# VARICELLA ZOSTER VIRUS PROMOTER SEQUENCES

1994

KANTAKAMALAKUL

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**Abstract**

Title of dissertation : Varicella Zoster Virus Promoter Sequences

Wannee Kantakamalakul, Doctor of Philosophy, 1994

Dissertation directed by : John Hay, Ph.D., Professor, Department of Microbiology

The human herpesviruses control the expression of their genes during viral growth in a tightly regulated fashion, and the major factor in this control is believed to be the use of specific promoter elements. To develop an understanding of how varicella zoster virus (VZV) regulates its growth cycle, we have experimentally defined the promoters for three different classes of VZV genes.

By characterizing the sequences upstream from the transcriptional start site of each gene, the functional promoter regions have been identified. For gpV (a late gene), a 580 base pair fragment of VZV DNA was cloned upstream from a reporter gene, and subsequent mutational analysis showed that a short sequence containing the "TATA" box (TTTAAATT) is sufficient for promoter activity, although it is much weaker than the equivalent promoter for its homologue, herpes simplex virus gC (TATAAATT). We suggest that the genomic environment, as well as the nucleotide sequence, is important

for this promoter function.

The gpIV (early:late) and ORF61 (early) gene promoters have been similarly dissected. Their "TATA" box sequences alone in transient assays allowed transcription, but at a low level. However, the presence of activating upstream sequences (AUS) have marked upregulatory effects. The AUS of gene 61 is different from that of gpIV, and the gpIV AUS is capable of upregulate the gpV "TATA" element defined above.

We have also examined the functional relationship of the VZV-encoded regulatory proteins from ORFs4, 61, 62, and 63 on the promoter regions of the VZV genes gpIV, gpV and ORF61, as well as on the HSV gC promoter. The ORF62 protein is clearly the major transactivator, and can be substantially augmented by the ORF4 product, while the ORF61 and ORF63 products have more minor influences.

Thus, we have defined the promoters for three kinetic classes of VZV gene, and have shown that they are markedly different. We now have a basis for understanding the genetic control of this important human pathogen.

Varicella Zoster Virus Promoter Sequences

by

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Dissertation submitted to the Faculty of the Department of Microbiology Graduate Program of teh Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1994.

TO MY FAMILY

'KANTAKAMALAKUL'

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# Introduction

Seven human herpesviruses are known to exist. All of them are members of the *herpesviridae* family based on the architecture of the virion, and they are further classified as alphaherpesvirinae, betaherpesvirinae, or gammaherpesvirinae based on biological and genetic properties (Roizman *et al.*, 1981). Varicella zoster virus (VZV) is a member of the human alphaherpesvirus subfamily, which includes VZV and herpes simplex virus types 1 and 2 (HSV-1 and HSV-2). The alphaherpesvirinae are grouped on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infectious primarily, but not exclusively, in sensory ganglia (Roizman *et al.*, 1981).

The remaining four human herpesviruses are cytomegalovirus (CMV, a betaherpesvirus), Epstein-Barr virus (EBV, a gammaherpesvirus), human herpes virus 6 (HHV-6, a betaherpesvirus) and human herpes virus 7 (HHV-7, unclassified) (Roizman *et al.*, 1981; Roizman and Sears, 1990). Other mammalian alphaherpesviruses which share many behavioral traits with VZV are, pseudorabies virus (PRV) and equine herpesvirus type 1 (EHV-1).

## VZV Disease

Varicella-zoster virus causes both chickenpox

(varicella) and shingles (zoster). The word "chickenpox" may be derived from the French *chiche* (meaning "chickpea"), an illusion to the characteristic varicella pock, or may be derived from the old English *gican* (meaning "itch" (Scott-Wilson, 1978)). The word "shingles" probably comes from the latin equivalent *cingere* (meaning "a belt or girdle" (Christie, 1974)).

Chickenpox is the primary VZV infection which usually develops in children aged younger than 10. Chickenpox is highly contagious, spreading by the airborne route (Leclair *et al.*, 1980). The respiratory tract of varicella patients has been assumed to be the source for VZV shedding (Grose and Giller, 1988), however VZV is rarely isolated from the respiratory tract during chickenpox. It is easier to isolate VZV from varicular lesions of the skin (Grose and Giller, 1988; Asano *et al.*, 1985; Trlifajova *et al.*, 1986). VZV is also naturally shed from skin lesions, which is probably the major source for transmission of virions in infected people (Tsolia *et al.*, 1990). However, some transmission may also occur by the respiratory route.

Chickenpox is characterized by a generalized examthem with a pruritic vesicular skin rash. In addition, nonspecific symptoms of infection, such as fever, also occur. Chickenpox is a disease of marked seasonality, usually occuring during the months of March until May in temperate climates (Preblud *et al.*, 1984).

VZV is species-specific. Thus, there is no good animal

model to produce infection that is clinically similar to human chickenpox (or shingles, for that matter). Therefore, the mousepox model (Fenner, 1948) is used as the current animal model for chickenpox. In this model, the virus enters through the mucosa of the upper respiratory tract by inhalation, then replicates and disseminates via the bloodstream and lymphatics in a primary viremia. The virus enters the cells of the reticuloendothelial system, where it undergoes several rounds of replication resulting in a secondary viremia (Feldman and Epp, 1979). Initially, viral replication is limited by nonspecific and developing, specific immune responses. However, these defense mechanisms are often overcome, leading to extensive secondary viremia. In immunocompetent individuals, the viremic phase is usually over by 3 days, as a result of humoral and cellular immune responses (Arvin *et al.*, 1983). In immunocompromised individuals, however, for example, in the leukemic child, viremia continues for many days. The secondary viremia is associated with and followed by infection of capillary endothelial cells which spread virus to epithelial cells of the epidermis, then produce cutaneous and mucosal lesions called a papulovesicular rash. The incubation period before eruption of the rash is 2 weeks, and the varicella rash is the most typical feature of the disease. It is characterized by a rapid progression from macules to papules to vesicles to crusts, appearing in distinct waves, or crops, and a marked geographic distribution (Gelb, 1993). Crusts fall off in 1 to

3 weeks without scarring. Lesions tend to appear first on the scalp and trunk and then spread to the extremities. The distribution is predominantly central, with the highest density of lesions found on the trunk, which correlates with later presentations of herpes zoster (Stern, 1937). Vesicles are also found on all mucosal surfaces, including the respiratory and gastrointestinal tracts.

Bacterial superinfection of the skin lesions is the most frequent complication of varicella in the normal host (Bullowa and Wishik, 1935). Pneumonia is the most common serious complication in adults (Guess, 1986). Neonatal varicella can develop in an infant whose mother had varicella between 5 days before and 2 days after delivery; the onset of illness is usually between 5 and 10 days of age. Virus is often widely dissiminated, with pneumonia and visceral disease (Arvin *et al.*, 1983; Brunell, 1983). In immunocompromised patients, particularly those with leukemia, the severity and complications of varicella are significantly increased (Balfour, 1988). Chickenpox has been implicated in more than 20% of Reye's syndrome cases reported in the United States (Hurwitz *et al.*, 1982).

In 1909, Von Bokay (1909) was the first to suggest that both chickenpox and shingles are caused by the same etiologic agent. He noticed that household susceptibles contracted chickenpox shortly after exposure to zoster. In 1921, Lipschutz (1921) showed histologic similarities in tissues from varicella and zoster skin lesions. Subsequently, Weller

(1953) demonstrated the same growth characteristics and plaque morphology in tissue culture of material from both varicella and zoster lesions. Later studies using convalescent antisera from both varicella and zoster patients were equally effective in detecting antigens to both varicella and zoster-derived virus in immunoflorescence, complement fixation and neutralization assays (Weller and Coons, 1954; Weller and Witton, 1958). The final proof of the identity of varicella and zoster viruses came from restriction endonuclease analysis, showing that the VZV isolate recovered from a patient with zoster was identical to that recovered from his primary varicella lesions 3 months earlier (Straus *et al.*, 1984).

Shingles, therefore, is a secondary (or recurrent) VZV infection. It appears to result from the reactivation of latent VZV, the result of a previous attack of chickenpox. It is characterized by a painful vesicular eruption, usually limited to a single dermatome. The lesions usually resolve in 2-3 weeks, but complications can result, and their severity is usually related to the immune condition of the host. Complications include postherpatic neuraglia, myelitis, and encephalitis (Watson and Evans, 1986; Reichman, 1978; Hogan and Krigman, 1973; Mc Cormick *et al.*, 1969). The pathogenesis of herpes zoster is not well understood. The current model (Hope-Simpson, 1965) is based primarily on clinical and epidemiologic data, as well as on analogy with recurrent HSV infections. VZV is thought to pass centripetally from skin

and mucosal lesions to the corresponding sensory ganglia via the contiguous sensory nerve endings and sensory nerve fibers (Gelb, 1993). However, the virus may also seed the ganglia hematogenously. Once in the ganglion, the virus sets up a latent infection without virus replication or cell damage. The cell type involved in latency is the subject of controversy. *In situ* hybridization localizes VZV nucleic acid to either neuronal cells (Gilden *et al.*, 1983; Hyman *et al.*, 1983) or surrounding satellite cells (Croen *et al.*, 1988; Straus, 1989). It is also unclear whether this is truly a latent infection or is actually a persistent infection in which some VZV is constantly being produced. In either case, the infection remains quiescent until reactivation as herpes zoster, and these reversions are sporadic and infrequent.

The mechanism of reactivation is also unclear. Many clinical conditions have been associated with the appearance of herpes zoster, including Hodgkin's disease and other lymphomas, immunosuppressive drugs, trauma to the spinal cord, adjacent structures, and heavy metal poisoning (Head and Campbell, 1900; Hope-Simpson, 1965; Juel Jenson and Mac Callum, 1972; Schimpff *et al.*, 1972). Shingles can occur at all ages; however, there is a direct correlation between increasing age and increasing incidence (Hope-Simpson, 1965). Changes in cell mediated immune (CMI) responses are apparently more important than those in the humoral immune (HI) response (Diaz *et al.*, 1989). Most zoster patients have significant levels of antibody at the onset of disease, but

their T-lymphocyte activity against the virus is often low.

Finally, VZV has been modified by prolonged passage in cell culture to develop a live attenuated vaccine product (Oka strain) (Takahashi *et al.*, 1974). This vaccine is licensed in Japan, Korea and some European countries, and it is now clear that it is highly effective in preventing varicella in both healthy and immuno-compromised populations, although it is able to establish latency and to be reactivated. It is anticipated that licensure will occur soon in the United States (Gershon *et al.*, 1993).

The Infectious Agent

Our knowledge of VZV growth and pathogenesis has been based primarily on clinical descriptions, because the virus propagation in tisssue culture systems is very inefficient and leads to low levels of largely cell-associated progeny virus. Weller *et al.* (1953) were first to propagate VZV successfully in human foreskin fibroblasts (HFF) and in human skin muscle tissue in the laboratory. Even today, human diploid cells such as HFF or human embryonic lung fibroblast (MRC-5, WI-38) are the cells of choice for growing VZV. Subsequently, other cell lines such as primary cultures of human thyroid cells (Caunt, 1963), human embryonic lung fibroblasts (Brunell, 1977; Dumas *et al.*, 1980), human melanoma cells (Grose *et al.*, 1979) have also been described as permissive for VZV. Cell-free VZV can be obtained from these cell lines in sufficient quantity to study the virus

particle itself, and to obtain sufficient DNA to be amplified in bacteria and be studied directly. However, it is still not possible to make synchronous infections with VZV using a large enough number of cells to study classical viral genetics and biochemistry.

Using light microscopy, VZV was first visualized in chickenpox vesicle fluid (Aragão, 1911). The virus particle was shown to be 0.125-0.175 μm in diameter, and subsequent electron microscopy has confirmed this observation. Virions from both varicella and zoster vesicles are identical (Rake *et al.*, 1948) and the virion morphology resembles that of other members of the herpesvirus family (Almeida *et al.*, 1962). The DNA genome is organized in a 75 nm toroidal core within the nucleocapsid, while the icosahedral nucleocapsid is approximately 100 nm in diameter and consists of 162 hexagonal capsomers. Around the nucleocapsid is a granular proteinaceous material called tegument and the outermost virus surface is a lipid bilayer membrane containing glycoprotein projections about 8 nm long.

The glycoproteins of VZV are the only structural proteins that have been studied in any detail; they have many important functions throughout the replication cycle (Grose, 1990). For example, they are sites of attachment and fusion of virus to the cell and, thus, help to specify the host range for the virus; they are also required for the formation of the complete virion (the infectious particle). Not surprisingly, viral glycoproteins can stimulate the host

immune responses (both humoral and cellular) resulting in resolution of the acute infection, and make the Oka vaccine functional.

At least 5 glycoprotein (gp) genes have been identified in VZV: gpI (encoded by gene68, homologous to HSV gE); gpII (gene31, HSV gB-like); gpIII (gene37, HSV gH-like); gpIV (gene67, HSV gI-like) and gpV (gene14, HSV gC-like).

The VZV Genome

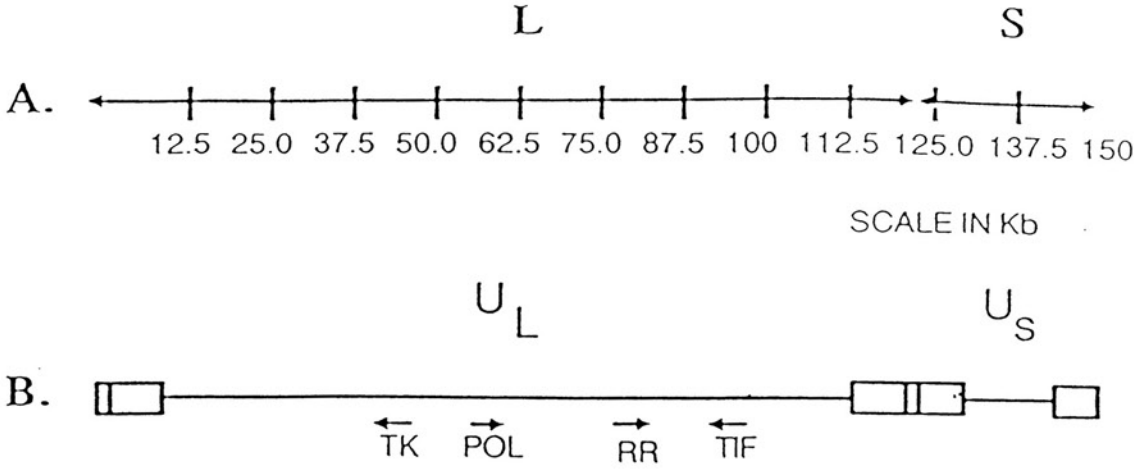
Several different approaches have been used to isolate and purify VZV DNA (Rapp *et al.*, 1977; Dumas *et al.*, 1980; Straus *et al.*, 1981). Using electron microscopy (Dumas *et al.*, 1980) and restriction endonuclease profiles (Dumas *et al.*, 1981; Mishra, 1984; Straus *et al.*, 1981, 1982), the VZV genome was shown to be about 85 megadaltons long. In 1986, Davison and Scott (1986) published the complete sequence of the VZV (Dumas strain) genome. It contains 124,884 base pairs in a linear double-stranded DNA, with an overall G+C content of 46%. The genome contains 2 unique segments: the unique long sequence, $U_L$ (104,836 bp) and the unique short sequence, $U_S$ (5232bp). Small inverted terminal repeats of $U_L$ named $TR_L$(terminal repeat long) and $IR_L$(internal repeat long) of 88.5 bp are found at the termini of $U_L$. Large inverted terminal repeats of 7319.5 bp named $TR_S$ and $IR_S$ are found at the $U_S$ termini. VZV DNA exists in multiple isomers (Iltis *et al.*, 1977; Oakes *et al.*, 1977; Richards *et al.*, 1979). Each isomer is packaged individually into virions and is

apparently infectious. However, two isomeric forms always predominate (instead of four isomeric forms as HSV) because while the $U_S$ segment can invert 50% of the time, the $U_L$ segment inverts much less freqently (2-5% of the time; Davison, 1983, 1984; Davison and Scott, 1985, 1986). The small 88.5 bp size of $TR_L$ and $IR_L$ alone does not seem to explain the infrequent isomerization of UL (Hayakawa and Hyman, 1987). One thing to keep in mind is the designation of prototype arrangements of the $U_L$ segments of HSV and VZV which are in the opposite orientation as shown in Figure 1. Repetitive DNA sequences are present in the VZV genome (Hyman, 1981; Mishra *et al.*, 1984). Five regions (but only four unique - the one in $TR_S$ is a duplicate of that in $IR_S$), of the genome contain reiterations of short GC-rich sequences (Davison and Scott, 1986). They are named R1, R2, R3, and R4 (2 copies). Each of the reiterated regions has its own structure. For example, R2 (from bp sequence 20692 to 21017) of the Dumas strain has the structure ABABAAAX, where A and B are 42 bp elements differing in a single base pair and X is a partial copy of 32 bp of B (Davision and Scott, 1986). Variation in the copy number of the 42 bp elements results in size heterogeneity in this region of the genome in different isolates (Kinchington *et al.*, 1986). R4 (109762 to 109907 and 119990 to 120135) has the structure AAAAAX, where A is a 27 bp element and X is a partial copy of 11 bp of A (Davison and Scott, 1986). The copy number of the 27 bp element varies between virus isolates (Casey *et al.*, 1985). R1, R2 and R3

