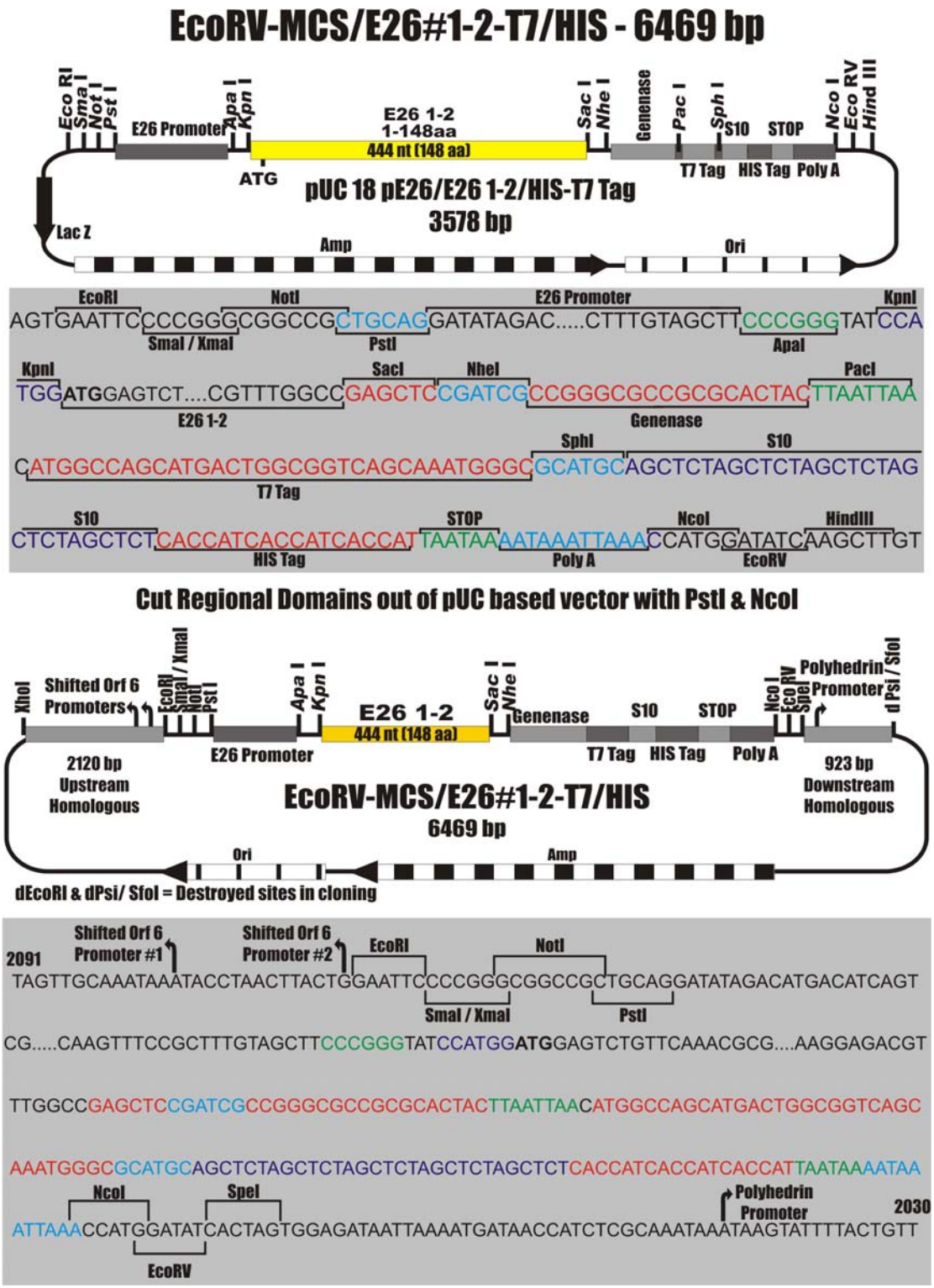
B44 : pEcoRV-MCS/E26 3/HIS-T7



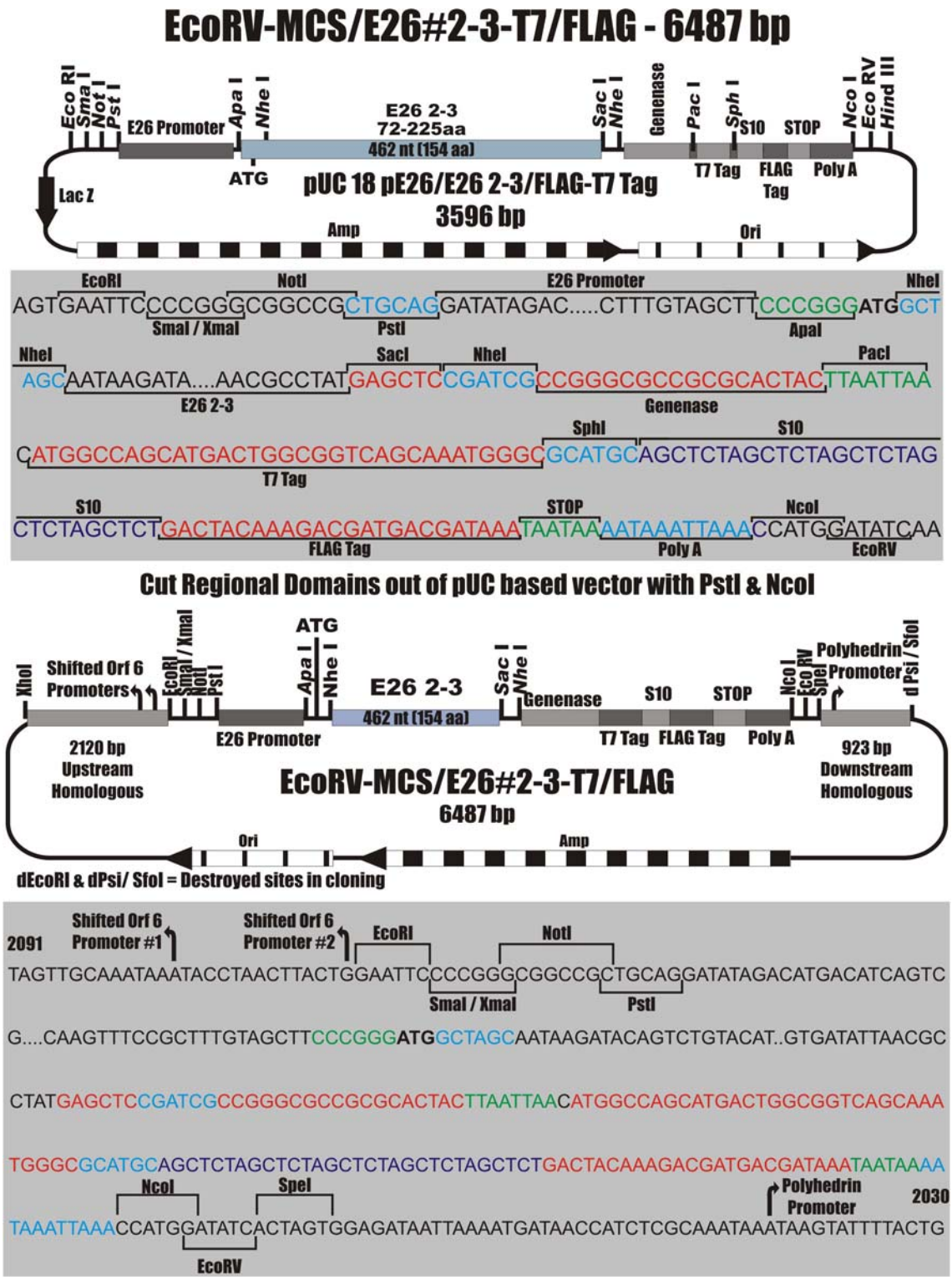
B45 : pEcoRV-MCS/E26 1-2/FLAG-T7



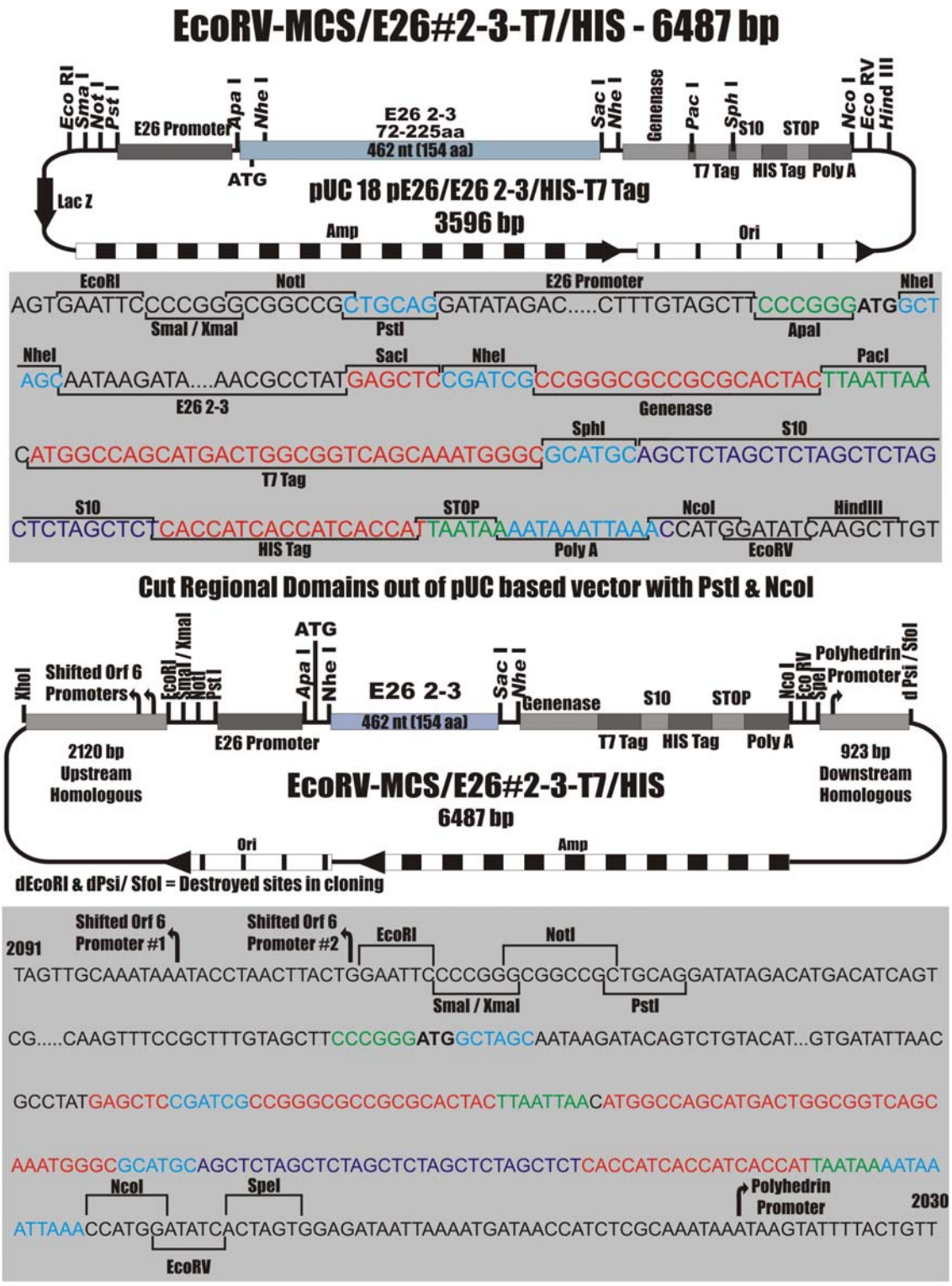
B46 : pEcoRV-MCS/E26 1-2/HIS-T7