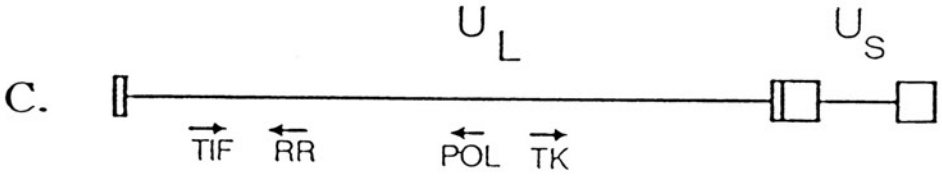
Figure 1



Illustration of the structures, relative sizes, and gene arrangements of the HSV and VZV genomes. A) the HSV genome, consisting of a long segment (L) and a short segment (S). Its size is shown in kilobases (Kb). B) lines indicate unique sequences: unique long sequence ($U_L$) and unique short sequence ($U_S$) and boxes designate inverted repeat regions. Arrows show the direction of ORFs (open reading frames); TK:thymidine kinase gene, POL: DNA polymerase gene, RR: ribonucleotide reductase gene, TIF: trans inducing factor gene. C) the VZV genome which also consists of $U_L$, $U_S$ and inverted repeat regions; however, the size of the genome is smaller than that of HSV. Because of the "prototype" configurations of the genomes, the gene arrangements in the $U_L$ regions of the genomes are reversed relative to each other. (reproduced from Ruyechan *et al.*, 1990)


L
S
A.
12.5 25.0 37.5 50.0 62.5 75.0 87.5 100 112.5 125.0 137.5 150
SCALE IN Kb
U L
U S
B.
TK
POL
RR
TIF

HSV


U L
U S
C.
TIF
RR
POL
TK

VZV

appear in the ORFs of gene 11, 14 and 22, respectively and thus all of them encode repeated amino acid sequences in their genome products. Whatever function this may have is unknown at present.

Sequence analysis of the VZV genome indicates 71 open reading frames (ORFs) that encode 68 unique proteins (some ORFs are diploid) (Davison and Scott, 1986). These ORFs are numbered, starting at #1 from the left hand end of the $U_L$ segment to the right hand end of the $TR_S$ of $U_S$ segment. The whole VZV genome has been cloned as discrete fragments in prokaryote vectors (Straus *et al.*, 1982; Ecker and Hyman, 1982; Mishra *et al.*, 1984). These cloned VZV DNA fragments are the best source of hybridization probes for the study of VZV replication, latency, and potential oncogenicity because they are uncontaminated by host cell DNA sequences. They also allow the precise mapping of mutants, RNA transcripts, and functional genes. Preliminary studies suggested that VZV DNA is homologous to EHV-1 (Atherton *et al.*, 1982), HSV, and PRV (Petrovskis *et al.*, 1986), and somewhat less to EBV (Davidson and Talor, 1987). The homology between the VZV and HSV short unique segments and their adjacent repeats is extensive (Davidson and McGeoch, 1986). These two viruses are grossly colinear in genome structure and location of homologous genes. Comparison of the primary amino acid sequences of ORFs from VZV with those of HSV reveals many similarities (Davidson and Scott, 1986; McGeoch *et al.*, 1988), and VZV can complement certain HSV-1 temperature sensitive mutants

(Felser *et al.*, 1987).

Herpesvirus gene expression

All herpes viruses share two basic properties: the patterns of gene expression during the lytic phase and the ability to establish and maintain a latent state of infection at a specific physiological site within an immunocompetent host. Herpesvirus genomes are promoter-rich; generally, the expression of a given protein is mediated by a specific promoter mapping to that gene (Wagner, 1991). Thus, there is no strict constraint on a precise genomic order of genes or of genomic organization. These genes are expressed in temporally regulated phases during the productive replication cycle in an infected cell (Roizman and Sears, 1990). This temporal cascade of expression has been divided into 3 classes: the immediate early (IE or α), the early (E or ß) and the late (L or γ) genes (Honess and Roizman, 1974). The immediate early (IE) genes are defined by their ability to be transcribed in the absence of cellular and viral protein synthesis, e.g., in the presence of cycloheximide; removal of cycloheximide will allow translation of IE genes. These IE gene products modify the host cell so that it can efficiently express a second set of viral genes (E), followed by the replication of the viral genome. Finally, the third set of genes (L), which is required for the formation, assembly and egress of the virion, are fully expressed. This set of genes requires viral DNA synthesis for its expression, in that L

proteins are not made in the presence of viral DNA synthesis inhibitors such as phosphonoacetic acid (PAA). The L class has been further subdivided into L1 (early/late) and L2 (true late) genes. Only the L2 class expression has an absolute requirement for viral DNA synthesis. The primary point for regulation of gene expression is at the level of transcription initiation (Wagner, 1991).

To determine the pattern of protein synthesis in HSV, classical kinetic labeling experiments at high multiplicities of infection were performed (Honess and Roizman, 1974). However, the full characterization of temporally regulated polypeptide synthesis has yet to be completed. In the case of VZV, it is very difficult to obtain enough cell free virions to study classical kinetics. Nevertheless, to attempt extensive, synchronous infections with VZV, Asano and Takahashi (1979) and Ruyechan *et al.* (1991) have infected cell monolayers with high ratios of infected cells. Cells were labeled with $^{35}$S-methionine and harvested at various intervals over a 72 hour time period. Although the number of observed polypeptides and their appearance in infection are slightly different from both laboratories, which may be due to an inability to control the initial infection inoculum accurately, these results indicate that VZV does encode three temporal polypeptide classes. Asano and Takahashi (1979) also showed that a number of specific novel polypeptides observed in infected cells were not observed in PAA-treated infected cell. Shiraki and Hyman (1987) observed four putative IE

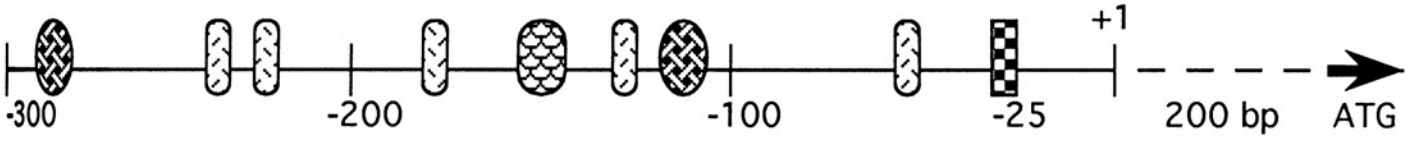
proteins from VZV infected cell which had been treated with cycloheximide followed by actinomycin D (this blocks RNA synthesis of E gene products). These results confirm that VZV gene products can be conventionally classified as in other herpesvirus, based on metabolic conditions.

Although the detailed mechanisms controlling the onset of gene expression in different herpesviruses are probably different, the close interaction between immediate early proteins and cellular transcription factors seems to be a common scenario. Herpesvirus-modified transcription factors mediate the expression of different temporal classes of viral genes via the cellular RNA polymerase II enzyme (Costanzo *et al.*, 1977). HSV RNAs, in contrast to host cell RNAs, are generally not spliced, but they are capped and polyadenylated (Bachenheimer and Roizman, 1972; Bartkoski and Roizman, 1976). Potential HSV promoter regions of all kinetic classes of genes have been predicted from transcriptional data and the minimum promoter domains and cis-acting regulatory elements have been analyzed. Generally, HSV promoters contain sequence elements similar to host cell promoters together with some viral specific sequence elements. The simplest comparison of the structure of some promoters are shown in Figure 2. For example, the IE gene promoter of Vmw175 (or ICP4) contains a TATAA box, a GC-rich binding region (CCCGCC), a TAATGARAT motif (R= any purine), and sequences that bind to the Vmw175 protein itself (ATCGTCnnnnYCGRC) (Kristie and Roizman, 1984, 1986; Mackem and Roizman, 1982a,

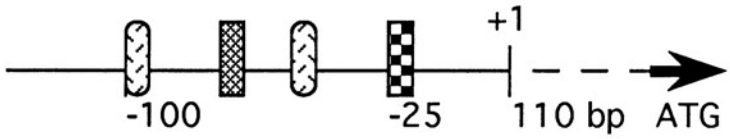
Figure 2

Diagram of examples of different kinetic classes of HSV gene promoters. The translational start site is at codon "ATG", the transcriptional cap site is at +1 and the leader sequence is shown in base pairs between them. Varieties of sequence elements identified as being important for each promoter are shown in different shaded boxes. The distance from the cap site is shown as a negative number of base pairs. TFIID, SP1 and C/EBP (CCAAT box binding protein) are cellular transcription factors; VP16 and Vmw175 are HSV transcription regulators.

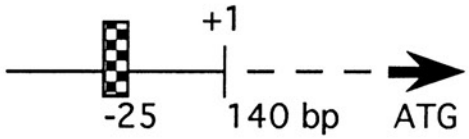
immediate early gene : Vmw175




early gene : thymidine kinase




late gene : gC




| | | responsive to |
|---|---|---|
|  | TATAA | TFIID factor |
|  | GC box | SP 1 |
|  | CCAAT | C/EBP |
|  | TAATGARAT | VP16 (α TIF, Vmw65) |
|  | ...ATCGTC... | Vmw175 |

1982b; Jones and Tjan, 1985; Jones *et al.*, 1985). For an early gene promoter, such as the one for thymidine kinase, data show the presence of a TATAA box, a CCAAT box, two GC-rich binding regions and possibly an octamer motif (ATTGCAT) (Mcknight *et al.*, 1981, 1982a, 1982b, 1984). In the case of late gene promoters such as that of glycoprotein C (gC), only a TATAA box and probably some 5' untranslated regions are enough for regulated transcription (Homa *et al.*, 1986a, 1988). However, there appear to be many heterogeneities among the promoters of each of the kinetic classes, and relying only on a DNA sequence examination to predict and categorize promoters is not feasible at present.

In HSV infected cells, cellular proteins binding to promoter region sequences have been identified: for example, TFIID (TATAA box), SPI (GC-rich binding regions), CTF/NFI, C/EBP (CCAAT box), and αH1/Oct-1/NFIII (TAATGARAT) (Schmidt *et al.*, 1989; Horikoshi *et al.*, 1989; Santoro *et al.*, 1988; Sturm *et al.*, 1988; Kadonaga *et al.*, 1987; Mitchel *et al.*, 1989). However, the precise role of herpesvirus-modified transcription factors interacting with the promoter and/or the cellular polymerase II enzyme is unclear. Vmw175, which is a strong transcriptional transactivator for early and late genes has been demonstrated to bind to these promoters (Michael *et al.*, 1988). It has also been shown to bind to its own promoter for autoregulation. However, the binding sites are not well characterized. Vmw65 (VP16), another strong transactivator, forms a complex with Oct-1, then this complex

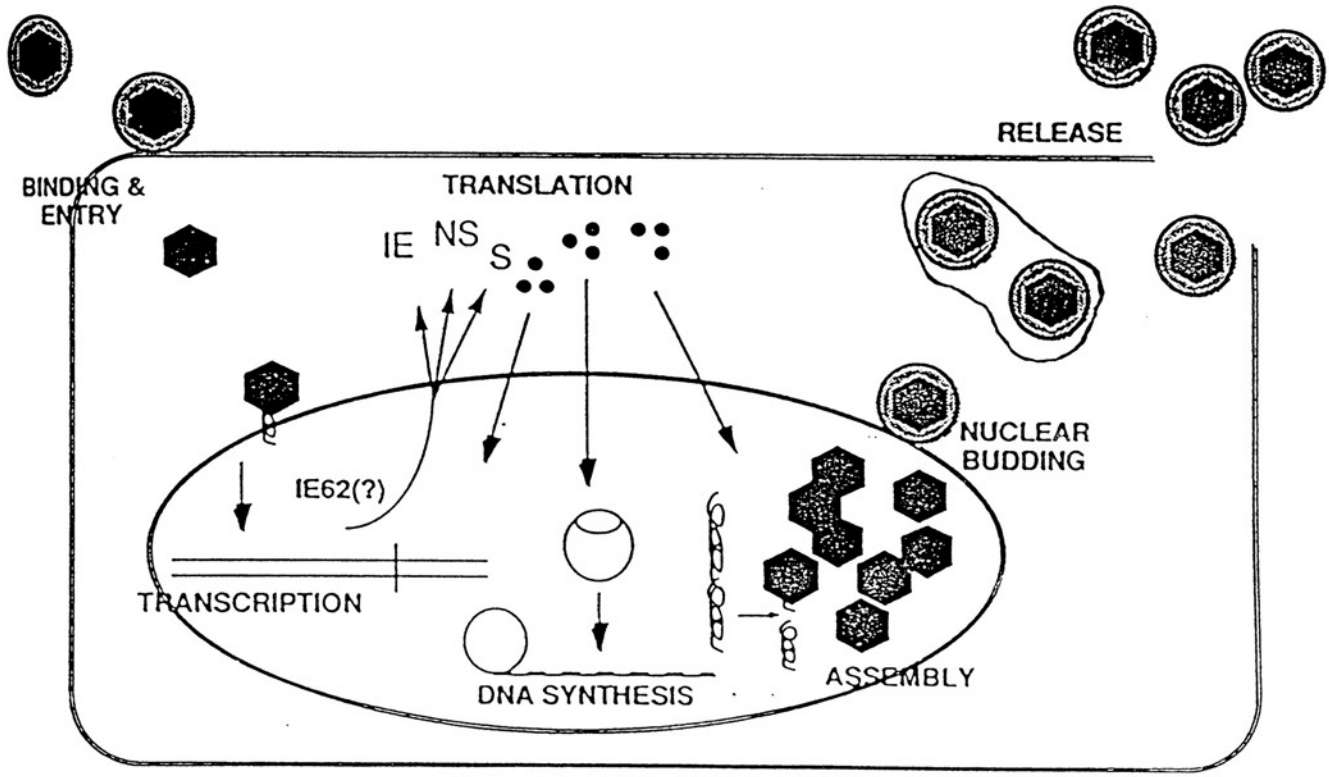
interacts with the TAATGARAT motif to enhance transcription; Vmw65 itself does not bind to DNA directly (Preston *et al.*, 1988; Triezenberg *et al.*, 1988). Several other viral IE proteins: Vmw110 (ICP0), Vmw68 (ICP22), Vmw63 (ICP27) and one E gene, ICP8 (the major DNA binding protein), also seem to regulate transcription of viral genes, but the mechanisms of their actions are still not precisely defined (Stow and Stow, 1986; Sacks *et al.*, 1985; Sacks and Schaffer, 1987; O'Hare and Hayward, 1985; McCarthy *et al.*, 1989; Everett, 1986; Godowski and Knipe, 1983; Post *et al.*, 1981; Roizman and Sears, 1990).

VZV gene expression

The lytic replication cycle of VZV (Fig. 2.1) is the same as that of other herpesviruses. The characterization of VZV transcription and gene regulation has only recently started. Using overlapping DNA restriction fragments to detect the total infected cell RNA by Northern blotting, at least 78 transcripts appear to be present in the VZV-infected cell (Ostrove *et al.*, 1985; Reinhold *et al.*, 1988). The directionality of these transcripts correlates roughly with the predicted ORFs from the gene layout of VZV reported by Davison and Scott (1986). Most of these transcripts are polyadenylated; however two species were not (Maguire and Hyman, 1986). ORFs42 and 45 are likely to be spliced during VZV gene expression based on the presence of consensus donor and acceptor sites (Davison and Scott, 1986). A detailed

Figure 2.1

Schematic diagram of the proposed infectious cycle of VZV during lytic infection. Infection is initiated by binding of the virus to the plasma membrane of susceptible cells followed by fusion of the viral envelope and entry of the virus. Following uncoating, the viral DNA enters the nucleus where three temporal classes of proteins, IE, E and L are produced. Transcription of IE mRNA may be enhanced by the IE62 protein which is carried into the cell as a major virion component. DNA synthesis takes place during the E phase, possibly utilizing both bidirectional and rolling circle mechanisms. During the L phase, progeny DNA are packaged into preformed nuclear capsids which, in turn, are enveloped by budding through the nuclear membrane. Enveloped particles acquire a second envelope by budding into cytoplasmic vesicles derived from the Golgi. The membranes surrounding these vescicles ultimately fuse with the cell membrane resulting in release of the virus. (reproduced from Ruyechan and Hay, 1994)


RELEASE
BINDING &
ENTRY
TRANSLATION
IE
NS
S
NUCLEAR
BUDDING
IE62(?)
TRANSCRIPTION
DNA SYNTHESIS
ASSEMBLY

mapping of each transcript and identification of the minimum cis-acting elements needed for regulating expression of these transcripts are available in only a few cases.

The 1.8 kb transcript of the VZV pyrimidine deoxynucleoside kinase (dPyK) gene was the first VZV transcript identified (Sawyer *et al*; 1986). Davison and Scott (1986) used S1 nuclease to map the 5' end of its mRNA and an interesting point arose from these studies; that the true 5' end of the dPyK mRNA could not be predicted from the nucleotide sequences alone. For example, the putative 'TATA' box (TATTAA) resides 25 bp upstream of the RNA initiation site, but three other AT-rich consensus 'TATA' boxes also appear between the RNA cap site and the AUG initiation codon. Similarly, the 3' terminus occurs just downstream from a AGTAA sequence, and this is 119 bp and 266 bp upstream from other canonical polyadenylation sequences (AATAAA and ATTAAA). The leader sequences (5' untranslated region) are also a surprising 420 bp in length (larger than the leader sequences of the homologous HSV gene which is 110 bp).

The 4.3 kb transcript of VZV ORF62 was first identified by Felser *et al.* (1988) and they predicted the putative regulatory elements (TATAA box, CCAAT box and TAATGARAT sequences) somewhere between -374 to -787 upstream from the predicted VZV ORF62. By using S1 nuclease and primer extension, McKee *et al.* (1990) mapped the 5' terminus. A 'TATA'-like box (TTTTAA) appears at -25 and a TAATGARAT-like element at -255 bp upstream from the transcriptional

initiation site.

The two major mRNAs of VZV ORF14, which encodes glycoprotein V of VZV, are 1.95 kb and 2.5 kb in length (Ling *et al.*, 1991). The putative 'TATA' box appears at -25 bp and the 'CCAAT' box at -55 bp upstream from the 5' end of the mRNAs.

There is a major 1.65 kb transcript which maps to the ORF67 gene encoding VZV gpIV (Ling *et al.*, 1992). The putative 'TATA' box appears at -30 bp and a 'CCAAT' box at -67 bp upstream from the 5' end of mRNAs.

A 1.8 kb transcript has been mapped to the ORF61 gene (Nagpal and Ostrove, 1991; Stevenson *et al.*, 1992). The putative 'TATA' boxes lie at -29 bp and -58 bp which is downstream from two potential 'CCAAT' boxes. The HSV homolog of ORF61 (Vmw110) has HSV IE upstream elements such as TAATGARAT. There is no report for such an element in the upstream region of VZV gene ORF61.

A major 1.8 kb and a minor 3.0 kb polyadenylated RNA from VZV ORF4 (the HSV ICP27 homologue) and a 1.3 kb and a 1.9 kb polyadenylated RNA of VZV ORF63 (the HSV ICP22 homologue) were recently identified (Kinchington *et al.*, 1994). The putative 'TATA' boxes of both genes are found approximately 25 to 35 bp upstream from the transcriptional start site. Unlike the HSV ICP27 & ICP22 genes, once again the TAATGARAT motif was absent from the upstream region of these two VZV genes, implying that VZV may have only one true IE gene (IE62). Although comparisons of the VZV gene

arrangement with published HSV-1 transcript mapping data indicate that both viruses have the same general layout, the transcriptional control of gene expression of VZV and HSV homologous genes may be not the same (Davison and Scott; 1986).

Although the transcriptional starting sites of above genes have been reported, only the cis-acting sequences that regulate the expression of VZV ORF 62 have been investigated to any extent. The minimum cis-acting elements which control gene expression of the other kinetic classes of VZV genes still have to be explored.

VZV gene regulatory proteins

VZV ORF62 was predicted to be the HSV Vmw175 homologue by its ability to complement HSV *ts* mutants in the Vmw175 gene, and this was confirmed by amino acid homology and genome location (Felser *et al.*, 1987, 1988). The VZV ORF10 protein is the homologue of HSV Vmw65 (VP16). Both of them are virion tegument proteins and, except for an acidic carboxy terminus that is critical for transactivation by Vmw65, the ORF10 protein and Vmw65 have similar amino acid sequences. In transient-expression assays in HeLa cells, McKee *et al.* (1990) reported that HSV Vmw65 can transactivate both VZV ORF62 and HSV Vmw175 promoters. Recently, Moriuchi *et al.* (1993a) have shown that the ORF10 protein can also transactivate VZV ORF62 and HSV ICP4 and ICP0 gene promoters in transient assays in Vero cells, but at a much reduced

level compared to Vmw175.

Inschuaspe *et al.* (1989a) demonstrated that the VZV ORF62 protein alone (or synergistically with the VZV ORF4 protein) could transactivate VZV dPyK and gpI promoters in transient-expression assays. These results showed the same pattern as had been demonstrated with the homologous genes in HSV (Vmw175 and Vmw63) in transactivating a variety of HSV promoters (Everett, 1986; O'Hare and Hayward, 1984; Shapira *et al.*, 1987). In the case of the VZV ORF61 protein, which is homologous to HSV Vmw110, Inchauspe *et al.* (1989b) also demonstrated that it can repress the expression of E genes and also modulated this E gene activation by ORF4 and ORF62 gene products. Nagpal and Ostrove (1991) also showed that it can repress the expression of IE, E, and L gene promoters in Vero cells in transient assays. On the other hand, Perera (1992a) showed that the ORF61 protein may play an accessory regulatory role in synergizing the activation of VZV genes induced by ORF62 protein in human T lymphocytes.

Jackers *et al.* (1992) reported that the ORF63 gene product expressed under the RSV (Rous Sarcoma Virus) LTR promoter strongly repressed the expression of ORF62 but activated the expression of the dPyK gene and had no significant effect on late genes (gpI and gpII) in Vero cells.

However, all of these VZV trans-acting elements which have been examined so far have been investigated using the "promoter-like" regions predicted from the transcriptional

starting sites. One of the aims of this dissertation will be to assess the influence of these trans-acting elements on authentic promoter regions as they are revealed by our investigations.

## Specific aims of the project

Although information on the detailed transcription strategy of the VZV genome is beginning to emerge, we know almost nothing about minimum cis-acting elements which control gene expresssion. Only the cis-acting sequences that regulate the expression of VZV ORF62 have been investigated to any extent. As in PRV and EHV-1, we propose that VZV has only a single IE gene, which is equivalent to Vmw175 of HSV (Fenwick and McMenamin, 1984; Gray *et al.*, 1987). The aims of this project, then, are to define the promoter elements for representatives of all of the other VZV kinetic classes of VZV genes and investigate the cis-acting sequences involved, and also to examine the functional relatioship of some of the VZV encoded regulatory proteins on these promoter regions. We have chosen three genes for our study, representing the E class (gene61), the L1 class (gpIV), and the L2 class (gpV). When the promoters for these genes have been defined, we will then be able to describe the control elements for all kinetic classes of VZV genes and the functions of tran-acting elements on these promoters, and develop an understanding of how the complex process of VZV gene regulation is controlled.

## Materials and Methods

### Cells

Human foreskin fibroblasts (HFF; strain USU 521) were obtained from Monroe Vincent, Department of Pediatrics, USUHS. HFF cells were grown in 175 $cm^2$ flasks (FALCON or NUNC) in Eagle's Minimum Essential Medium (GIBCO-BRL) supplemented with 5% (v/v) fetal bovine serum (GIBCO), 5%(v/v) Serum Plus (HAZLETON), and 100 u/ml penicillin G and 100 mg/ml streptomycin at 37°C and 5% $CO_2$. They were trypsinized (0.25% trypsin-EDTA in HBSS, JRH BIOSCIENCE, Inc.) when monolayers were confluent, resuspended in growth medium, and seeded into new flasks or new plates which were used for transfection assays.

A CD4-positive, continuous human T cell line, A3.01, was obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases. They were grown in 75 $cm^2$ flasks in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, and 100 u/ml penicillin G and 100 mg/ml streptomycin at 37°C and 5% $CO_2$. They were seeded into new flasks by simply transferring 10% of the cells from the previous flask.

### Viruses

Varicella zoster virus (VZV) strain Scott was obtained from Monroe Vincent, Department of Pediatrics, USUHS. VZV was grown in HFF cells, by trypsinizing infected cell cultures

and adding them to uninfected cell monolayers at a ratio of 1 to 5 infected to 10 uninfected cells. The infected cells were then incubated at 37°C for 3-5 days until 80% of cytopathic efffect (CPE) was seen under the light microscope. Cells were then harvested for experimental uses or for infecting other monolayers.

Herpes simplex virus (HSV) type 1 strain KOS was obtained from Dr. William Ruyechan, Department of Microbiology, SUNY @ Buffalo and was also grown in HFF cells. The infected cells were incubated at 37°C for 2-4 days until 80% CPE were seen under the light microscope. Cells were then harvested for experimental uses.

Gel Electrophoresis

Agarose gel electrophoresis:

Agarose slab gels were prepared by calculating the desired percentage of agarose (w/v). The agarose powder was added to 1XTAE running buffer (TAE: 0.04 M Tris-acetate, 0.001M EDTA) and was dissolved by boiling in a microwave oven, cooled to 60°C, poured in a plastic tray, and then the desired comb inserted. When the gel was solid, the comb was removed and the gel with the tray was submerged in an electrophoresis rig (BRL, Gaithersburg, MD; model H5) containing 1XTAE buffer. Samples and a 1 Kb DNA standard ladder (GIBCO, BRL) were then loaded into the wells in 1x loading buffer (10x loading buffer: 40%(w/v) bromphenol blue, 40% (w/v) xylene cyanol, and 25%(w/v) ficoll type 400) and

electrophoresed at constant voltage. To visualize nucleic acid samples, the entire gel was immersed in an ethidium bromide solution (50 µg/ml ethidium bromide in $H_2O$) for 15 min. A permanent picture of ethidium bromide-stained gels was recorded by photography using Polaroid type 667 film with an Electrophoresis Systems Photo-Documentation HOOD (FISHER-SCIENTIFIC) camera.

Purification of DNA fragments

DNA fragments were purified by excising the appropriate bands with a razor blade from the agarose gel after electrophoresis. If the DNA fragments were >400 bp, the agarose pieces were dissolved in 6M NaI from a GENE CLEAN II kit (BIO 101) and the DNA fragments recovered following the manufacturer's protocol. If the DNA fragments were <400 bp, they were purified using MILLIPORE ultrafree-MC filters (cat. no. UFC3 OHV and UFC3 TTK) following the manufacturer's protocol.

Molecular cloning of DNA fragments

Most of the recombinant plasmids that were generated in this study came from the pCATBasic vector (purchased from PROMEGA) and the insertion of the desired fragments of the putative promoter regions of ORF67, ORF14 or ORF61 which encode glycoproteinIV (gpIV), glycoproteinV (gpV), and ORF61 proteins, respectively.

For gpIV and gpV, approximately 430 base pair and 580

base pair *Bgl*II fragments were excised from pG4-3 and pS6 plasmids (obtained from Dr. Paul Ling, USUHS), respectively. The protruding 5'ends were blunt-ended by filling in with DNA polymerase I large fragment (Klenow) enzyme and 4 dNTPs. For the ORF61 gene, an approximately 930 base pair *Mlu*I fragment was excised from a p*Hin*dIII-CAT plasmid (this plasmid was made by cloning a 4.63 Kb.*Hin*dIII fragment which had been excised from the p*Eco*RI'A' plasmid {containing *Eco*RI'A' fragment of the VZV genome, obtained from Dr. L.P.Perera} into the *Hin*dIII site of pCATBasic). The protruding 5'ends were changed to a blunt-ended fragment to get rid of the "ATG" starting codon for the ORF61 gene by excising with $T_4$ polymerase enzyme and 4 dNTPs.

Each of the blunt-ended fragments was cloned into the *Xba*I site of the pCATBasic vector which had already been cut with *Xba*I enzyme and blunt-ended. We named them: pgpIV-CAT, pgpV-CAT and pMluI-CAT for the gpIV, gpV and gene 61 promoter fragments, respectively.

For the second gpV recombinant promoter plasmid, which contained the upstream sequences as well as part of the gpV ORF in a LAC fusion construct, the pBS(-) vector (purchased from STRATEGENE) was used. A 1.16 Kb *Nde*I/*Eco*RI fragment which included the 580 base pair promoter-like region and 7.76 copies of the 42 base pair repeated inframe sequences was excised from the p*Pst*I-BS plasmid (made from the 3.5 Kb *Pst*I fragment which had been excised from a pAT153 plasmid {containing the *Kpn*I'A' fragment of the VZV genome, obtained

from Dr. A.J. Davison} into the *Pst*I site of pBS(-) ). The protruding 5' ends were blunted by Klenow enzyme and 4 dNTPs. This fragment was then cloned into *Sma*I site of pBS(-). A 3.08 Kb *Bam*HI fragment of ß-galactosidase excised from the pDp503 plasmid (obtained from Dr. Frank Jenkins, USUHS) was also cloned into the *Bam*HI site of the pBS(-) plasmid above. We named it the pgpV-42-ßgal plasmid.

A further construct, pgpV120(3-3.4)-CAT, was made by reannealing the 3-3.4U oligonucleotide and the 5' phosphorylated 3-3.4L oligonucleotide and cloning into the *Sph*I site of pgpV120-CAT plasmid (see Figure 20).

Generation of deleted promoter constructs

Fragments from each of the recombinant plasmids were excised to determine the optimal sequences used for promoter function. The ExonucleaseIII/Mung Bean Nuclease enzyme deletion approach (STRATAGENE, La Jolla, CA) was used. All procedures followed the manufacturer's protocol.

GpV: A set of plasmids containing deletions in the promoter sequences of the gpV gene in the pgpV-CAT plasmid (that contained sequences from -546 to +35 from the transcriptional starting site) were constructed. All the deletion plasmids were cut from nucleotide -546 downstream (towards the transcriptional start site) as follows: pgpV120-CAT(-43), pgpV100-CAT(-87), pgpV60-CAT(-135) (see Figure 3).