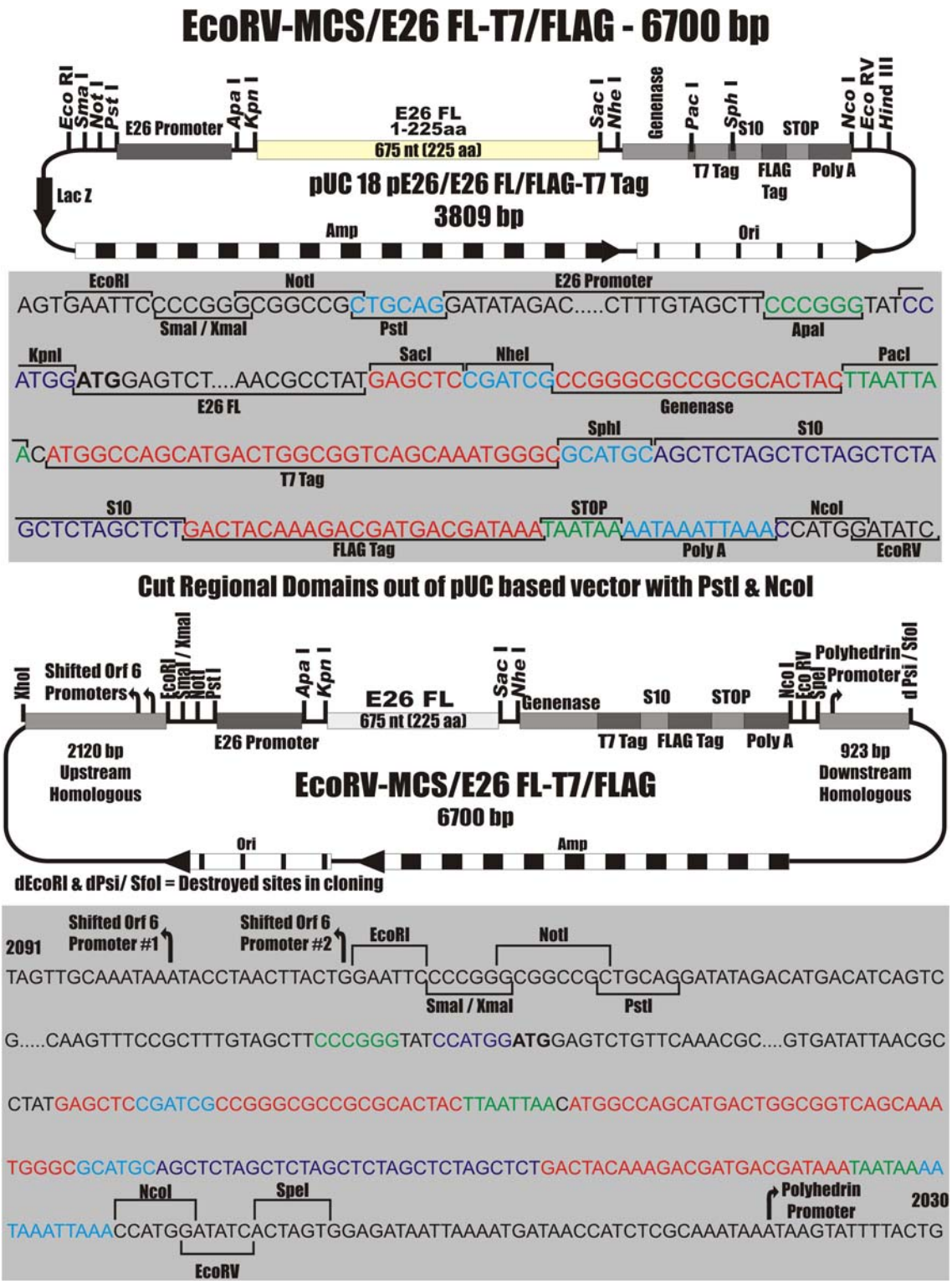
B47 : pEcoRV-MCS/E26 2-3/FLAG-T7



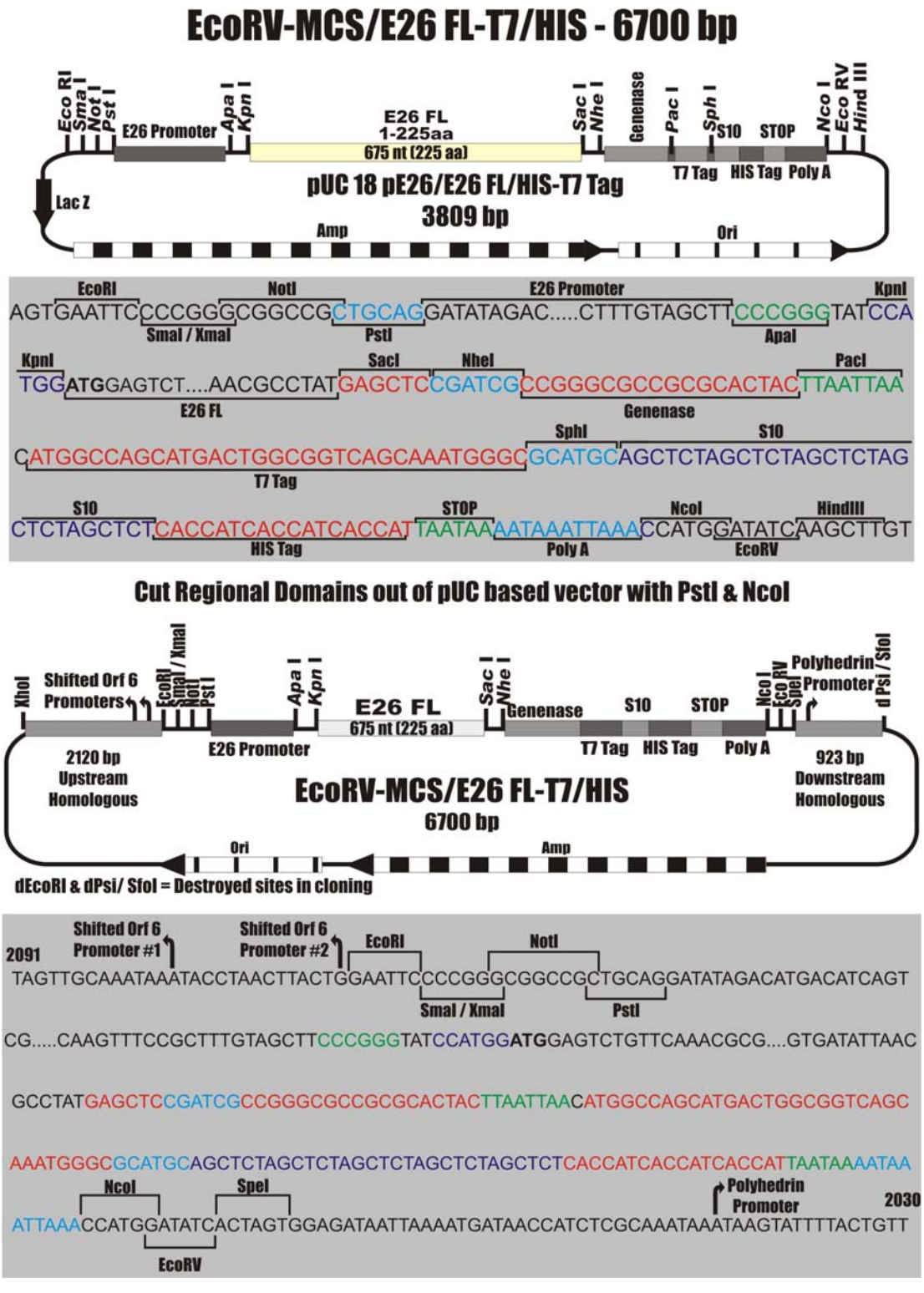
B48 : pEcoRV-MCS/E26 2-3/HIS-T7



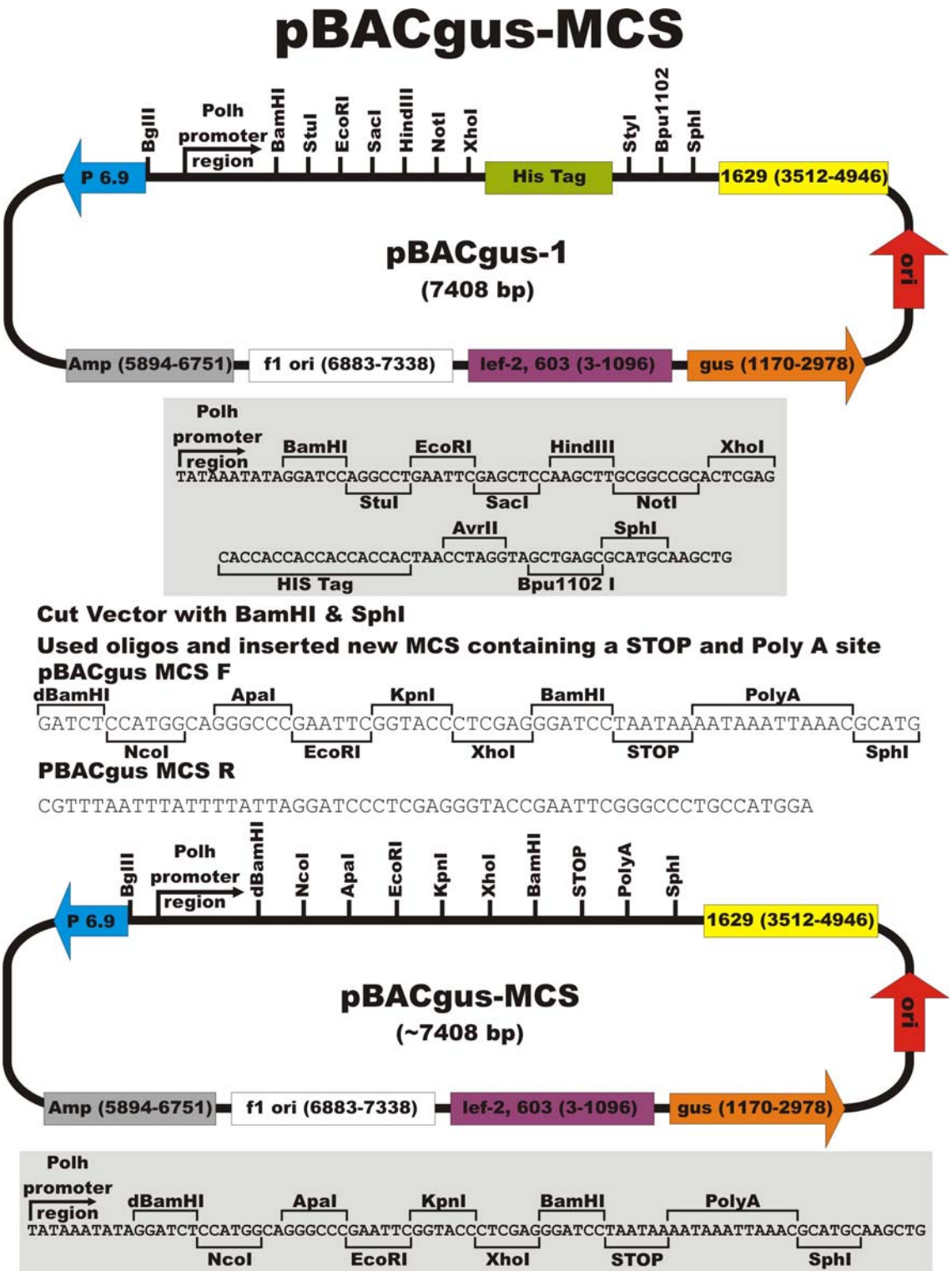
B49 : pEcoRV-MCS/E26 FL-FLAG-T7