GpIV: Deletions from the gpIV promoter in pgpIV-CAT plasmid which contained sequences from -363 to +63 relative

to the transcriptional start site were also carried out. All plasmids in this series were cut from sequence -363 downstream as follows: pgpIV1-CAT(+16), pgpIV2-CAT(-12), pgpIV3-CAT(-34), pgpIV3.1-CAT(-39), pgpIV3.2-CAT(-42), pgpIV3.3-CAT(-46), pgpIV3.4-CAT(-53), pgpIV4-CAT(-57), pgpIV5-CAT(-60), pgpIV6-CAT(-70), pgpIV6.1-CAT(-86) (see Figure 13).

Gene 61: Since there was the same compatible restriction enzyme cutting sites on both pCATBasic and the *Mlu*I fragment of the gene 61 promoter, one deletion plasmid (p61-WK10) was constructed by removing a 692 bp *Acc*I fragment from pMluI-CAT, and then re-ligating the remaining sequences of the plasmid. Thus, the p61-WK10 plasmid contained sequences from -179 to +64. All other deletion constructs except p61-WK6 and p61-WK7 were deleted from pMluI-CAT which contained sequences from -871 to +64 relative to the transcriptional start site. These were deleted from sequence -871 downstream as follows: p61-WK9(-123), p61-WK8(-80), p61-WK5(-33), p61-WK4(-23), p61-WK3(-16), p61-WK2(-5), p61-WK1(+34) (see Figure 23).

<u>The polymerase chain reaction (PCR) method for site directed mutagenesis</u>

For changing the sequence 'in' or 'in&around' the "TATA" box of the VZV gpV promoter to be the same as that of the HSV gC promoter, we used the PCR method. Oligo gpV-C1 or Oligo gpV-C14 (for 1 base or 14 base similarities, respectively)

and Oligo gpV-*Bst*XI were used as primers to make fragments of approximately 480 bp from the pgpV-CAT plasmid. The reactions were amplified by a programmable DNA Thermal Cycler (PERKIN-ELMER). These PCR products were then run on agarose gel electrophoresis and the bands were excised and gene cleaned. Each of PCR products was cut with *Sac*II and *Bst*XI and then recloned into the pgpV-CAT from which the fragment of *Sac*II and *Bst*XI had already been removed. Both clones were reconfirmed by sequencing. We named them pgpVCm1 and pgpVCm14, respectively.

The Polymerase chain reaction (PCR) method for cloning of DNA fragments.

We used the PCR method for constructing plasmids p61-WK6 and p61-WK7 from gene 61, which contained sequences from -57 to +64 and from -72 to +64 from the transcriptional start site, respectively. Oligonucleotide G61-WK6 or G61-WK7 and oligonucleotide G61-*Nco*I were used as primers to make fragments of 133 bp or 148 bp, respectively, from the p61-WK10 plasmid. All oligonucleotides had been designed to have *Xba*I cut sites at their 5′ ends. The reactions were amplified by a programmable DNA Thermal Cycler (PERKIN-ELMER, Foster City, CA). The PCR products were then run on agarose gel electrophoresis and the bands were excised and purified by MILLIPORE ultrafree-MC filters. Each of PCR products was cloned into the pCR™Vector (TA Cloning™ Kit, INVITROGEN, San Diego, CA). We named them pCRII-WK6 and pCRII-WK7,

respectively. The needed fragments were excised from both plasmids using *Xba*I and then cloned into the *Xba*I site of pCATBasic. The orientation and presence of DNA inserted in both clones were confirmed by sequencing; we named them p61-WK6 and p61-WK7, respectively.

Bacterial transformation

The DH5α strain of *E. coli* was made competent by either the standard Hanahan method (Hanahan, 1983) or by the calcium chloride method, which is a variation of that of Cohen *et al.* (1972) as described by Sambrook *et al.* (1989). Ten µl of the ligation reaction was added to 100 µl of competent bacteria for half an hour on ice. The cells were then heat-shocked at 42°C for 1-2 min and put back on ice for 2 min. S.O.C. medium (900 ml; 2%(w/v) tryptone, 0.5%(w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl 10 mM $MgCl_2$ ,10 mM $MgSO_4$, 20 mM glucose) was added and the cells were incubated on a shaker at 37°C for 1 hour. The bacteria were spun at 3000 rpm for 5 min. and resuspended in 100 µl of S.O.C. medium. This suspension was then spread onto sterile LB agar plates containing 100 µg/ml ampicillin (since the vectors contained an ampicillin resistance gene). In some cases, plates also contained XGal (2% v/v) and IPTG (25 µg/ml) for selection of positive bacterial colonies (blue color) or negative bacterial colonies (white color) relevant to the production of ß-galactosidase (ß-gal). Plates were incubated overnight at 37°C.

Identification of recombinant plasmids

Bacterial colonies were screened by picking blue colonies (ß-gal positive) in cases where a sequence was cloned into the pBS(-) plasmid. In case of the pCATBasic vectors, however, all colonies had to be tested. Each of the colonies was cultured in 5 ml LB broth containing ampicillin and then incubated overnight at 37°C to allow growth. Plasmid DNA was prepared using the "Super Quick Plasmid DNA Preparation" (this method was adapted from the Lysis Boiling Procedure described by Sambrook *et al.* (1989)) by spinning down 1.5 ml of each bacterial preparation. Bacteria were resuspended in 0.4 ml of STET/lysozyme buffer (STET: 8% w/v sucrose, 5% v/v Triton-X100, 50 mM Tris-Cl pH8.0, 50mM EDTA, lysozyme 10mg/ml), boiled for 60 seconds, spun at 10,000 rpm for 15 min. at room temperature, and then the pellet removed. The 0.4 ml of isopropanol was added to each tube, mixed, incubated at -20°C for 5 min., spun for another 5 min, and the pellet dried at room temperature and resuspended with 50 µl distilled water. The plasmid DNA was cleaved by restriction enzymes, electrophoresed on agarose gels and visualized by staining with ethidium bromide to confirm the orientation and presence of DNA inserted. In case of pgpV-42-ß-gal, sequencing of DNA was done to confirm the correct frame of the ß-gal ORF. For deletion sets of each recombinant plasmid, DNA sequencing was also done to determine the extent of sequence removal. For all PCR clones, the sequences and orientations were confirmed by sequencing.

If large amounts of recombinant DNA were required, bacteria were grown in 500 ml media and plasmid DNA was prepared using the "Lysis by Alkali" procedure and purified by CsCl-ethidium bromide centrifugation, as described by Sambrook *et al.* (1989).

DNA Sequencing

To determine or confirm the DNA sequences of genomic segments that had been cloned or deleted, chain-termination DNA sequencing reactions were performed by the method of Sanger *et al.* (1977) using the Sequenase V2.0 sequencing kit (USB, Cleveland, Ohio).

Plasmids

All plasmid vectors used in this study are either commercially available or are well documented in the literature. The pCATBasic, pCATControl (PROMEGA Biotech, Madison, WI), pUC18 (BRL, Gaithersburg, MD), pBS(-), $pSV_2$ß-gal (STRATAGENE, La Jolla, CA) were all purchased from the manufacturers. The $pSV_2$CAT plasmid was generously provided by Dr. George Khoury (NIH). The pGC-CAT plasmid and its deletion plasmids were generously provided by Dr. Fred L. Homa (University of Michigan). The pDp503 plasmid was kindly provided by Dr. Frank Jenkins (USUHS). The pCMV62, pCMV4, pCMV63 and pCMV61 plasmids were kindly donated by Dr. Majid Sadeghi-Zadeh (Université de Paris) and Dr. Paul Kinchington (University of Pittsburgh). The p*Eco*RI'A' was kindly provided

by Dr. L.P. Perera (NIH). The pAT153 VZV clones were generously provided by Dr. A.J. Davidson (University of Glasgow). Maps of the more important plasmids are shown in the Appendix. Schematic representations of recombinant and deletion sets of plasmids generated for use in this study, as well as pertinent data regarding orientation and confirmation of DNA insertion are shown in the Appendix and in the "Results" section of this dissertation.

Restriction enzyme digestion of DNA

Purified plasmid DNA was stored in sterile double-distilled $H_2O$ and diluted in restriction enzyme buffer supplied by the manufacturer. The amounts of enzyme used, temperature and time of digestion followed the manufacturer's protocol. In some cases, when other restriction enzyme digestions or other modifications of DNA were needed, the first reaction was stopped by heat inactivation or phenol-chloroform extraction before subsequent procedures. Once all the manipulations were complete, the reaction was stopped by addition of 10X loading buffer and the sample electrophoresed on agarose gels as described above.

Oligonucleotides

Oligonucleotides used in these studies were synthesized by the Oligonucleotide Synthesis Facility, Department of Biochemical Pharmacology, SUNY @ Buffalo. They are listed by complementary gene, name, and sequences.

| gene | name | sequences |
|---|---|---|
| gpV | gpV-C1 | 5′ CACCGCGGAATTTATATGATGGAAAC 3′ |
| gpV | gpV-C14 | 5′ CCACCGCGGAATTTATACCCTGGAAAC 3′ |
| gpV | gpV-*Bst*XI | 5′ ACCCCATTAATGTGGGCA 3′ |
| gpIV | 3-3.4L | 5′ GTAATGGGGCGTGACTCTGT 3′ |
| gpIV | 3-3.4U | 5′ ACAGAGTCACGCCCCATTAC 3′ |
| gene61 | G61-*Nco*I | 5' TCTAGAGTAACAACTGGCTGTAT 3' |
| gene61 | G61-WK7 | 5′ TCTAGAGGGTGGAGGGATATA 3' |
| gene61 | G61-WK8 | 5′ TCTAGAGGGGTGTGTCTTCGTTG 3' |
| CAT | CAT-Ba | 5′ CTCGCCAAGCTCAGATCC 3′ |

Plasmid transfection of cells

For DNA transfections in Human Foreskin Fibroblast (HFF) cells, the DEAE-Dextran method was used, following the detailed procedure in "Protocols in Molecular Biology" (Ausubel *et al.*, 1992). Ten µg of target plasmid was used to transfect HFF cells in 10 cm tissue culture plates. To infect transfected cells with VZV, VZV infected monolayers were trypsinized, then added to transfected cells at a ratio of 1 to 10 infected to uninfected cells. When co-transfections with effector plasmids were carried out, each was added at 5 µg per plate. The total DNA amount was kept constant by adding pUC18 plasmid DNA. The cells were harvested at 48 hours after VZV infection or co-transfection.

For DNA transfections in the human T cell A3.01 line, the electroporation method was used. The cells were washed with PBS and resuspended in serum-free RPMI media without

glutamine or antibiotics at a density of $2x10^7$ cells per ml. The 0.4 ml cell suspension was mixed with 10 µg of target plasmid and 5 µg of each effector plasmid in an electroporation chamber. The amount of total DNA was kept constant by adding pUC18 plasmid DNA. The chamber was incubated on ice for 10 min. The cells were then electroporated with one pulse by using a Electro cell manipulator 600 (BTX Inc, CA) with a setting of mode T = 500 V/ CAPACITANCE & RESISTANCE, C = 700 µF, R = R1(13 ohm), chamber gap = 2 mm, S = 150 V, desired field strength = 0.75 kV/cm, desired pulse length = ~4 msec, then put on ice for another 10 min. The cells were transferred to 24 well tissue culture plates and 1 ml of growth medium was added. The cells were harvested at 48 hours after plating.

For harvesting the cells, both HFF and A3.01 cells were washed with PBS. For A3.01 cells, the pellet was resuspended in 100 µl of 0.25M Tris HCl, pH 7.8. For HFF cells, the cells were scraped from the plate in 1 ml cold Tris/EDTA pH7.8, and spun before resuspending in 100 µl of 0.25M Tris HCl, pH 7.8. The cells were then disrupted by 3 cycles of freeze-thawing. At this point, the separated supernates were ready for ß-galactosidase assay. In the case of CAT assays, the supernates were further incubated in 60°C for 10 min before use.

ß-galactosidase assays

These assays were performed by following the method

described by Sambrook *et al.* (1989). Equal amounts of protein in each sample (as determined by the Bradford (1976) method) were used. The optical density of the reaction was read at 420 nm in the Du640 spectrophotometer (BECKMAN, Columbia, MD).

Chloramphenicol acetyltransferase (CAT) assays

CAT assays were performed by the method of Gorman *et al.* (1982). We used equal amounts of protein as determined by the Bradford (1976) method. At times, the CAT activity was quantitated with a PhosphorImager scanner with Image Quant software from Molecular Dynamics (Sunnyvale, CA), otherwise samples were counted in nonaqueous scintillation fluid on a Liquid Scintillation Counter (Wallac 1409; Wallac Inc, Gaithersburg, MD).

## Results

As pointed out in the introduction, VZV, like other herpesviruses, rigorously controls the sequence of production of viral proteins during the course of infection. It seems likely that this control is exerted at the level of promoter function; each VZV protein appears to have its own promoter, although only that for ORF62 has been described in any detail (Wagner, 1991; McKee *et al.*, 1990).

The overall aims of this study were to define the important cis-acting elements in the promoters for the four main kinetic classes of gene expression in VZV - immediate early (IE), early (E), early/late (L1) and late (L2). Since the only likely IE promoter (that for ORF62) has already been described (McKee *et al.*, 1990), we will focus on members of the remaining three classes in the work which follows. We will also show some VZV regulatory proteins that involve in the activation of these promoters.

### 1. Definition of the functional promoter region of the true late (L2) VZV protein, gpV.

**Construction of the gpV promoter construct, pgpV-CAT, and its deletion plasmids.**

Previous studies from our laboratory, using primer extension analysis, identified the transcriptional start (cap) site of the late (L2) VZV gene for glycoprotein V (gpV) (Ling *et al.*, 1991). Sequences upstream from the cap site

contained putative 'TATA' box and 'CCAAT' box elements at -25 bp and -55 bp, respectively. To determine the essential features of gpV promoter activity in transient assays, the putative promoter region and a number of deletion mutants constructed from it were fused to the CAT (chloramphenicol acetyltransferase) reporter gene of the plasmid pCATBasic. An approximately 580 bp *Bgl*II fragment of the pS6 plasmid (which includes a *Bst*NI-*Eco*RI fragment containing the 5' untranscribed and upstream sequences of the gpV gene) was cloned into pCATBasic to construct the pgpV-CAT plasmid. Using deletion procedures outlined in the methods, 3 pgpV-CAT deletion plasmids were also constructed (Fig. 3). All of these were deleted from nucleotide -546 to downstream sequences as follows:

1. pgpV120-CAT (contains the putative 'TATA' box),
2. pgpV100-CAT (contains the putative 'TATA' & 'CCAAT' boxes),
3. pgpV60-CAT (contains the putative 'TATA' & 'CCAAT' boxes + upstream sequences).

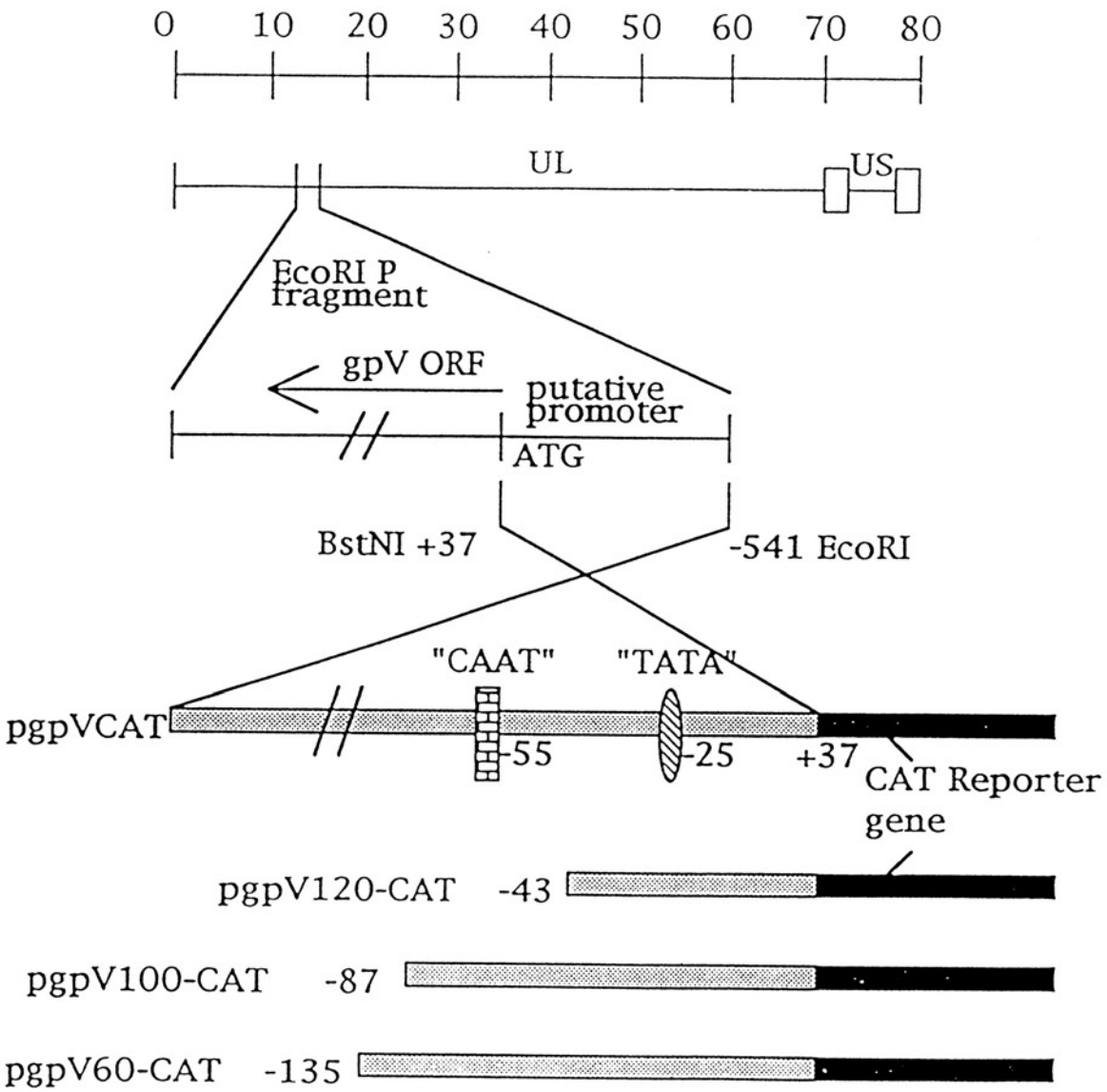
**Promoter activity of pgpV-CAT and its deletion plasmids.**

To test the expression from the CAT gene under the control of the putative gpV promoter, pgpV-CAT or each of its deletion plasmids was transfected into HFF cells by the DEAE-Dextran method, followed by infection with VZV. VZV infection was carried out to provide any viral products necessary for

Figure 3



Detailed structure of the gpV promoter construct, pgpV-CAT and its deletion plasmids. At the top, the VZV genome is presented. The upper line denotes the genome size in megadaltons ($80x10^6$). A *Bst*NI/*Eco*RI fragment was cut from the *Eco*RI P fragment and fused to the CAT reporter gene of the pCATBasic vector, to make pgpV-CAT. The pgpV120-CAT, pgpV100-CAT, pgpV60-CAT constructs were derived from pgpV-CAT by deletion mutation. The putative "TATA" and "CCAAT" boxes are located at -25 and -55, respectively. The VZV sequences are shown as dotted blocks and the CAT reporter sequences as solid blocks.


0
10
20
30
40
50
60
70
80
UL
US
EcoRI P fragment
gpV ORF
putative promoter
ATG
BstNI +37
-541 EcoRI
"CAAT"
"TATA"
pgpVCAT
-55
-25
+37
CAT Reporter gene
pgpV120-CAT -43
pgpV100-CAT -87
pgpV60-CAT -135

promoter activity. The results show that, while the putative gpV promoter sequences were inactive in HFF cells, VZV infection raised CAT activities from 4-12 fold above the constitutive levels (Fig. 4). This indicates that the region we have cloned is indeed able to function as a promoter and that it requires VZV infection for its activity. The behavior of the deletion plasmids (pgpV120-CAT is as active as the entire promoter region (pgpV-CAT)) showed that the minimal upstream region which confers activity is the 'TATA' box (Fig. 4). This leads to the conclusion that the promoter sequences essential for the expression of gpV are in the 'TATA' box region located between -25 and +37 from the transcriptional start site.

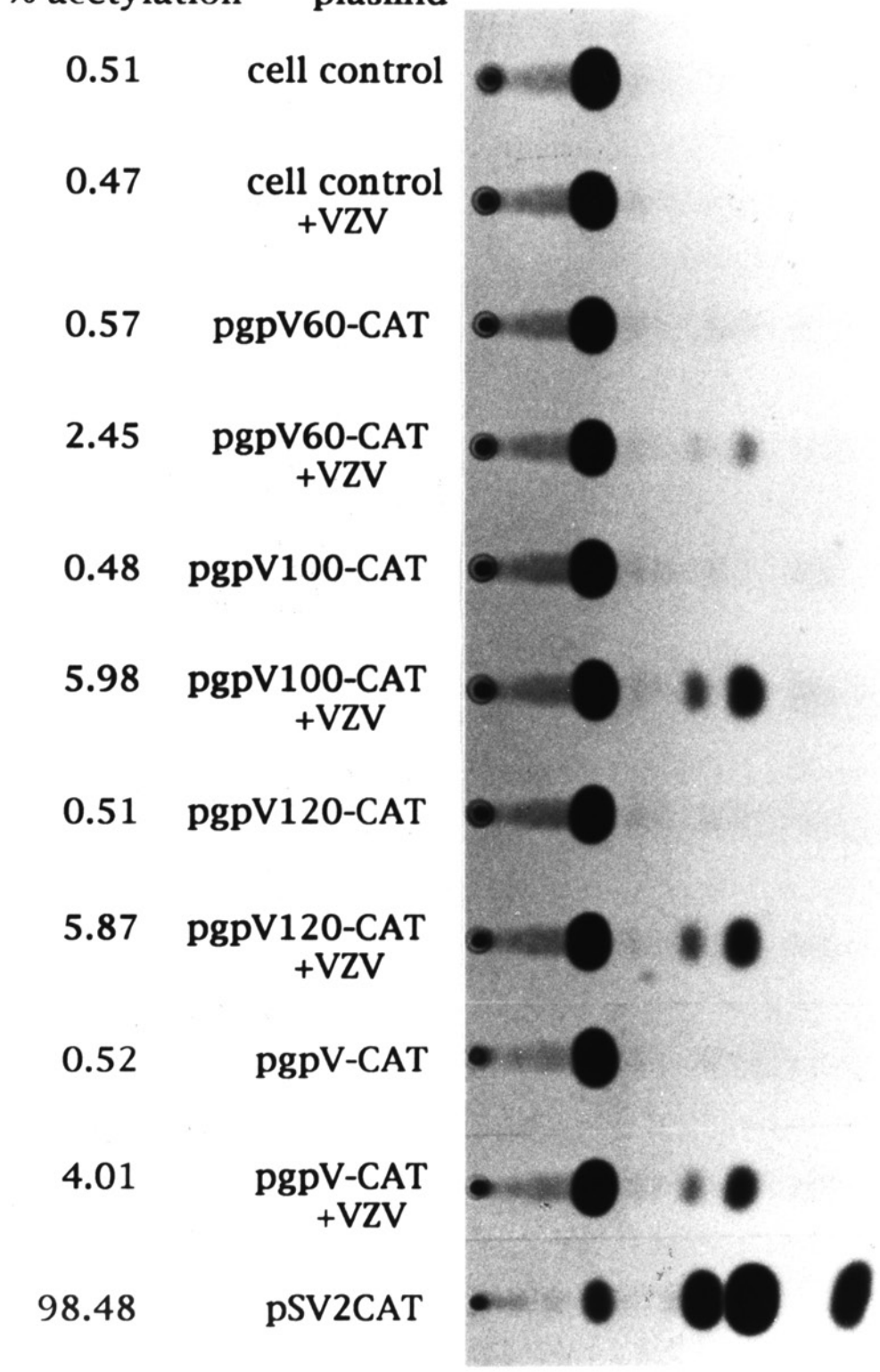
**Potential involvement of sequences 3' to the cap site in gpV promoter activity: construction of the pgpV-42-ßgal plasmid.**

The above data on the gpV promoter showed that the only upstream sequences needed for activity were around the 'TATA' box. However, the levels of activity seen were lower than expected and we considered the possibility that some downstream sequences may also affect promoter function. In particular, we had previously shown that variable copy numbers of a 42 bp repeated sequence were located close to the beginning of the ORF (14) which encodes gpV; these sequences have features similar to known enhancer elements (Kinchington *et al.*, 1986). Although these sequences had

Figure 4

Expression from the gpV promoter construct, pgpV-CAT and its deletion plasmids. Human foreskin fibroblast (HFF) cells were transfected with 10 μg of each plasmid by the DEAE-Dextran transfection method. Cells were either uninfected or infected with VZV on the following day. At 48 hours post infection, the cells were harvested, extracted and the levels of CAT activity were measured. The percentage acetylation is shown on the left column. The pSV2CAT plasmid was used as a positive control.

# pgpV-CAT and its deletion plasmids with VZV infection in HFF cells



already been examined for general enhancer activity by cloning them into the $pSV_2$-CAT plasmid (which has the SV40 E promoter) and we could not detect the activity (unpublished observations), we considered it important to retest them in the context of the gpV promoter. A 1.16 Kb fragment which included 581 bp of the (upstream) promoter and 7.76 copies of the repeat sequence was cloned inframe into pBS(-), containing a 3.08 Kb *Bam*HI fragment encoding the ß-galactosidase reporter gene (Fig. 5), to form a gpV-gal fusion protein.

**Expression from the pgpV-42-ßgal plasmid.**

To test the expression of the fusion protein, the pgpV-42-ßgal plasmid was transfected into HFF cells, followed by VZV infection. The ß-galactosidase activity level from the VZV-infected cell extract was 4-fold more than the constitutive level but was only about 1% of the control level (Fig. 6). Thus, this result showed that these 42 base pair repeated sequences had no obvious role in gpV promoter function, and suggested that only the 'TATA' box region was necessary for expression of gpV.

2. Comparison of the VZV gpV promoter with that of HSV gC.

**Expression from HSV pGC-CAT and its deletion plasmids.**

There are reports that VZV transactivating factors can complement that of HSV and *vice versa*: for example, VZV ORF62 can complement HSV-1 temperature sensitive mutants in the

Figure 5

Detailed structure of the gpV promoter construct, pgpV-42-ßgal. The *Nde*I/*Eco*RI fragment, which includes the in-frame 42 base pair repeats from the gpV ORF plus a 545 base pair upstream sequence was cloned into the *Sma*I site of the pBS(-) vector which contained a 3.08 Kb *Bam*HI fragment of the ß-galactosidase reporter gene. The VZV sequences are shown as dotted blocks and the CAT reporter gene as solid blocks.

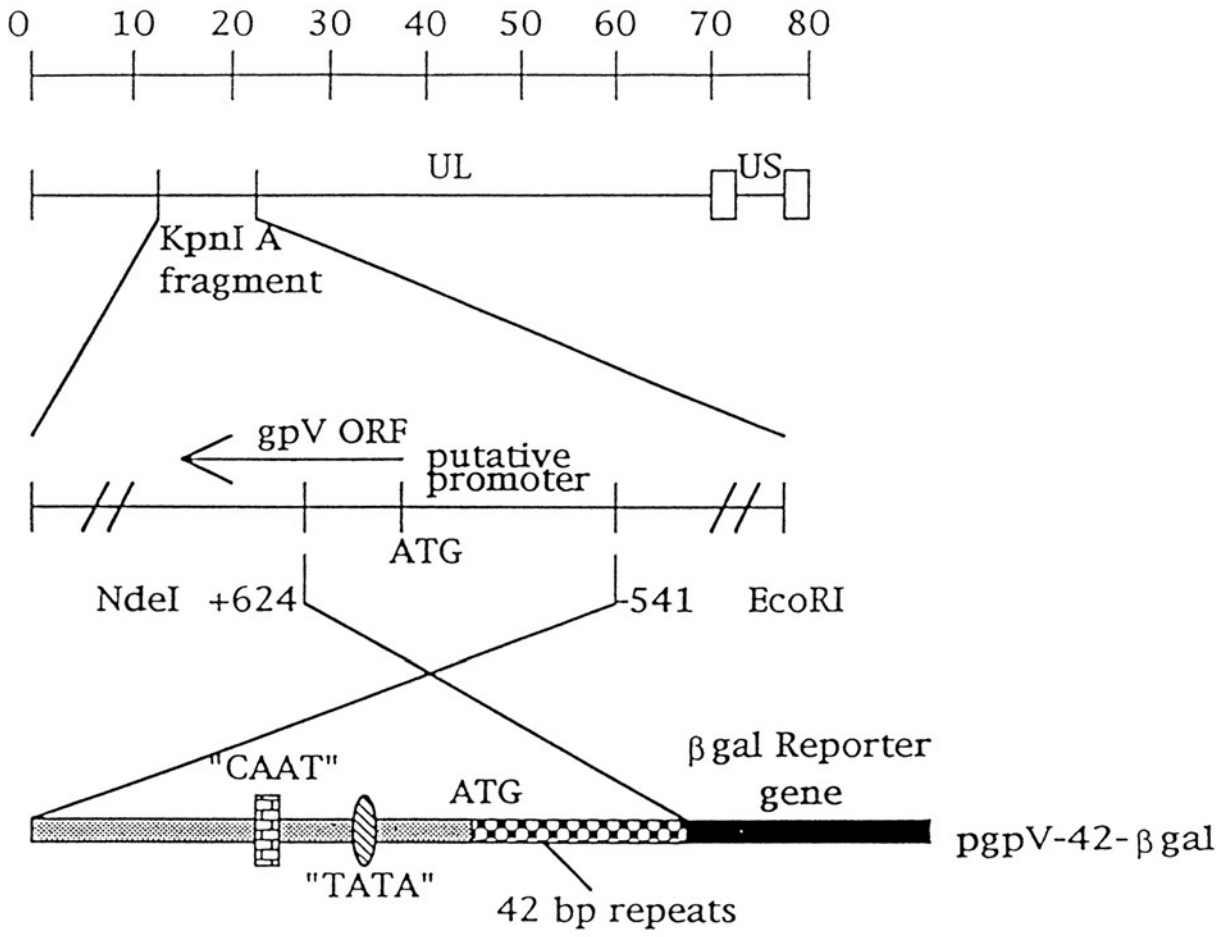
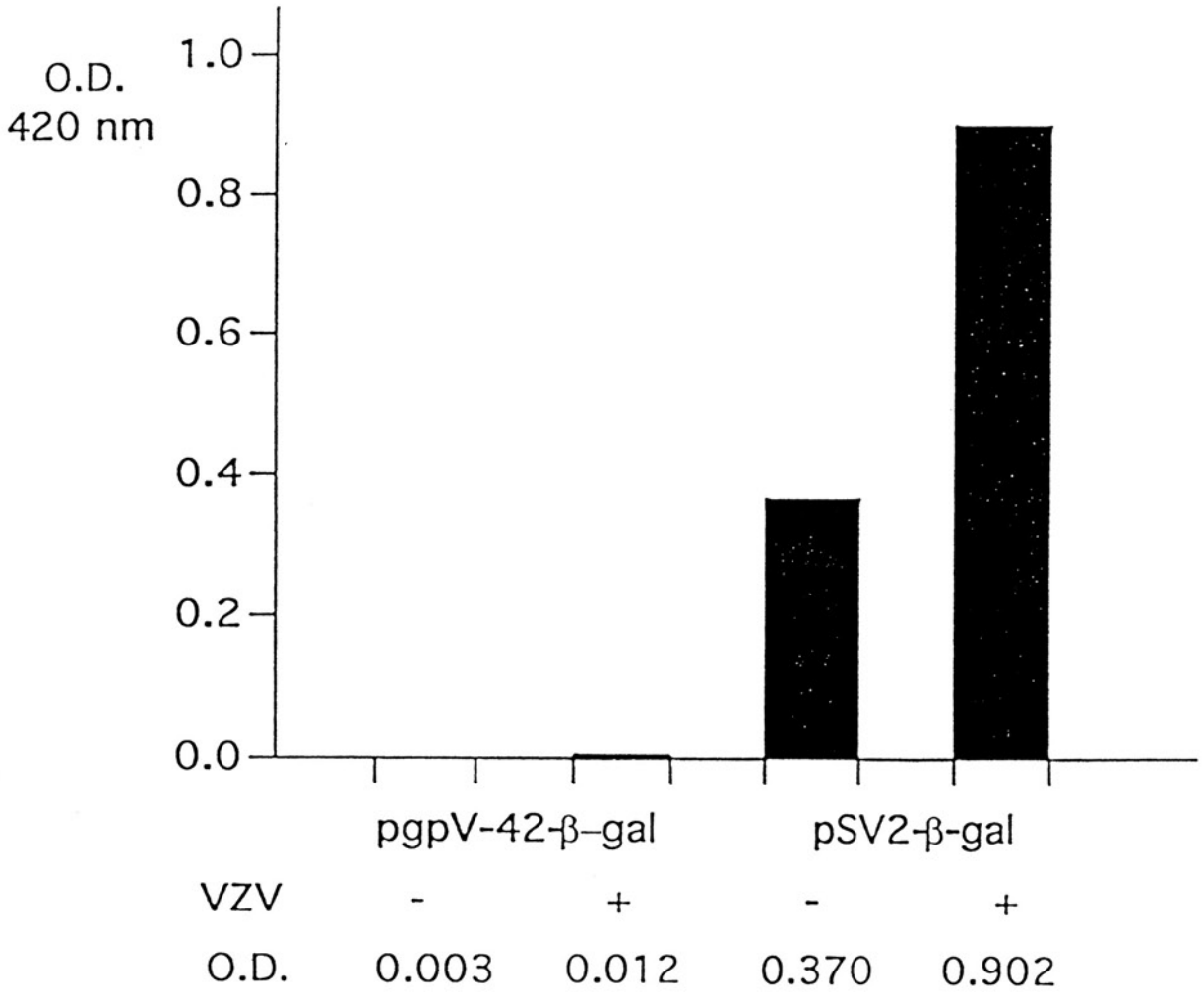