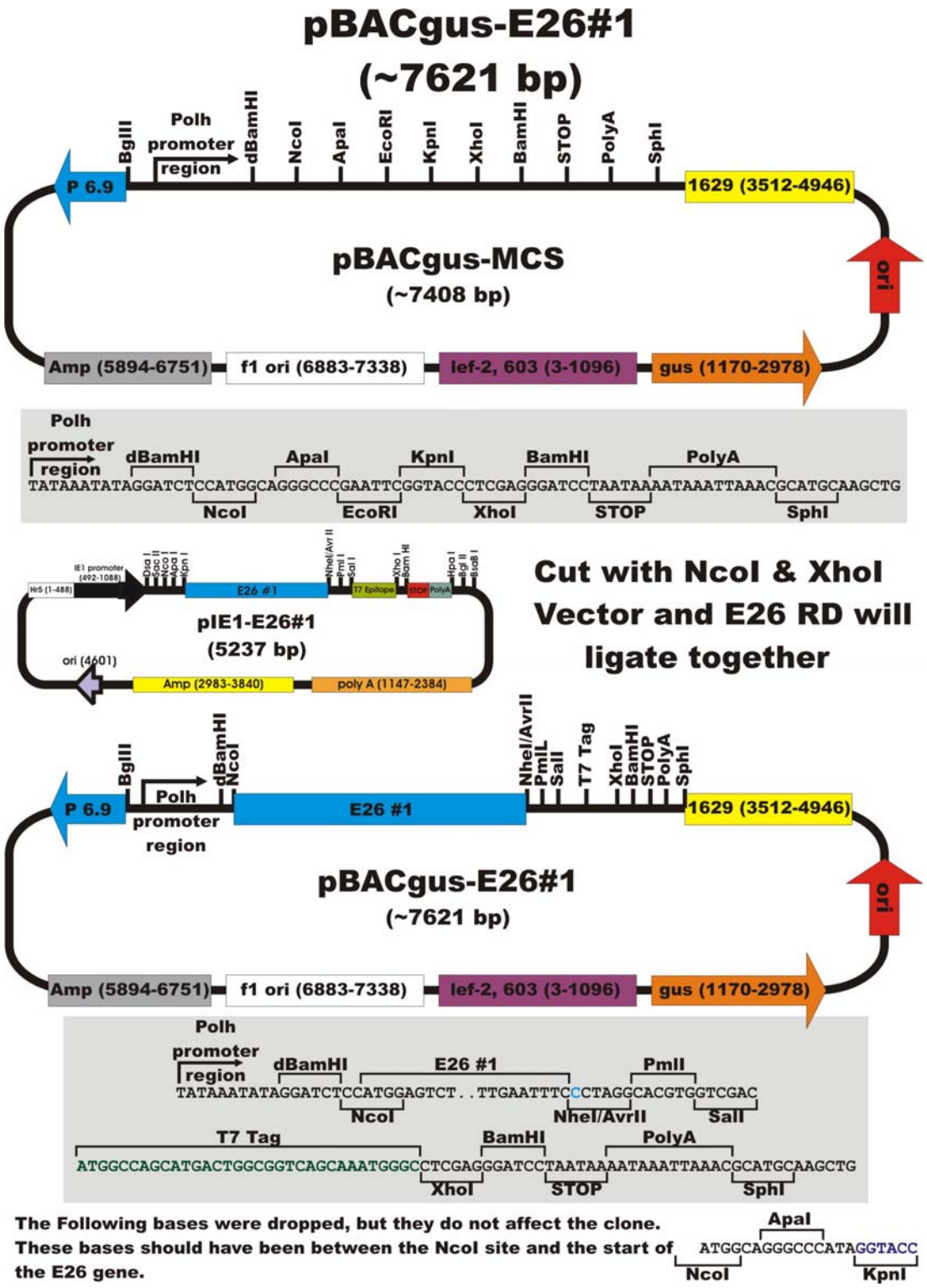
B50 : pEcoRV-MCS/E26 FL/HIS-T7



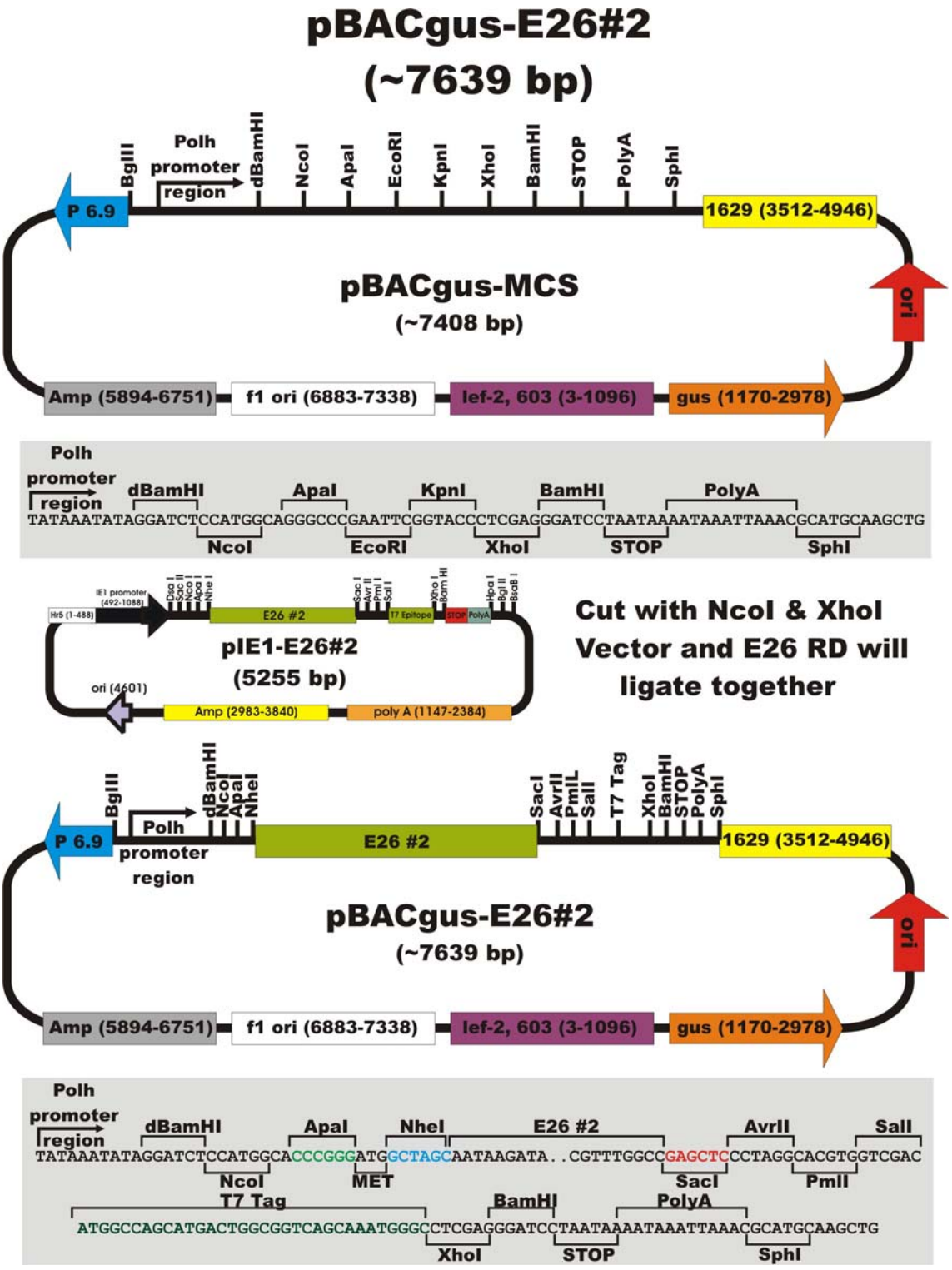
B51 : pBACgus-MCS



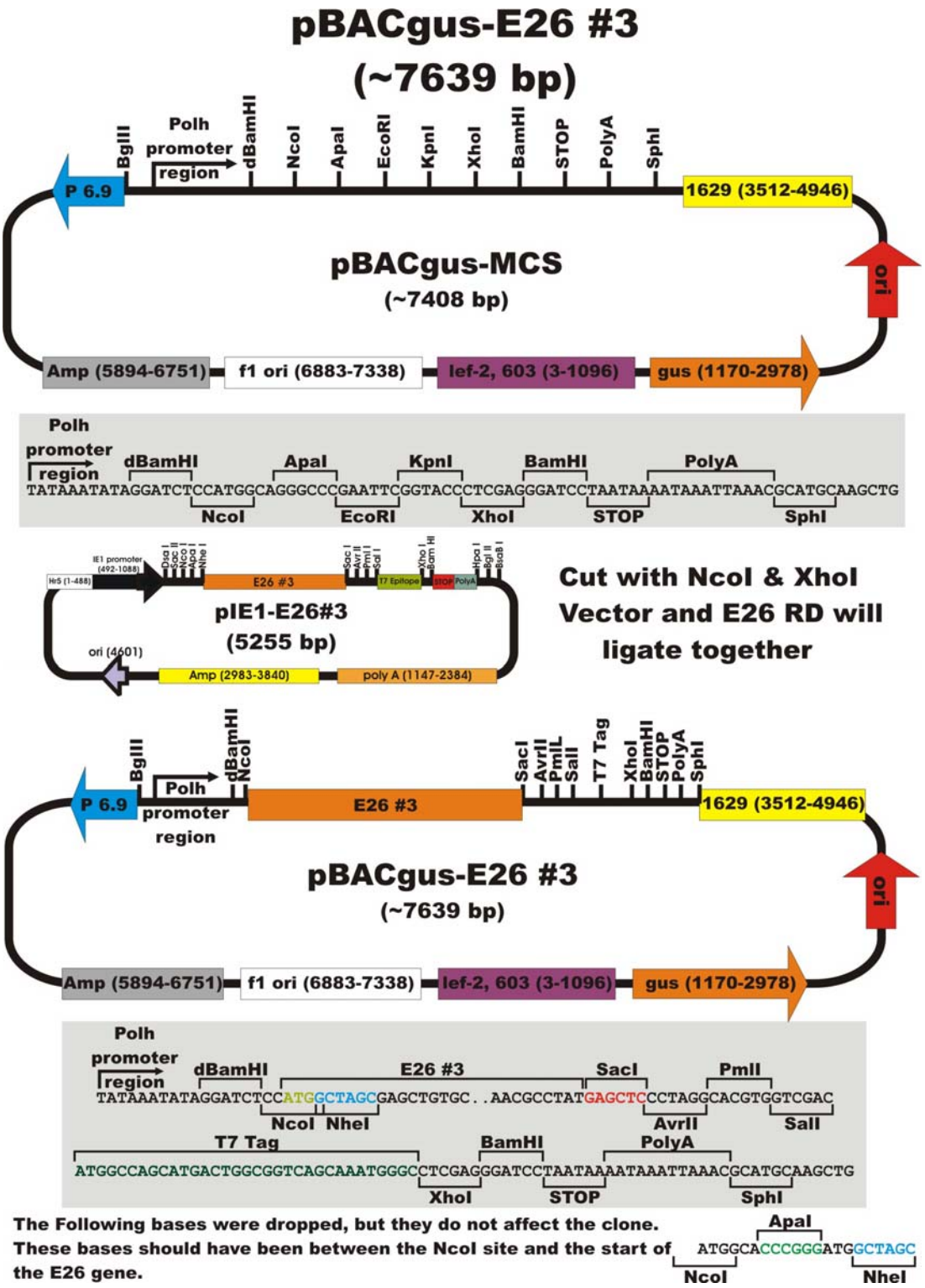
B52 : pBACgus-E26 1



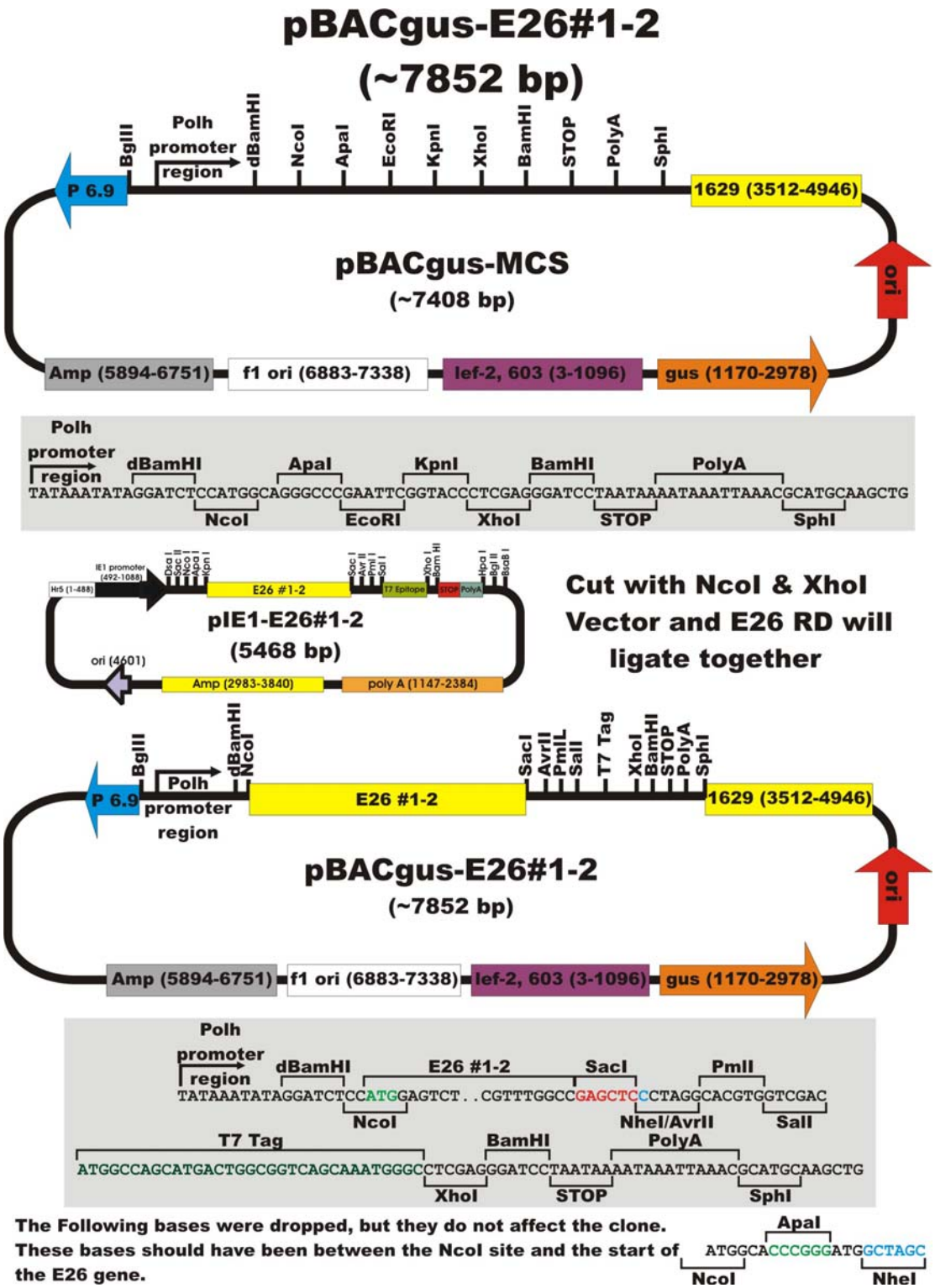
B53 : pBACgus-E26 2