0
10
20
30
40
50
60
70
80
UL
US
KpnI A
fragment
gpV ORF
putative
promoter
ATG
NdeI
+624
-541
EcoRI
"CAAT"
ATG
βgal Reporter
gene
pgpV-42-βgal
"TATA"
42 bp repeats

Figure 6

Expression from the pgpV-42-ßgal plasmid. HFF cells were transfected with 10 µg of plasmid by the DEAE-Dextran transfection method and were either uninfected or infected with VZV on the following day. At 48 hours post infection, the cells were harvested, extracted and the levels of ß-galactosidase were measured at 420 nm. The pSV2-ßgal plasmid was used as a positive control.

# β–galactosidase activity of pgpV-42- β gal with VZV infection



Vmw175 gene (Felser *et al.*, 1987; 1988); HSV Vmw65 can transactivate both VZV ORF62 and HSV Vmw175 (McKee *et al.*, 1990), while VZV ORF10 can transactivate VZV ORF62 and HSV Vmw175 & Vmw110 (Moriuchi *et al.*, 1993a). To determine whether VZV infection could induce the promoter for HSV glycoprotein C (gC, the homologue to VZV gpV), the gC promoter construct pgC-CAT and several of its deletion plasmids (see Fig. 7) were transfected into HFF cells, followed by infection with VZV. The CAT activities with the intact or deletion plasmids all showed more than a 175-fold increase above the constitutive level after VZV infection, except for the pGC^3-CAT construct which lacked the 'TATA' box and transcriptional start site (Fig. 8). The results indicate that VZV can effectively provide the transactivating factors necessary for expression from the HSV gC promoter, as well as confirming that only the 'TATA' box-proximal sequences are needed for this promoter activity. Interestingly, however, the levels of activity seen with the gC constructs were much higher than that of its VZV counterpart (compare Figs. 4 and 8).

**Construction of HSV gC-like mutants of the VZV promoter.**

From the results above, it was clear that the gC promoter is more effective than the gpV promoter. We have shown above that the minimal gpV promoter is at the 'TATA' box, within the sequence 5'..TCATTTAAATTCCGC..3', similar but

Figure 7

Detailed structure of the HSV gC promoter construct, pGC-CAT and pGC-CAT deletion plasmids*. At the top, the HSV-1 genome is presented in the prototype arrangement showing the location of the 4 kb *Sal*I/*Hind*III fragment (map units 0.620-0.647) present in the plasmid pGC. Plasmid pGC-CAT was derived from pGC by deleting the coding sequences of the gC gene from the *Bgl*II to the *Hind*III site followed by insertion of the coding sequences of the CAT gene. The CAT structural sequences are therefore fused to the gC promoter at base +124 (*Bgl*II site) relative to the 5' terminus of the gC mRNA. Plasmids pGC^3-CAT, pGC^8-CAT and pGC^10-CAT were constructed in a similiar manner using pGC deletion plasmids. The HSV sequences appear as dotted blocks and the CAT sequences as solid blocks.

* Plasmid pGC-CAT and its deletion plasmids were gifts from Dr. Fred L. Homa.

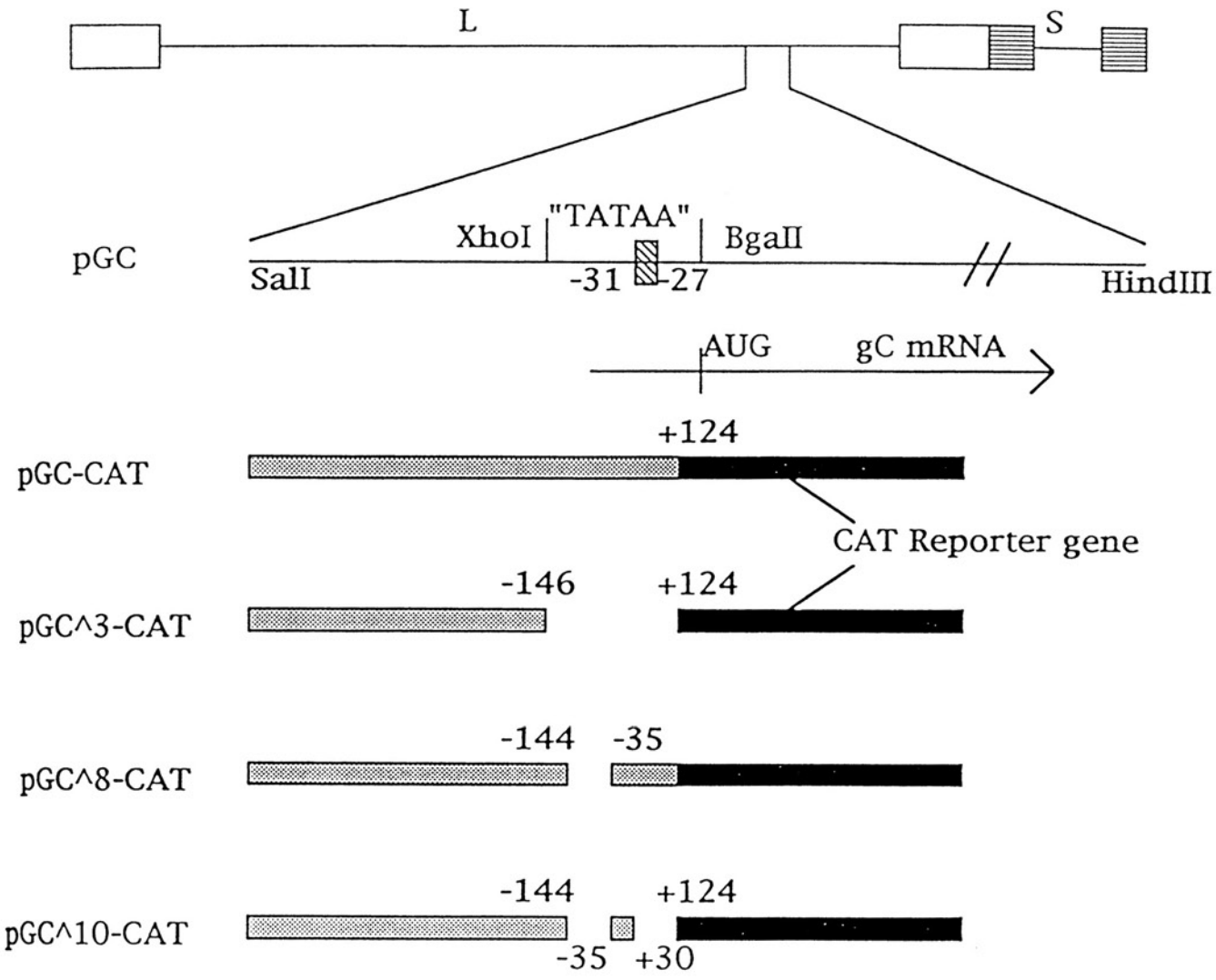
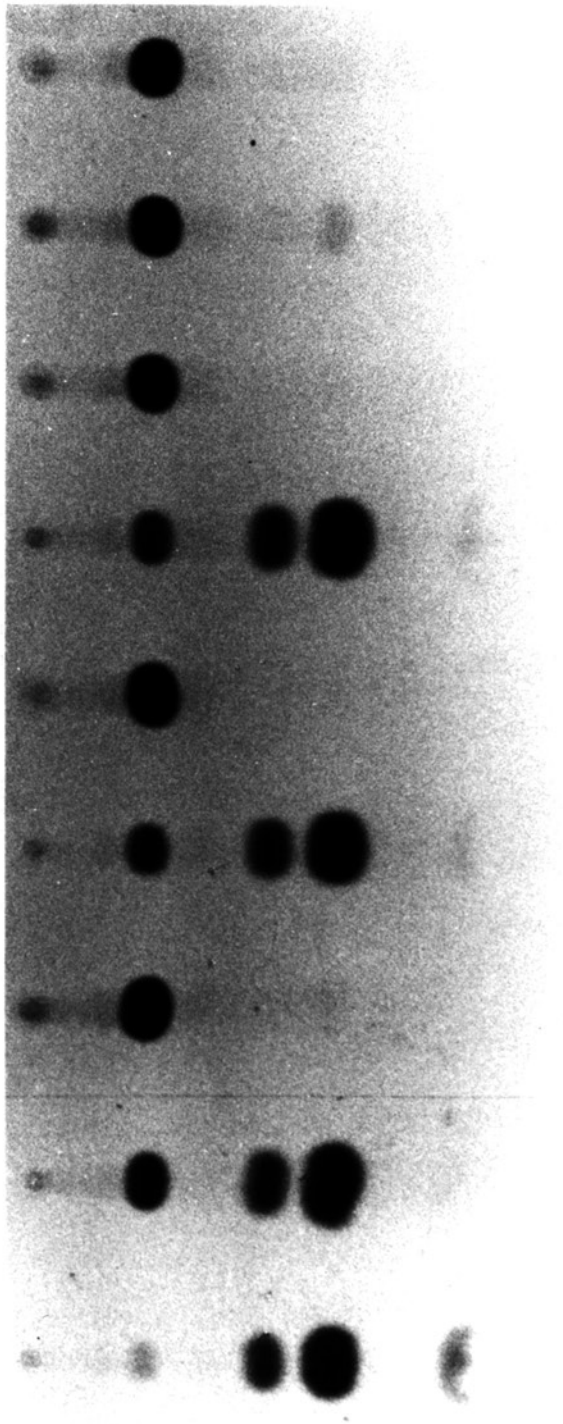

L
S
pGC
XhoI
"TATAA"
BgaII
SalI
-31
-27
HindIII
AUG
gC mRNA
+124
pGC-CAT
CAT Reporter gene
-146
+124
pGC^3-CAT
-144
-35
pGC^8-CAT
-144
+124
pGC^10-CAT
-35
+30

Figure 8

Expression of the HSV gC promoter construct, pGC-CAT and its deletion plasmids. HFF cells were transfected with 10 µg of plasmid by the DEAE-Dextran transfection method. Cells were either uninfected or infected with VZV on the following day. At 48 hours post infection, the cells were harvested, extracted and the levels of CAT activity were measured. The percentage acetylation is shown on the left column. The pSV2CAT plasmid was used as a positive control.

# HSV pGC-CAT and its deletion plasmids with VZV infection in HFF cells

| % acetylation | plasmid |
|---|---|
| 0.39 | pGC^3-CAT |
| 0.50 | pGC^3-CAT +VZV |
| 0.42 | pGC^8-CAT |
| 94.28 | pGC^8-CAT +VZV |
| 0.39 | pGC^10-CAT |
| 92.10 | pGC^10-CAT +VZV |
| 0.51 | pGC-CAT |
| 90.02 | pGC-CAT +VZV |
| 93.13 | pSV2CAT |



not identical to that in the HSV gC promoter (5'..GGGTATAAATTCCGG..3'), which has been shown to confer specific transcription regulation (Homa *et al.*, 1986a; 1988). Thus, we decided to change the sequence around the 'TATA' box of the VZV gpV promoter to resemble that of the HSV gC promoter (Fig. 9). Two constructs were made: pVCm1-CAT was mutated at 1 base in the 'TATA' box ("T" to "A"), and pVCm14-CAT was mutated at 4 bases which gives this plasmid an identical 14 base pair sequence to that of the HSV gC promoter.

**Expression from the pVCm1-CAT & pVCm14-CAT plasmids.**

Each of the plasmids was transfected into HFF cells, followed by HSV or VZV infection on the following day (Fig. 10). Upon VZV infection, the CAT activities from both pVCm1-CAT and pVCm14-CAT did not show significant increases over that of pgpV-CAT (less than 2 fold) (lanes 9, 10, 12). In addition, both activities are much less than that of pgC-CAT. We conclude that VZV transactivating factors could not activate the HSV gC 'TATA' box in the context of the gpV promoter as effectively as in the context of its own promoter. In HSV infection, (lanes 5-8) only the CAT gene in pgC-CAT was expressed and, thus, HSV transactivating factors could not activate the HSV 'TATA' box in the context of the gpV promoter (lanes 5, 6). Interestingly, pgpV-CAT also could not be activated by HSV infection (lane 8), showing that the HSV transactivation system is unable to use the VZV gpV

Figure 9

Description of "TATA" box mutants of the gpV promoter. The "TATA" box region of the pgpV-CAT plasmid was mutated to have the same characteristics as the HSV gC promoter, using PCR. The plasmid pVCm1-CAT has only 1 base pair change (from **T** to **A**). On the other hand, 4 base pairs were changed in pVCm14-CAT in order to have all 14 base pairs identical to that of the HSV gC promoter. The block shows the putative "TATA" box.