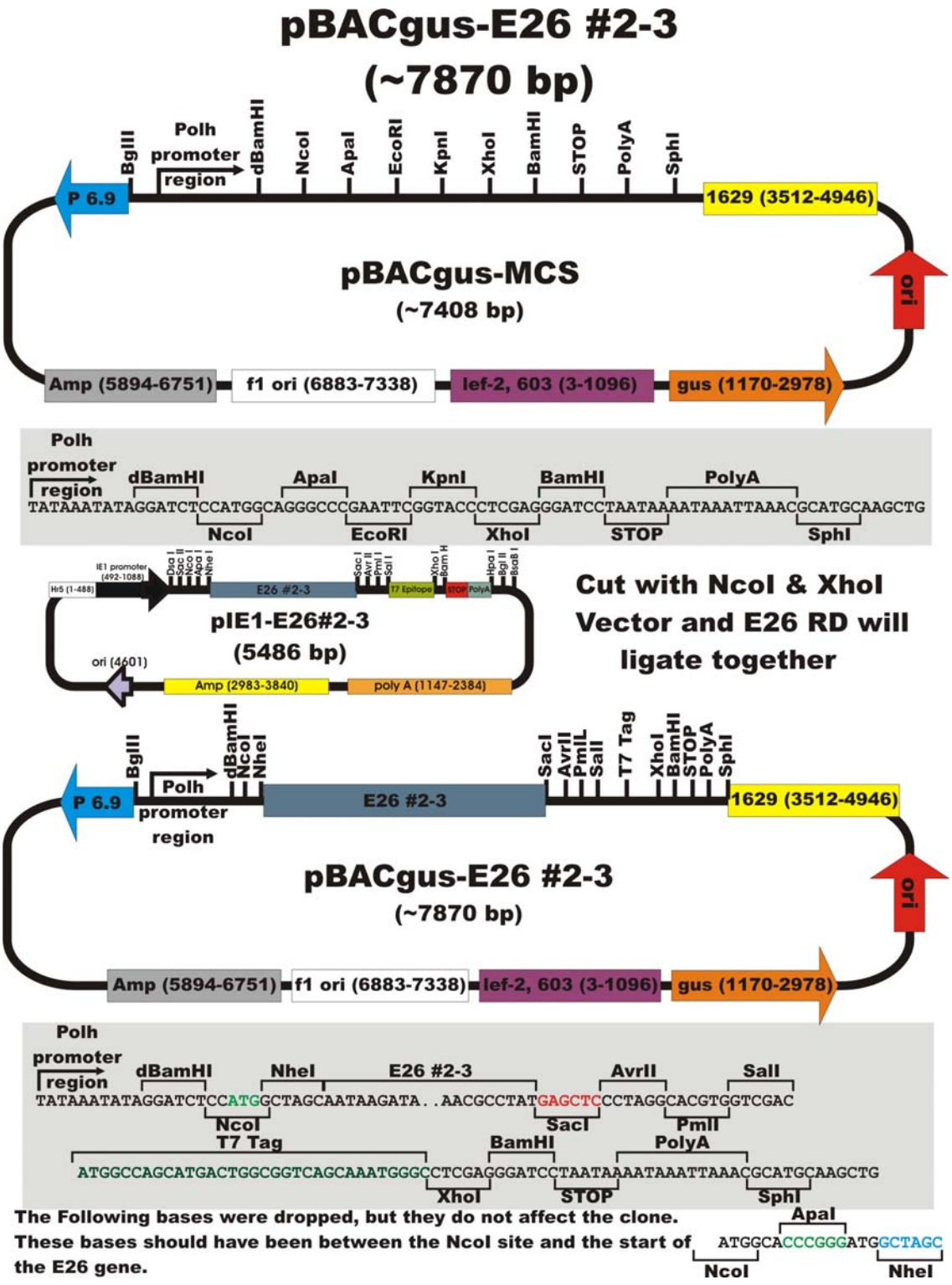
B54 : pBACgus-E26 3



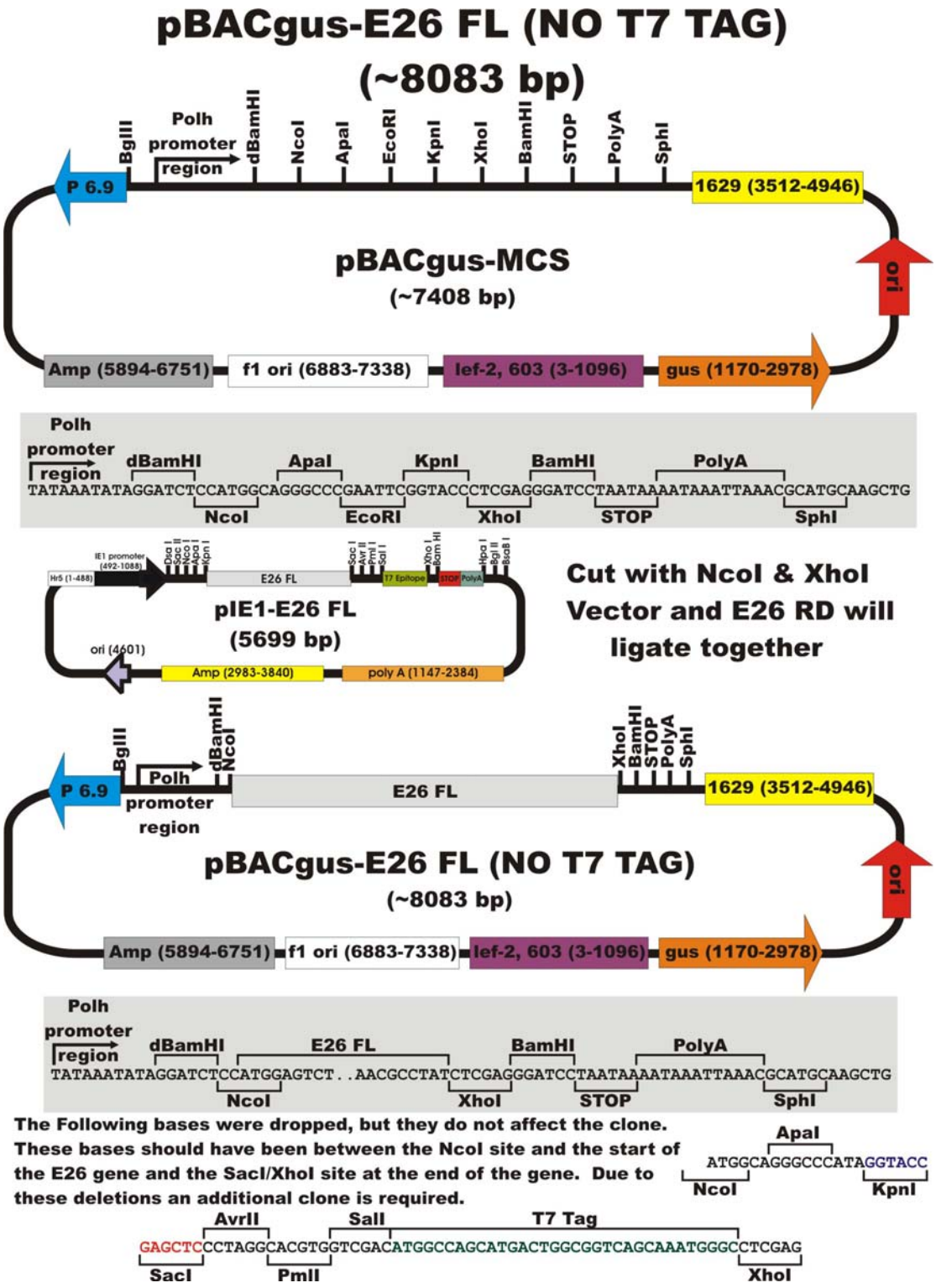
B55 : pBACgus-E26 1-2



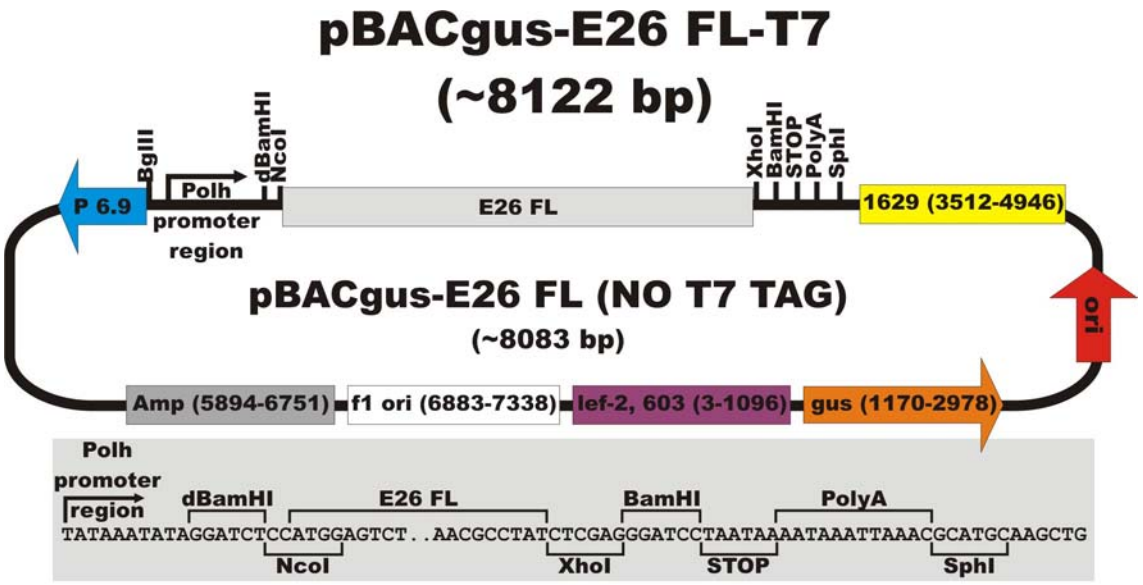
B56 : pBACgus-E26 2-3



B57 : pBACgus-E26 FL (NO T7 TAG)