HSV gC "TATA" : 5'..GGGTATAA ATTCCG..3'

VZV gpV "TATA" : 5'..TCATTTAA ATTCCG..3'

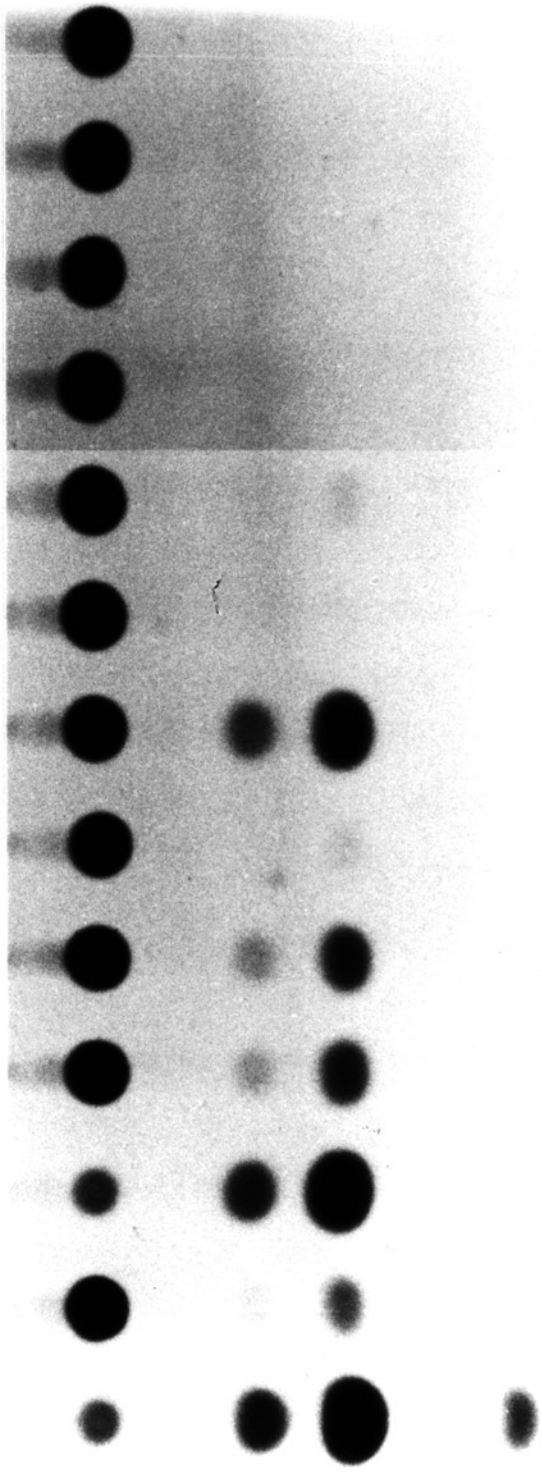
pVCm1-CAT 5'..TCATATAAATTCCG..3'

pVCm14-CAT 5'..GGGTATAAATTCCG..3'

Figure 10

Expression of the gpV promoter mutants, pVCm1-CAT and pVCm14-CAT, compared to pgpV-CAT and pGC-CAT. HFF cells were transfected with 10 µg of plasmid and were either uninfected or infected with HSV (###) or VZV (***) on the following day. At 48 hours post infection, the cells were harvested, extracted and the levels of CAT activity were measured. Lane 1; pVCm1-CAT, lane 2; pVCm14-CAT, lane 3; pGC-CAT, lane 4; pgpV-CAT. Lanes 1-4 were mock-infected. Lanes 5-8 and 9-12 correlate with lanes 1-4 but were infected with HSV (###) and VZV (***), respectively. The percentage acetylation is shown on the left column. The pCAT-Control plasmid was used as a positive control.

# pVCm1-CAT and pVCm14-CAT with HSV### VS VZV*** infection in HFF cells



| % acetylation | plasmid |
|---|---|
| 0.39 | pVCm1-CAT |
| 0.29 | pVCm14-CAT |
| 0.35 | pGC-CAT |
| 0.31 | pgpV-CAT |
| 0.76 | pVCm1-CAT ### |
| 0.30 | pVCm14-CAT ### |
| 29.36 | pGC-CAT ### |
| 1.18 | pgpV-CAT ### |
| 8.61 | pVCm1-CAT *** |
| 8.99 | pVCm14-CAT *** |
| 95.67 | pGC-CAT *** |
| 5.01 | pgpV-CAT *** |
| 97.33 | pCAT-Control |

promoter. In contrast, HSV pgC-CAT was activated by VZV infection more efficiently than by HSV infection (compare lanes 11 and 7).

3. Identification of the VZV proteins involved in activation of late promoters.

**Regulation of pgpV120-CAT expression by VZV gene product(s) in HFF cells.**

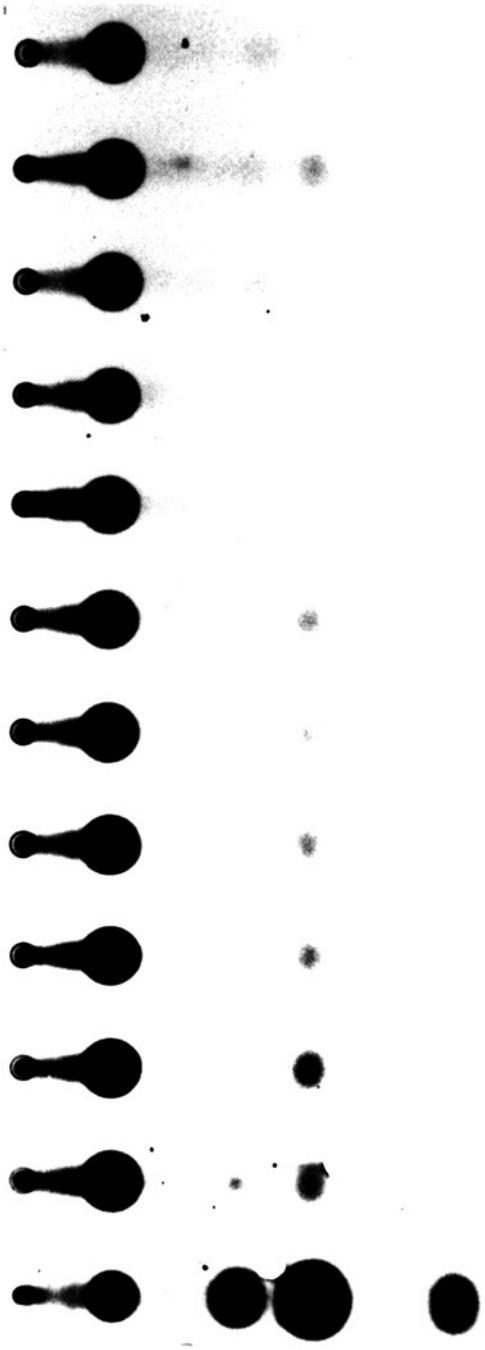
We know of several VZV genes that encode gene regulatory functions. The VZV ORF62 protein is a general transactivator (Inchauspe *et al.*, 1989a); the VZV ORF61 protein can repress the expression of IE, E or L gene promoters in Vero cells (Nagpal & Ostrove, 1991) but it can synergize ORF62 protein in the activation of VZV genes in human T lymphocytes (Perera *et al.*, 1992a). The VZV ORF63 protein can strongly repress the expression of ORF62 but has no significant effect on the late genes in Vero cells (Jackers *et al.*, 1992). Given that we have shown that VZV infection activates the gpV promoter, we now wish to define which of these VZV regulatory proteins is responsible for the activation. Accordingly, pgpV120-CAT was cotransfected with pCMV62, pCMV4, pCMV61 and pCMV63 (expressing VZV ORF62, 4, 61, and 63 products, respectively) alone or in combinations in HFF cells (Fig. 11). Only ORF62 protein alone (lane 2) showed activation; ORF4, or ORF61 or ORF63 proteins alone (lanes 3, 4, 5, respectively) did not. However, each plasmid showed synergism with the ORF62 protein to further activate CAT expression (lanes 6, 7, 8). The best

Figure 11

Effect of VZV gene product(s) on the expression of the gpV promoter construct, pgpV120-CAT in HFF cells. The pgpV120-CAT plasmid was cotransfected with effector plasmid(s) (5 μg) expressing VZV ORF 62, 4, 61, and 63 (pCMV62, pCMV4, pCMV61 and pCMV63, respectively) alone or in combinations. In each cotransfection experiment, the total DNA amount was kept constant at 30 μg by adding pUC18 plasmid DNA as a carrier. Cells were harvested 48 hours after DNA transfection and CAT assays were performed. The percentage acetylation is shown on the left column.

# Regulation of pgpV120-CAT with VZV gene product(s) in HFF cells

| % acetylation | effector(s) |
|---|---|
| 0.49 | - |
| 1.39 | pCMV62 |
| 0.50 | pCMV4 |
| 0.42 | pCMV61 |
| 0.54 | pCMV63 |
| 2.09 | pCMV62+4 |
| 1.89 | pCMV62+61 |
| 2.36 | pCMV62+63 |
| 2.21 | pCMV62+4+61 |
| 3.59 | pCMV62+4+63 |
| 3.01 | pCMV62+4+61+63 |
| 91.27 | positive control |



combinations were 'ORF62+ORF4+ORF61+ORF63' (lane 11), and 'ORF62 + ORF4 + ORF63' (lane 10), perhaps reflecting the situation in the VZV-infected cell.

**Regulation of pGC^8-CAT expression by VZV gene product(s) in HFF cells.**

From our earlier results (Fig. 8), we saw that VZV infection could induce the HSV gC promoter in pgC-CAT and its deletion plasmids much better than its own gpV promoter. To investigate whether the gene product(s) of VZV ORF62, 4, 61 and 63 were involved in this activation, the pGC^8-CAT plasmid was cotransfected with pCMV62, pCMV4, pCMV61 and pCMV63, as above, (Fig 12). Again only ORF62 protein alone (lane 2) showed activation, while ORF4, ORF61 and ORF63 proteins alone (lanes 3, 4, 5) did not. In other respects the data were similar to that for the gpV promoter (above). Thus the mechanisms of activation of these two herpesvirus late promoters by VZV is strikingly similar.

4. Definition of the functional promoter for the early/late (L1) VZV protein, gpIV.

**Construction of gpIV-CAT and its deletion plasmids.**

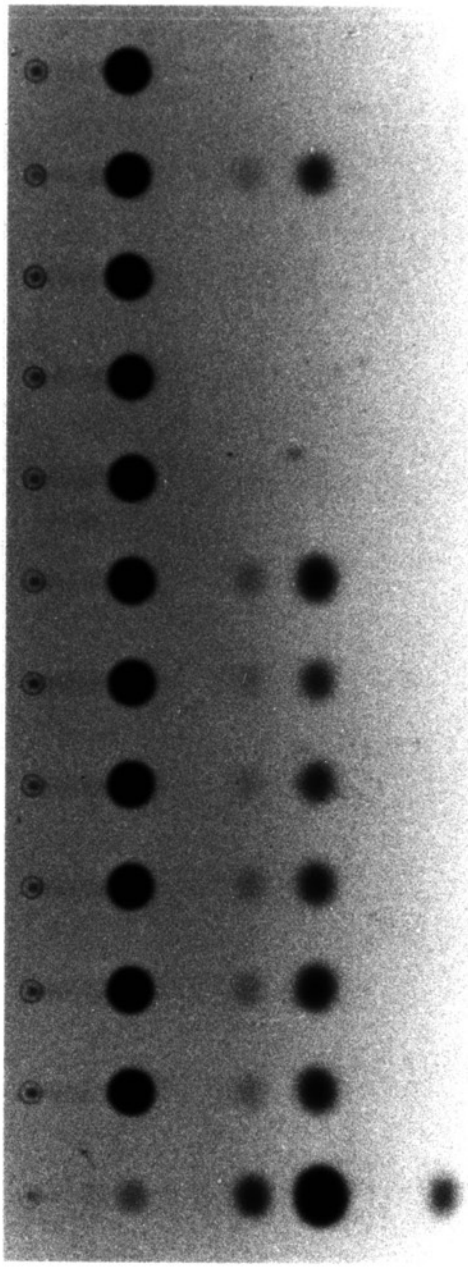
Studies from our laboratory had defined, using primer extension analysis, the transcriptional start site of glycoprotein IV (gpIV; an early/late, L1 gene) (Ling *et al.*, 1992). A putative 'TATA' box at -25 bp and 'CCAAT' box at -65 bp were apparent in sequences upstream from the cap site. To

Figure 12

Effect of VZV gene product(s) on the expression of the HSV gC promoter construct, pGC^8-CAT in HFF cells. The pGC^8-CAT was cotransfected with effector plasmid(s) (5 μg) expressing VZV ORF 62, 4, 61 and 63 (pCMV62, pCMV4, pCMV61 and pCMV63, respectively) alone or in combinations. In each cotransfection experiment, the total DNA amount was kept constant at 30 μg by adding pUC18 plasmid DNA as a carrier. Cells were harvested 48 hours after DNA transfection and CAT assays were performed. The percentage acetylation is shown on the left column.

# Regulation of HSV pGC^8-CAT expression with VZV gene product(s) in HFF cells

| % acetylation | effector(s) |
|---|---|
| 0.83 | - |
| 11.10 | pCMV62 |
| 0.73 | pCMV4 |
| 0.81 | pCMV61 |
| 0.78 | pCMV63 |
| 19.53 | pCMV62+4 |
| 8.55 | pCMV62+61 |
| 11.20 | pCMV62+63 |
| 12.59 | pCMV62+4+61 |
| 18.19 | pCMV62+4+63 |
| 16.19 | pCMV62+4+61+63 |
| 97.97 | positive control |



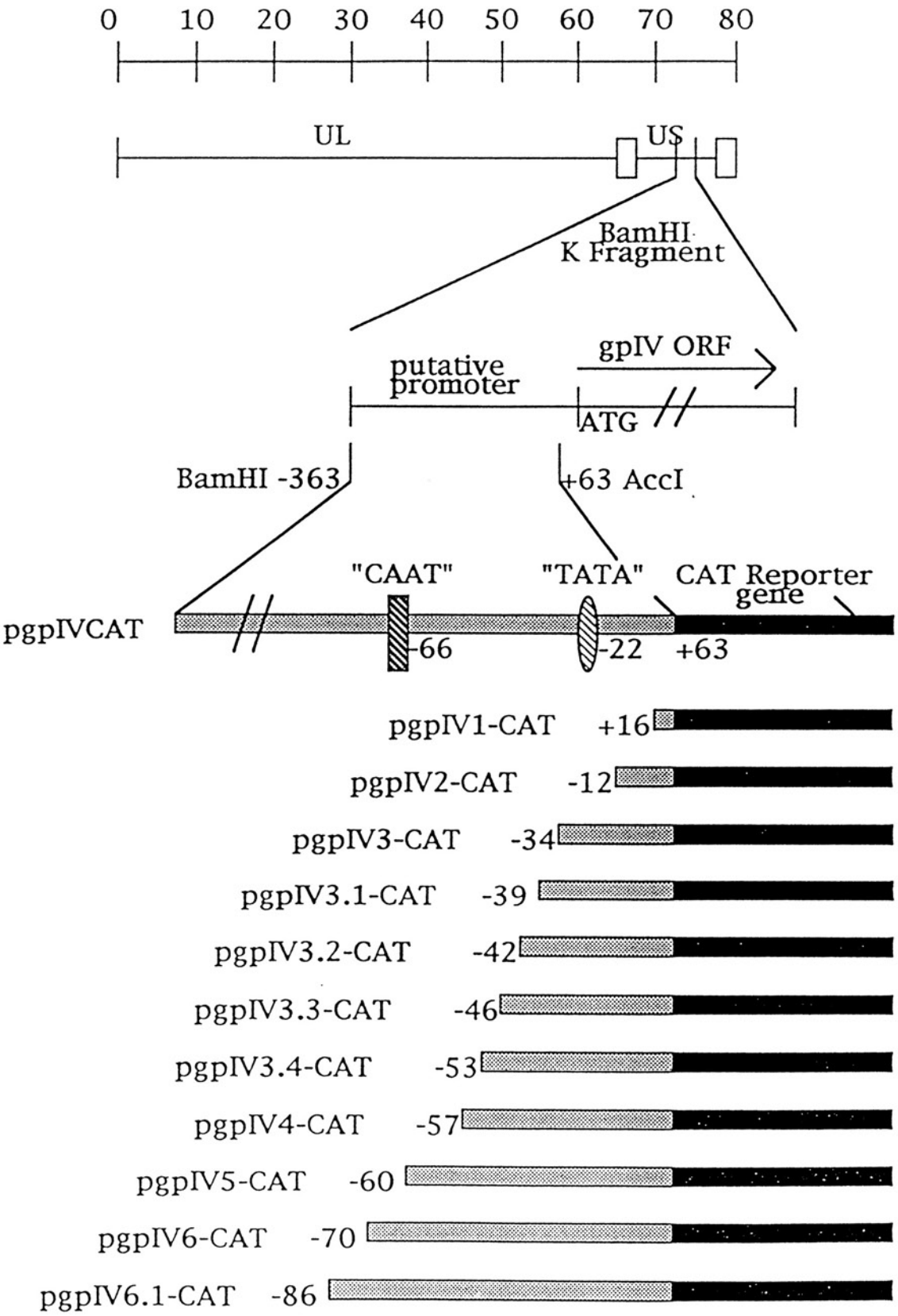
examine the minimal cis-acting elements which control expression of gpIV promoter in transient assays, the gpIV putative promoter region and deletion mutants derived from it were fused to the CAT reporter gene of pCATBasic (Fig. 13). An approximately 430 bp fragment of a *Bgl*II fragment of the pG4-3 plasmid (which includes the 5' untranscribed and upstream sequence of the gpIV gene) was fused to the CAT gene to construct the pgpIV-CAT plasmid. By deletion procedures outlined in the methods, 11 deletion plasmids were also constructed (Fig. 13). All of them were deleted from nucleotide -363 downstream as follows:
pgpIV1-CAT (no mRNA start site), pgpIV2-CAT (mRNA start site only), pgpIV3-CAT (with the 'TATA' box), gpIV3.1-CAT (with the 'TATA' box + upstream sequences), pgpIV3.2-CAT (-42), pgpIV3.3-CAT (-46), pgpIV3.4-CAT (-53), pgpIV4-CAT (-57), pgpIV5-CAT (-60), pgpIV6-CAT (-70), pgpIV6.1-CAT (-86); the last seven plasmids all have the 'TATA' box + upstream sequences.