B58 : pBACgus-E26 FL-T7



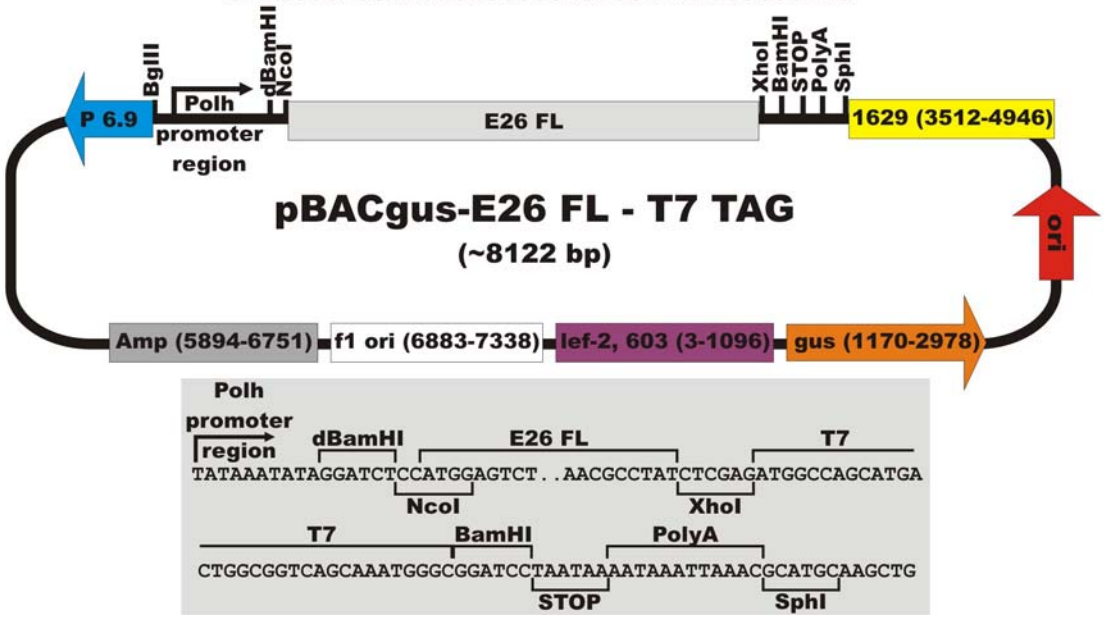
Oligo inserting a T7 epitope between the XhoI and BamHI sites
Digested vector with BamHI and XhoI and ligated insert

P1 - Xho-T7-Bam Forward

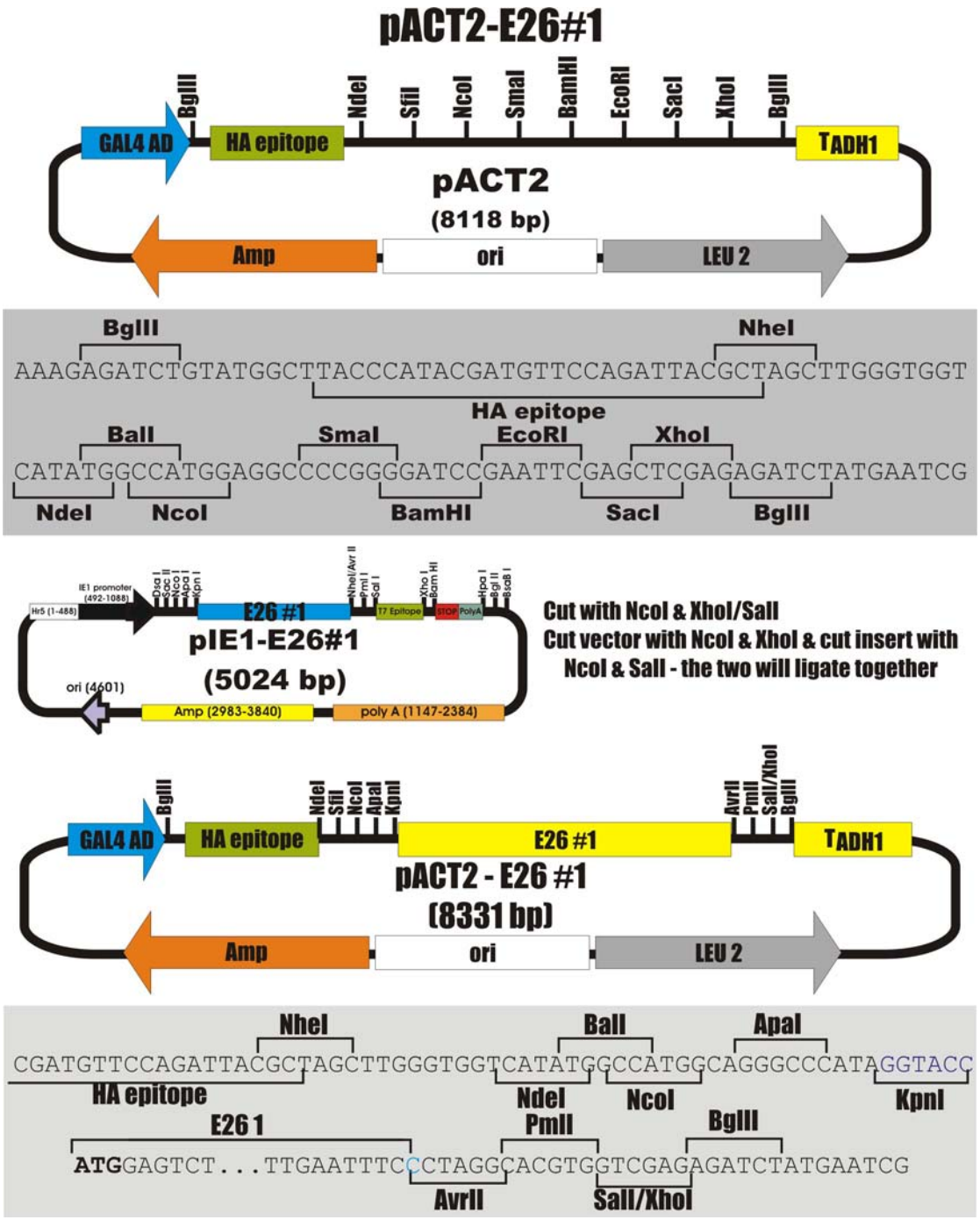
XhoI BamHI
TCGAGATGGCCAGCATGACTGGCGGTCAGCAAATGGGCG
T7

P2 - Xho-T7-Bam Reverse

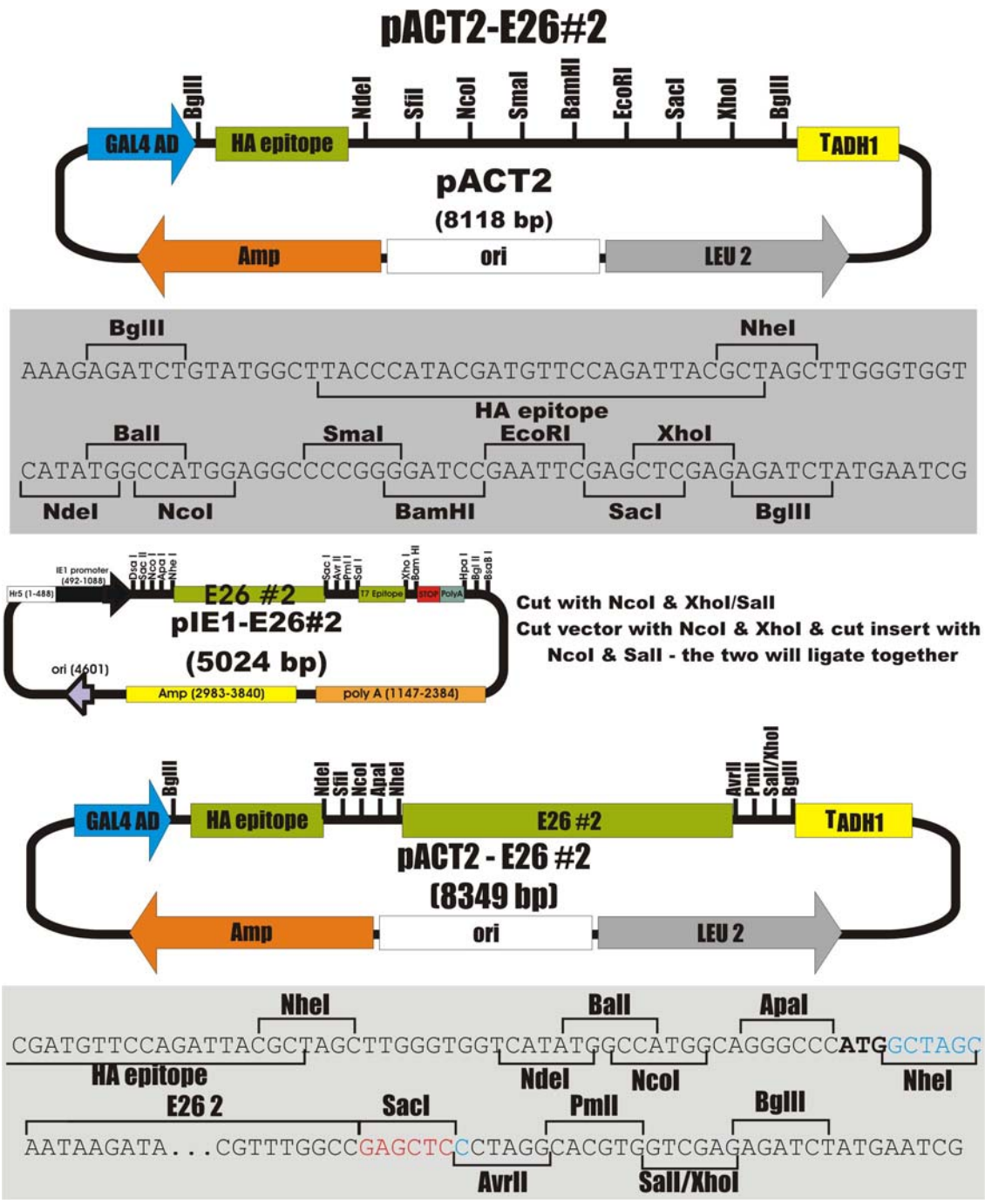
CTACCGGTCGTACTGACCGCCAGTCGTTTACCCGCCTAG



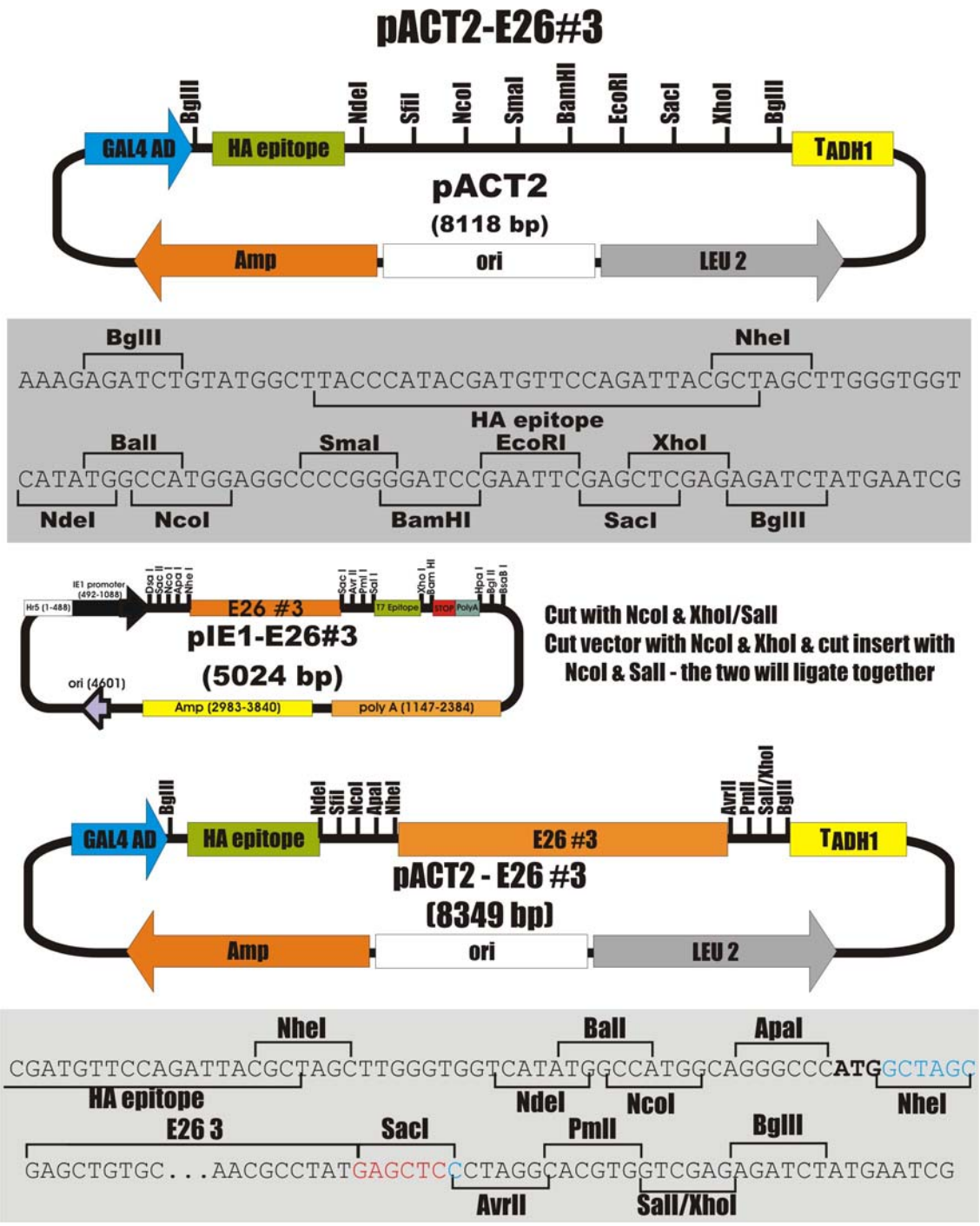
B59 : pACT2-E26 1



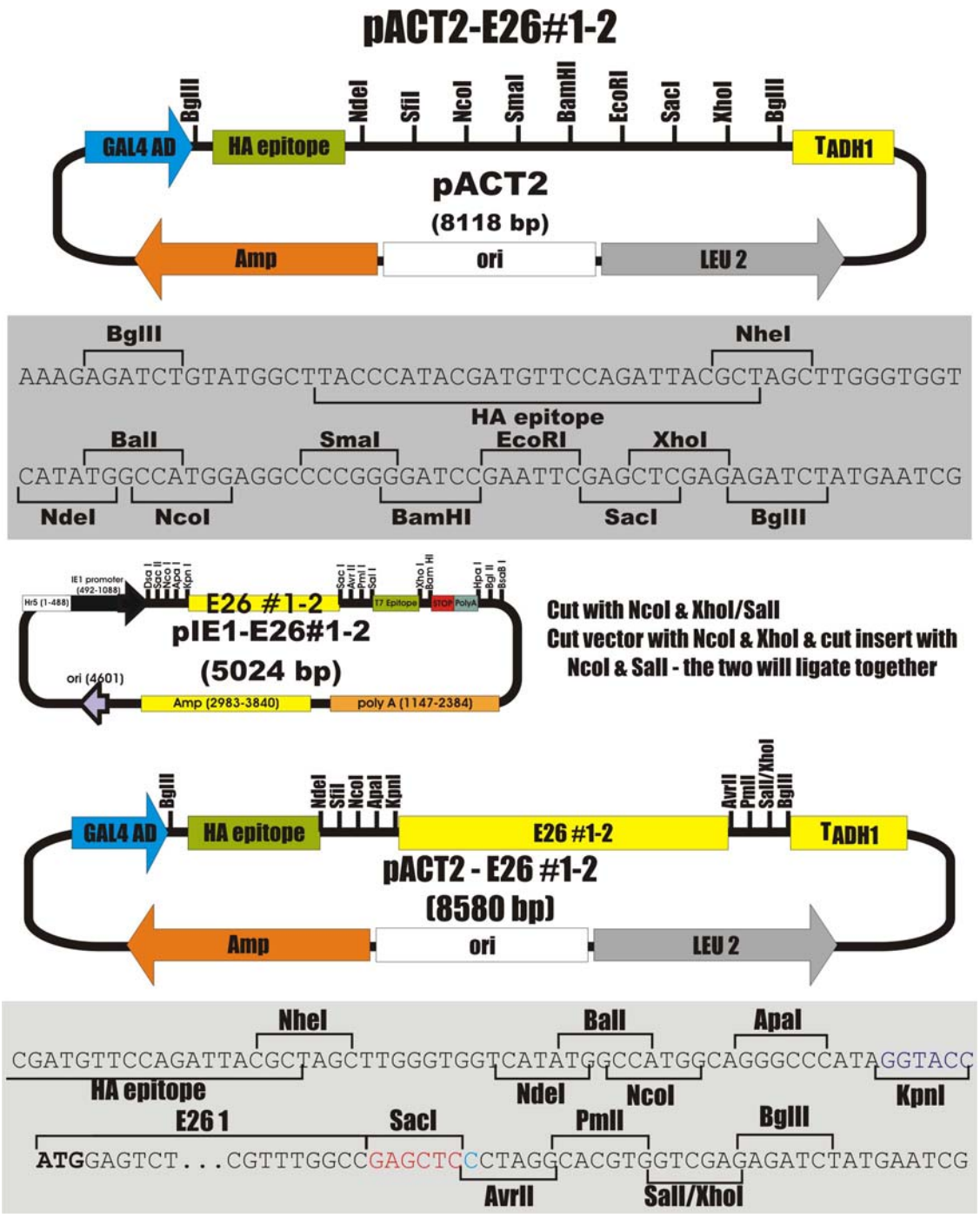
B60 : pACT2-E26 2



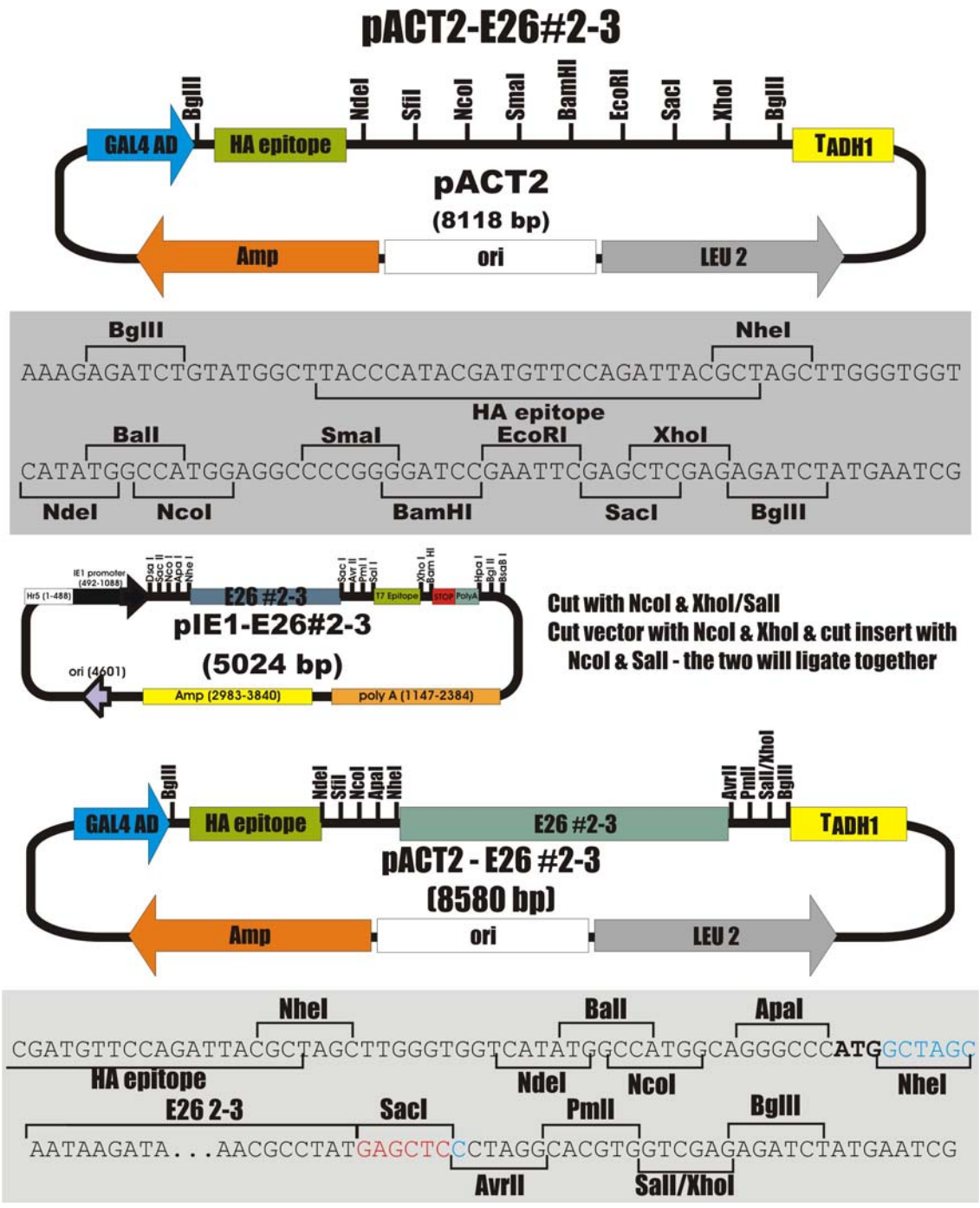
B61 : pACT2-E26 3



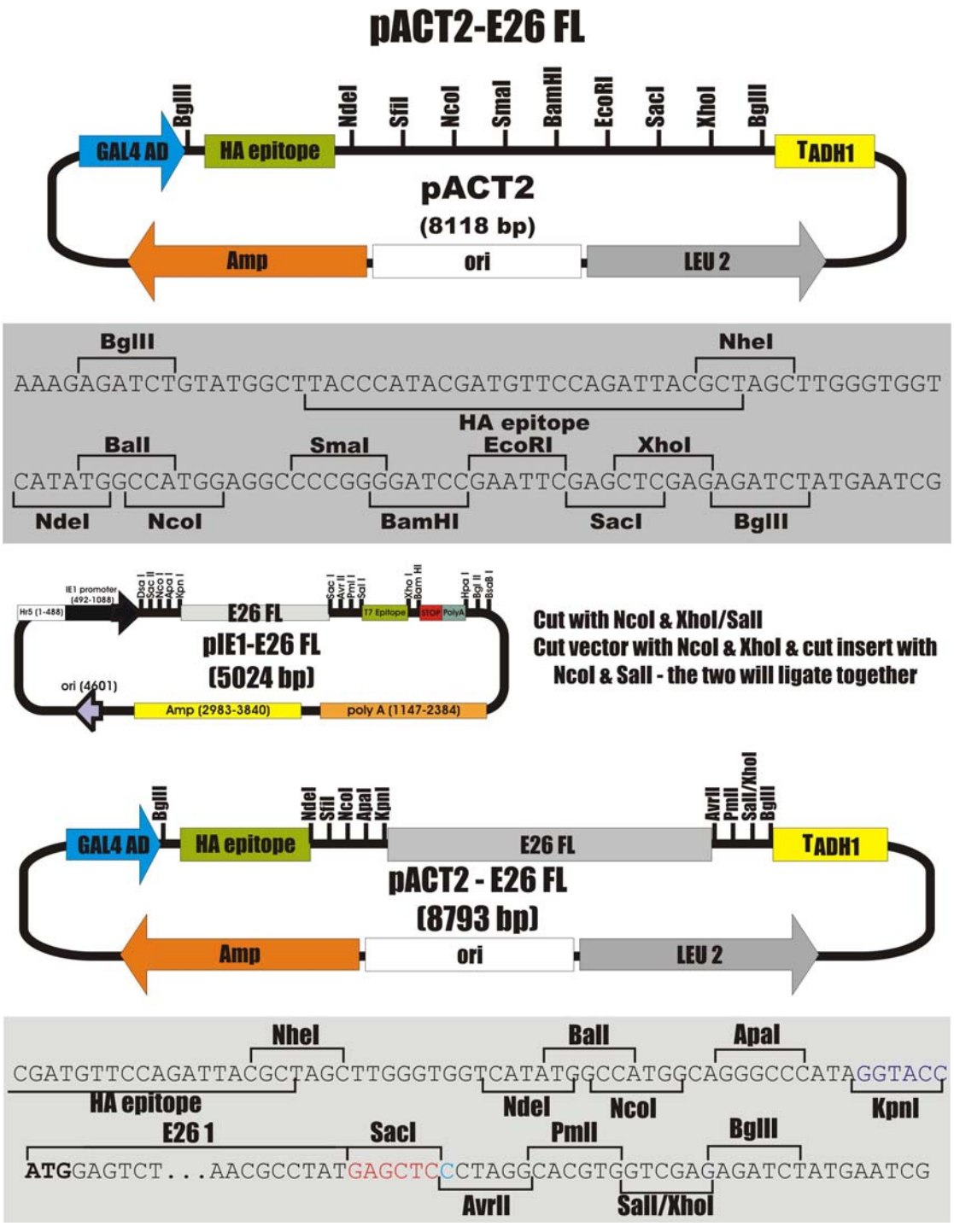
B62 : pACT2-E26 1-2



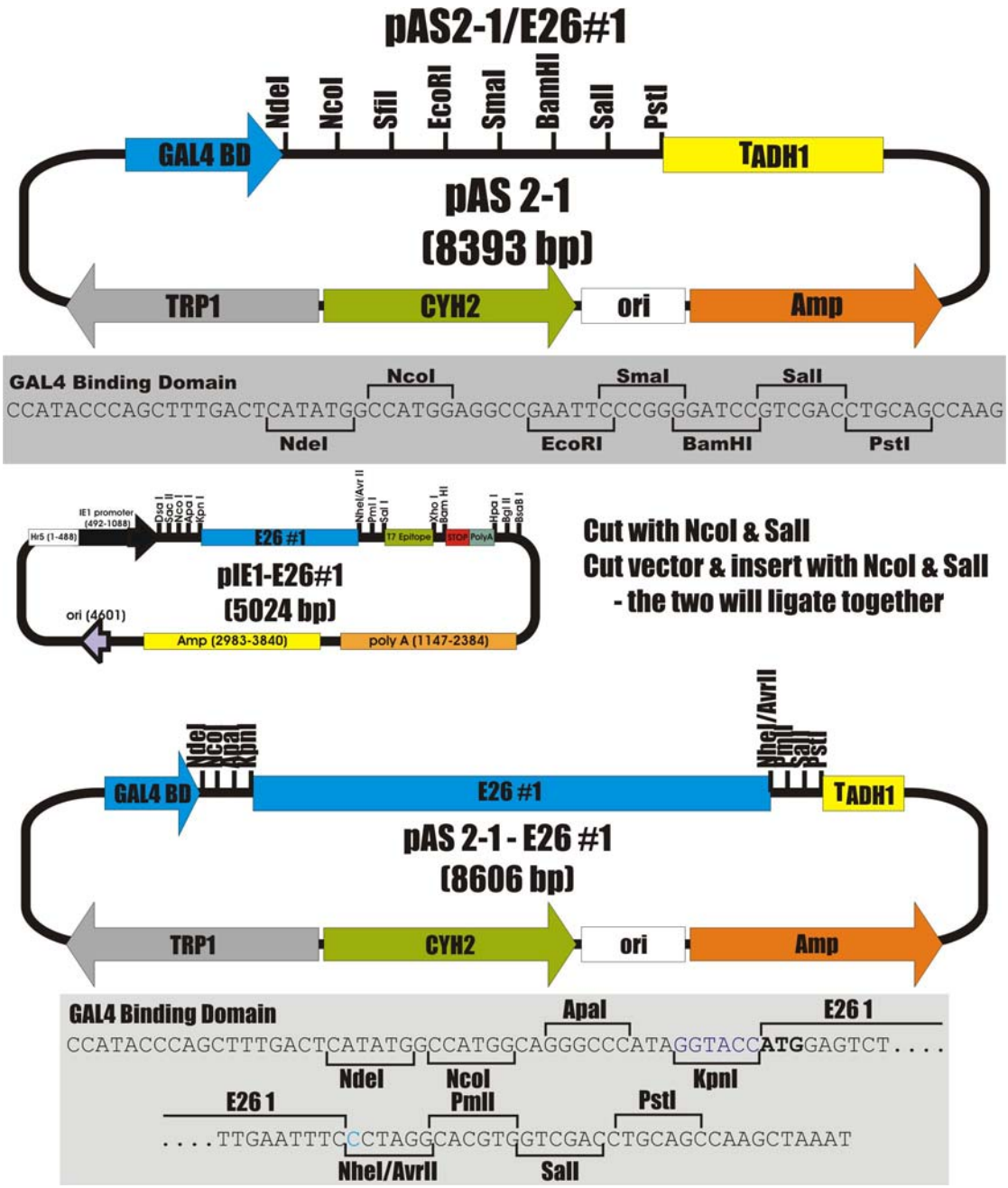
B63 : pACT2-E26 2-3



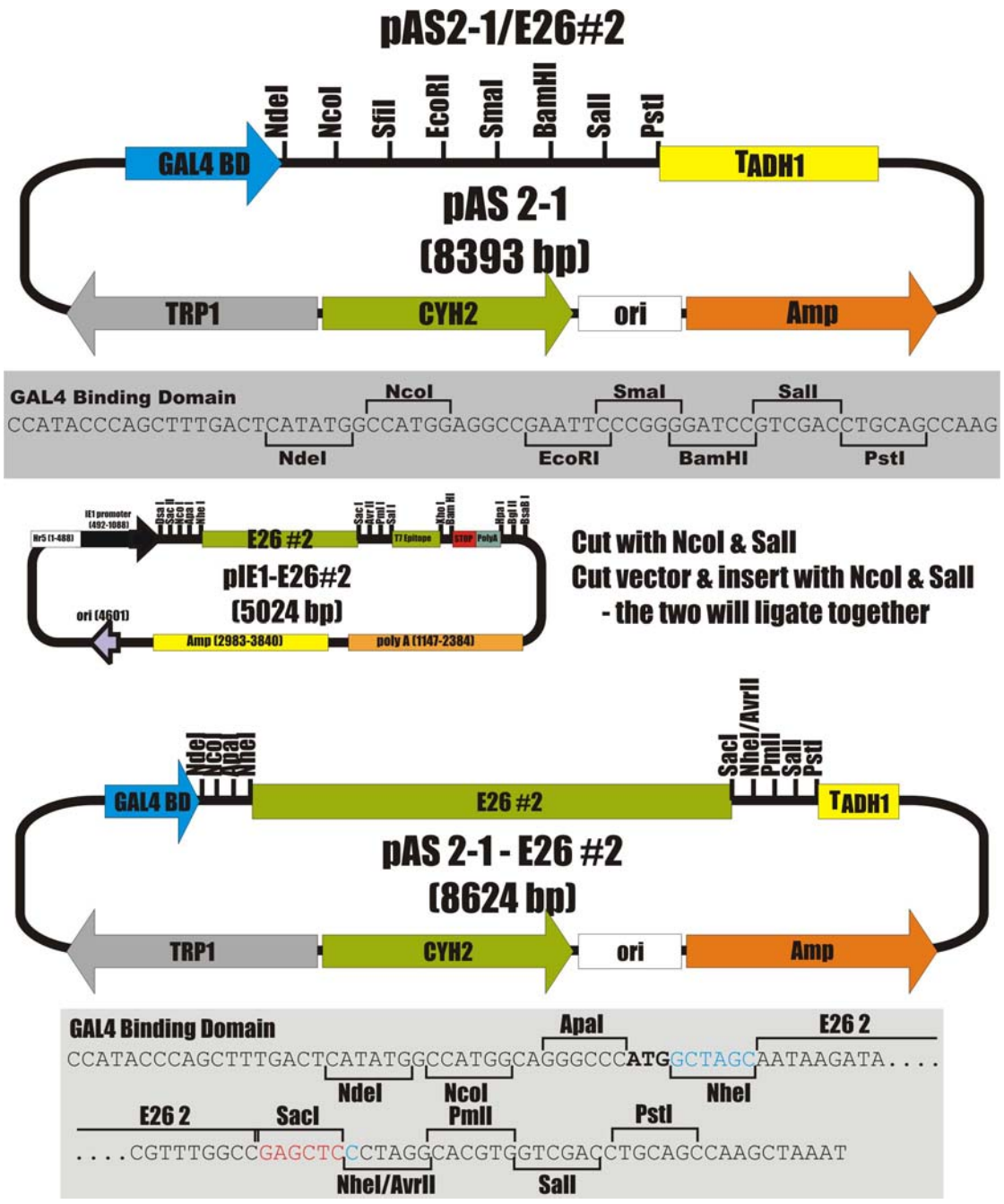
B64 : pACT2-E26 FL



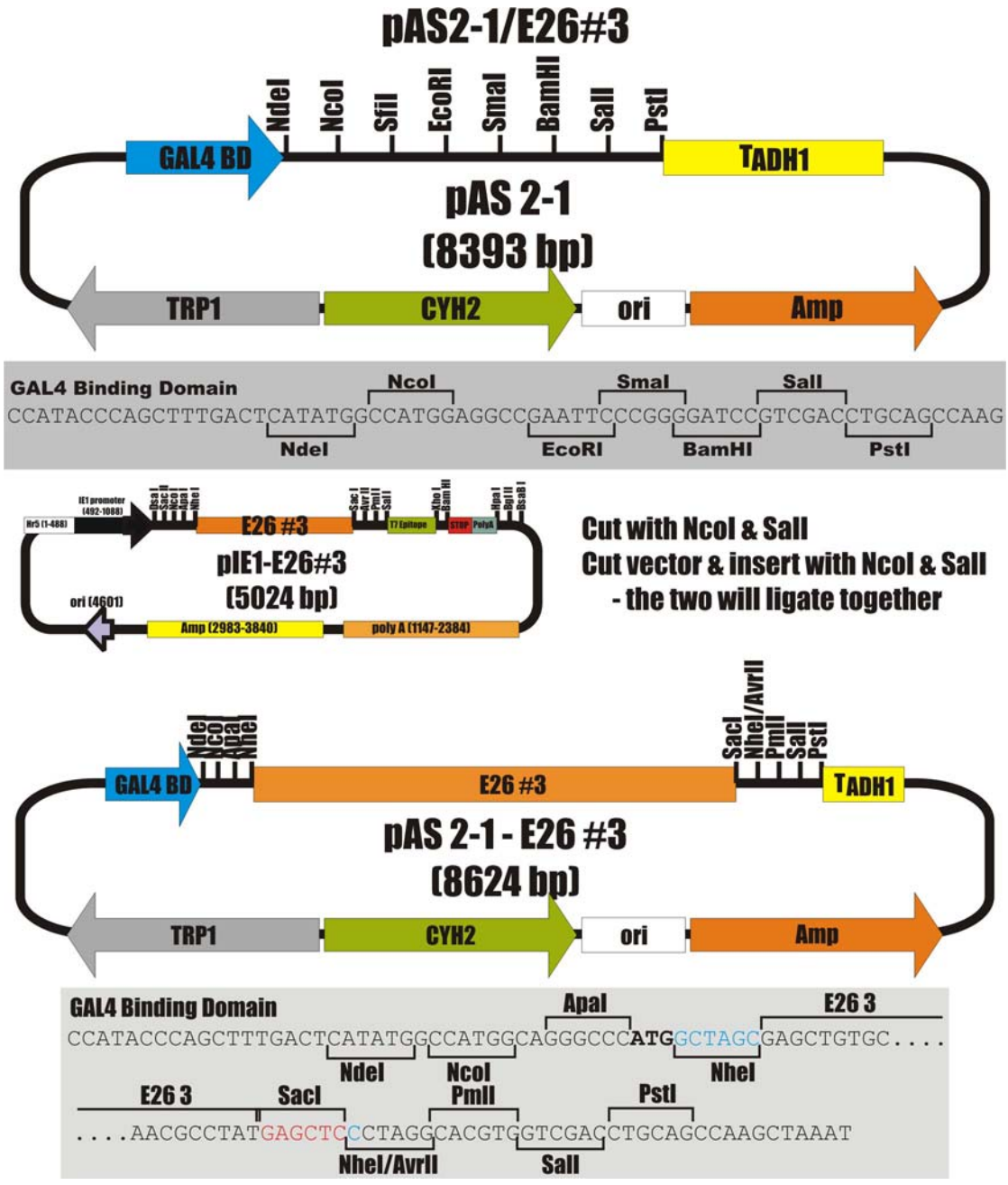
B65 : pAS2-1/E26 1



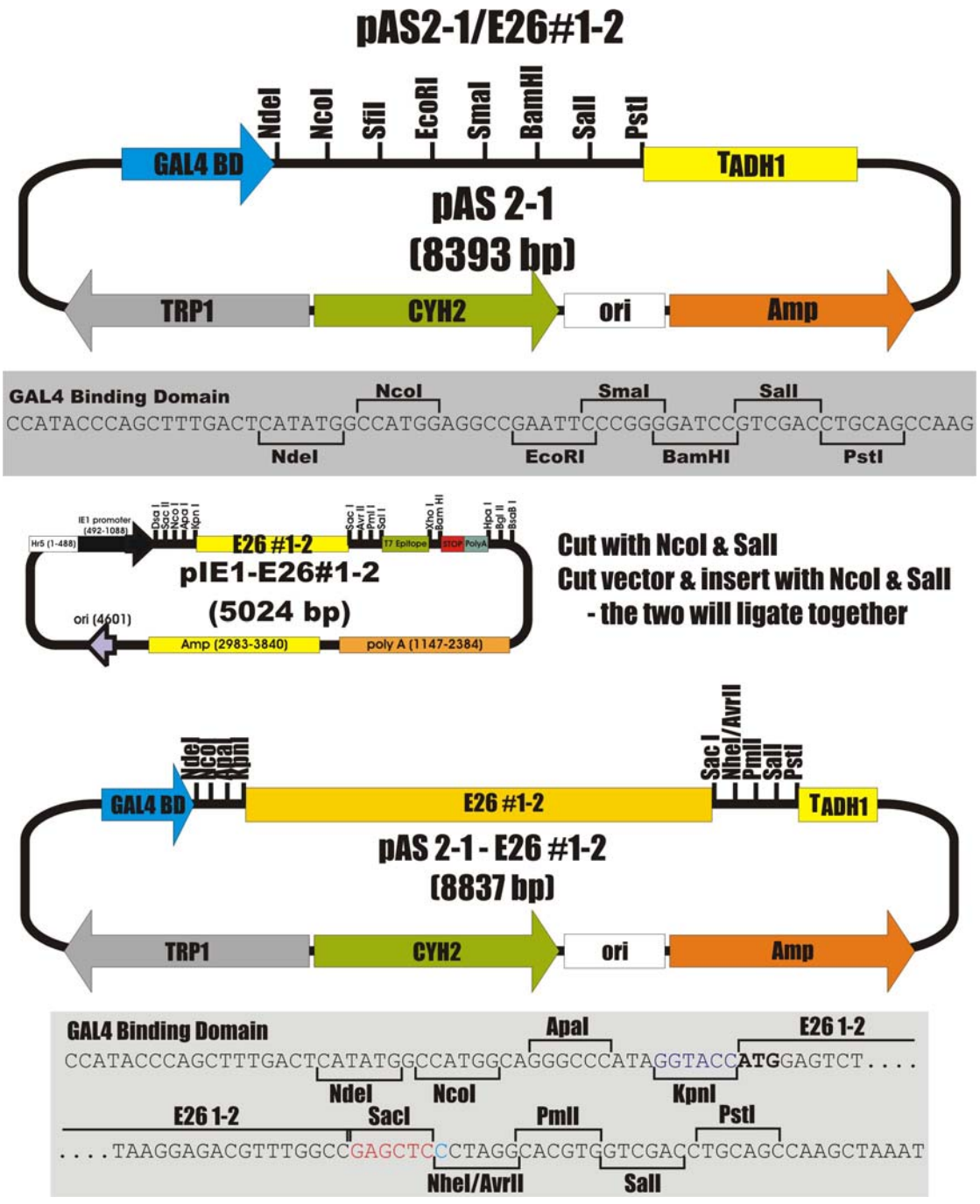
B66 : pAS2-1/E26 2



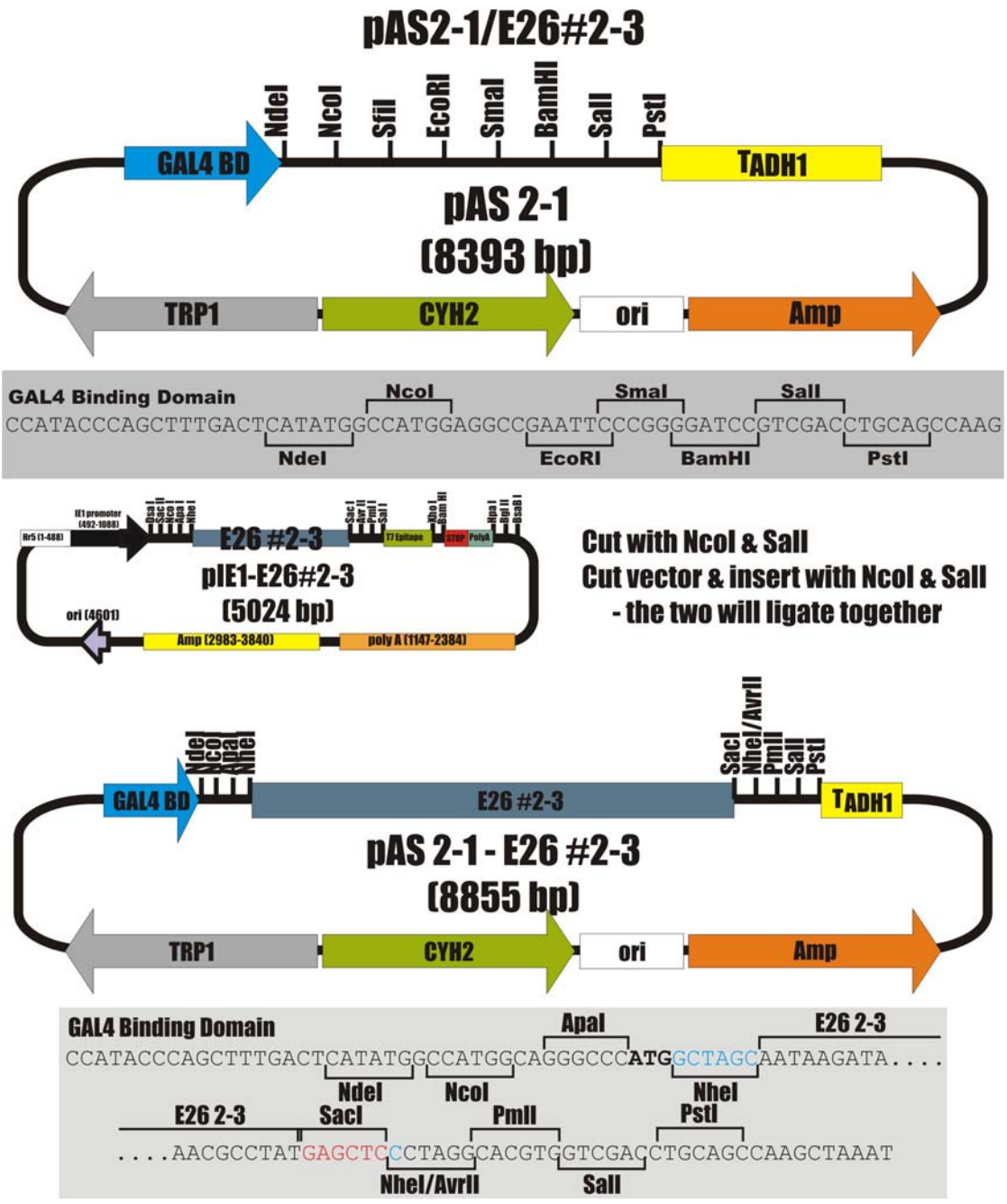
B67 : pAS2-1/E26 3



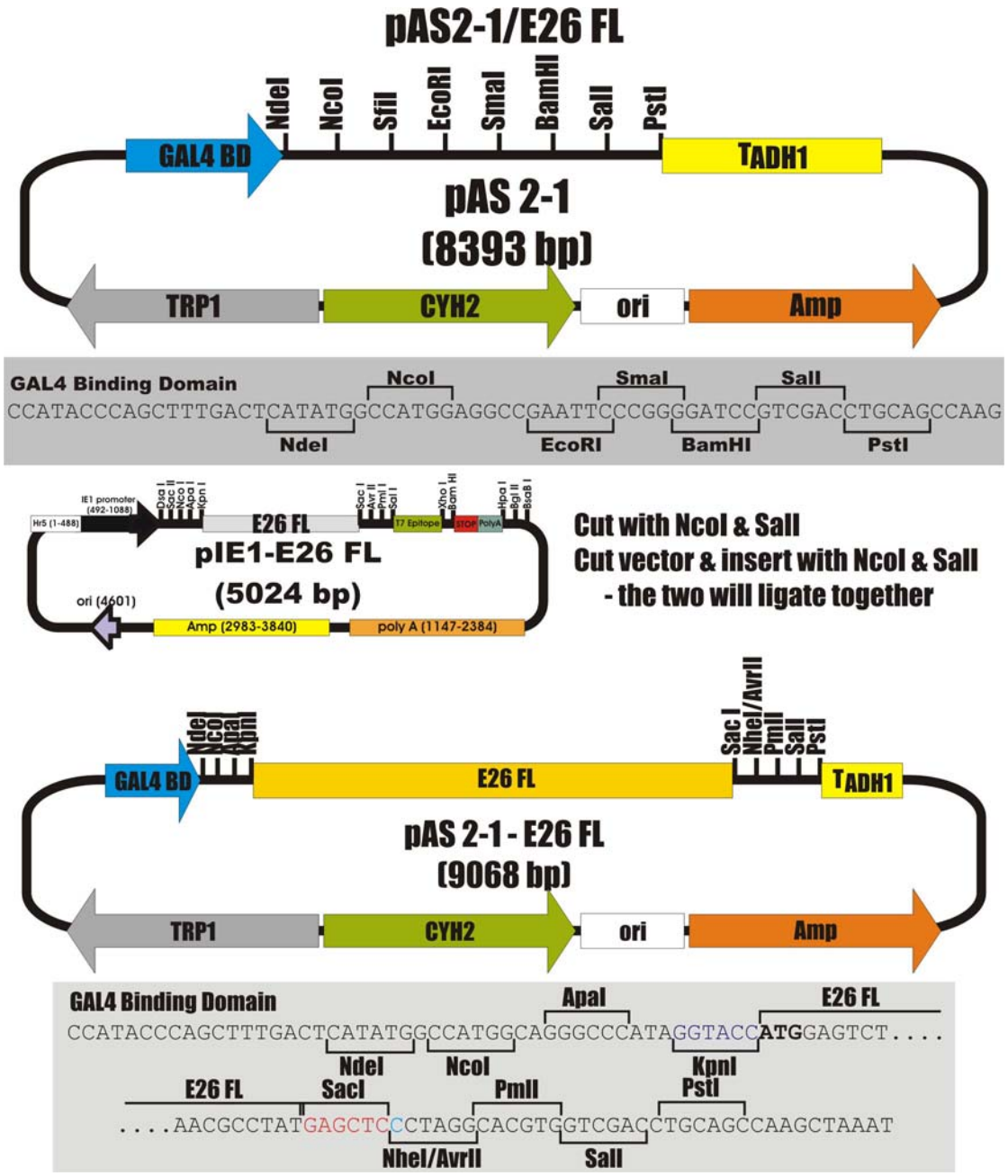
B68 : pAS2-1/E26 1-2



B69 : pAS2-1/E26 2-3



B70 : pAS2-1/E26 FL



VITA

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Bachelor of Science: Preprofessional Biology (1995)

Publications:

Braunagel, S.C., Burks, J.K., Harrison, R.L., Summers, M.D. July, 1997. The relationship between FP25K and ODV envelope proteins. American Society for Virology Meeting Abstract.

Braunagel, S. C., Burks, J. K., Rosas-Acosta, G., Harrison, R. L., Ma, H., and Summers, M. D. 1999. Mutations within the *Autographa californica* nucleopolyhedrovirus FP25K gene decrease the accumulation of ODV-E66 and alter its intranuclear transport. J Virol 73, 8559-8570.

Burks, J.K., Braunagel, S.C., Summers, M.D. July, 2004. BV/ODV E26: modification and membrane association. American Society for Virology Meeting Abstract.

Research Presentations:

Entomology Graduate Student Forum, August 2003 "BV/ODV E26 localization in *Spodoptera frugiperda* cells suggests two models for protein trafficking" Received 3rd place in presentation competition wining a monetary award.

American Society for Virology, 2004 Annual Meeting McGill University "BV/ODV E26: modification and membrane association." Received a travel grant.

Committee Membership:

Light Microscopy Users Group (Student Representative)

Professional Societies:

American Society for Microbiology
American Society for Virology