**Expression from pgpIV-CAT and its deletion plasmids.**

To examine expression, the pgpIV-CAT or each of its deletion plasmids was transfected into HFF cells, followed by VZV infection on the next day (Figs. 14, 15). The results from CAT assays showed that the promoter activity started with the pgpIV3-CAT construct (containing only the 'TATA' box) (Fig. 15, lane 3,), although at a low level. From pgpIV3.1-CAT to pgpIV3.4-CAT (lane 4-7), the more upstream

Figure 13

Detailed structure of the gpIV promoter construct, pgpIV-CAT and its deletion plasmids. At the top, the VZV genome is presented. The upper line denotes the genome size in megadaltons ($80x10^6$). A BamHI/AccI fragment was cut from the BamHI K fragment and fused to the CAT reporter gene of the pCATBasic vector, to make pgpIV-CAT. All the deletion plasmids were derived from pgpIV-CAT by deletion mutation. The putative "TATA" and "CCAAT" boxes are located at -22 and -66, respectively. The VZV sequences are shown as dotted blocks and the CAT sequences as solid blocks.

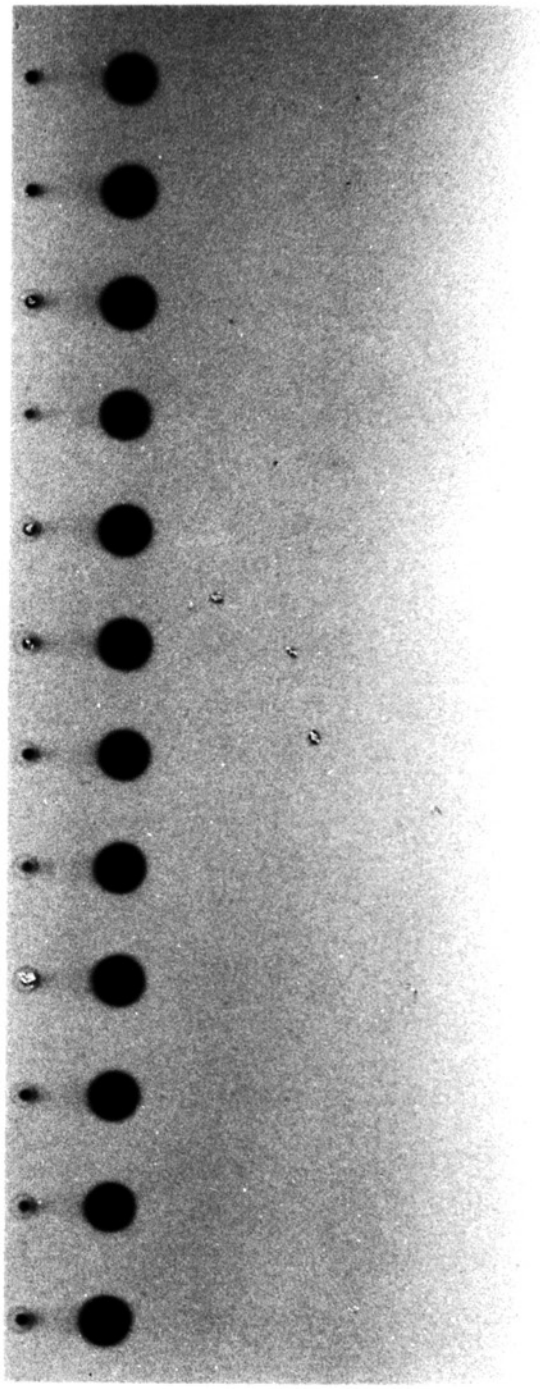

0
10
20
30
40
50
60
70
80
UL
US
BamHI
K Fragment
gpIV ORF
putative
promoter
ATG
BamHI -363
+63 AccI
"CAAT"
"TATA"
CAT Reporter
gene
pgpIVCAT
-66
-22
+63
pgpIV1-CAT +16
pgpIV2-CAT -12
pgpIV3-CAT -34
pgpIV3.1-CAT -39
pgpIV3.2-CAT -42
pgpIV3.3-CAT -46
pgpIV3.4-CAT -53
pgpIV4-CAT -57
pgpIV5-CAT -60
pgpIV6-CAT -70
pgpIV6.1-CAT -86

Figures 14-15

Expression from pgpIV-CAT and its deletion plasmids. HFF cells were transfected with 10 μg of plasmid by the DEAE-Dextran transfection method. Cells were uninfected or infected with VZV on the following day. At 48 hours post infection, the cells were harvested, extracted and the levels of CAT activity were measured. The percentage acetylations of mock and VZV infection are shown on the left columns of Fig 14. and Fig 15., respectively. The pCAT-Control plasmid was used as a positive control.

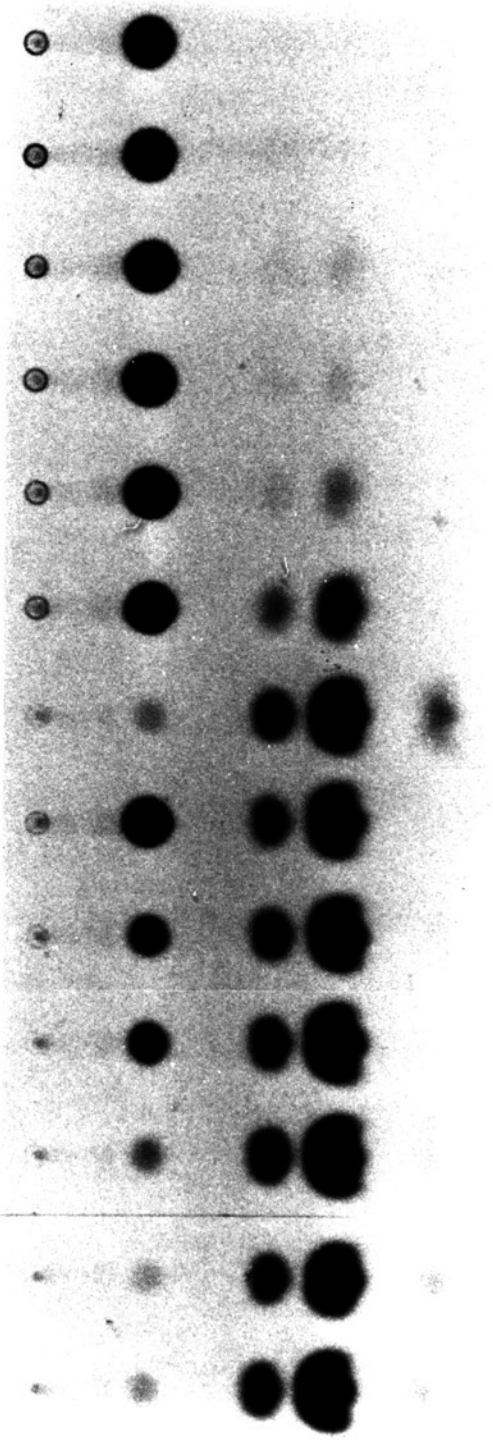
# pgpIV-CAT and its deletion plasmids in HFF cells (control set)

| % acetylation | plasmid |
|---|---|
| 0.48 | pgpIV1-CAT |
| 0.55 | pgpIV2-CAT |
| 0.63 | pgpIV3-CAT |
| 0.56 | pgpIV3.1-CAT |
| 0.67 | pgpIV3.2-CAT |
| 0.72 | pgpIV3.3-CAT |
| 0.65 | pgpIV3.4-CAT |
| 0.73 | pgpIV4-CAT |
| 0.65 | pgpIV5-CAT |
| 0.59 | pgpIV6-CAT |
| 0.77 | pgpIV6.1-CAT |
| 0.67 | pgpIV-CAT |



# pgpIV-CAT and its deletion plasmids with VZV infection in HFF cells

| % acetylation | plasmid |
|---|---|
| 0.38 | pgpIV1-CAT |
| 0.57 | pgpIV2-CAT |
| 1.89 | pgpIV3-CAT |
| 2.02 | pgpIV3.1-CAT |
| 4.01 | pgpIV3.2-CAT |
| 23.28 | pgpIV3.3-CAT |
| 92.21 | pgpIV3.4-CAT |
| 52.24 | pgpIV4-CAT |
| 72.88 | pgpIV5-CAT |
| 75.53 | pgpIV6-CAT |
| 94.42 | pgpIV6.1-CAT |
| 97.23 | pgpIV-CAT |
| 95.36 | pCAT-Control |



sequences were added to the 'TATA' box sequences, the more acetylation increased. These results lead to the conclusion that only the 'TATA' box is necessary for the minimal expression from the gpIV promoter but, to have full expression, the upstream sequences from the end of pgpIV3.2-CAT to pgpIV3.4-CAT had to be added to the 'TATA' box sequences. This 3.2-3.4 sequence is 5'...CAGAGTCACGC...3' (Fig. 16). Thus, in contrast to the situation with the true late gpV promoter, this early/late gpIV promoter requires upstream sequences for its full activity in addition to its 'TATA' sequences.

5. Identification of the VZV proteins involved in gpIV gene regulation in different cell types.

**Expression from pgpIV-CAT and its deletion plasmids by ORF62 and ORF4 gene products in HFF cells and T cells.**

We and others have shown that the VZV ORF62 and ORF4 gene products have synergistic transactivation effects on the expression of many of the putative promoters of VZV genes including that of gpIV (Inchauspe *et al.*, 1989a; Ling *et al.*, 1992; Perera *et al.*, 1992a). These two proteins appear to be able to substitute effectively for VZV infection in transient assays. To determine whether they might be responsible for the sequence-dependent activity of the promoter which we had demonstrated (Fig. 15), each plasmid was cotransfected with pCMV62 and pCMV4 into HFF cells by the DEAE-Dextran method (Fig. 17) or into T cells by electroporation (Fig. 18, 19).

Figure 16

Detailed map of the gpIV upstream sequences from the putative promoter. The numbers above the arrows were correlated to the gpIV deletion clone numbers shown in Fig. 13. 'ATAAA' & 'CCAAT' are the putative "TATA" and "CCAAT" boxes, respectively. 'T' is the mRNA start site. 'CAGAGTCACGC' and 'CCAATAGAAA' are the additional upstream sequences which confer marked upregulatory effects in pgpIV3.4-CAT and pgpIV6-CAT plasmids, respectively. The 'CCAATAGAAA' sequence appears as an upregulatory element chiefly in the presence of the ORF62 and ORF4 products alone (Fig. 17).

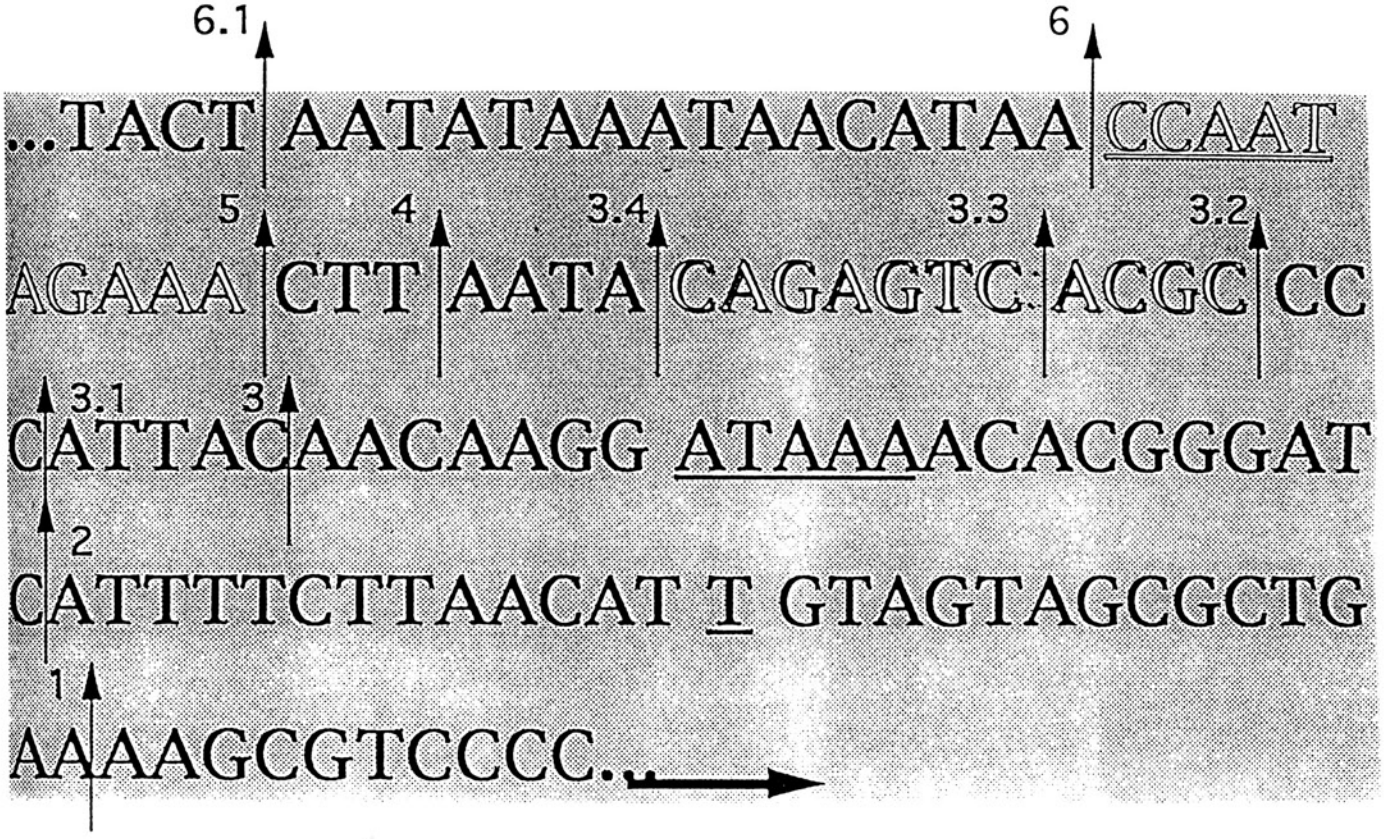
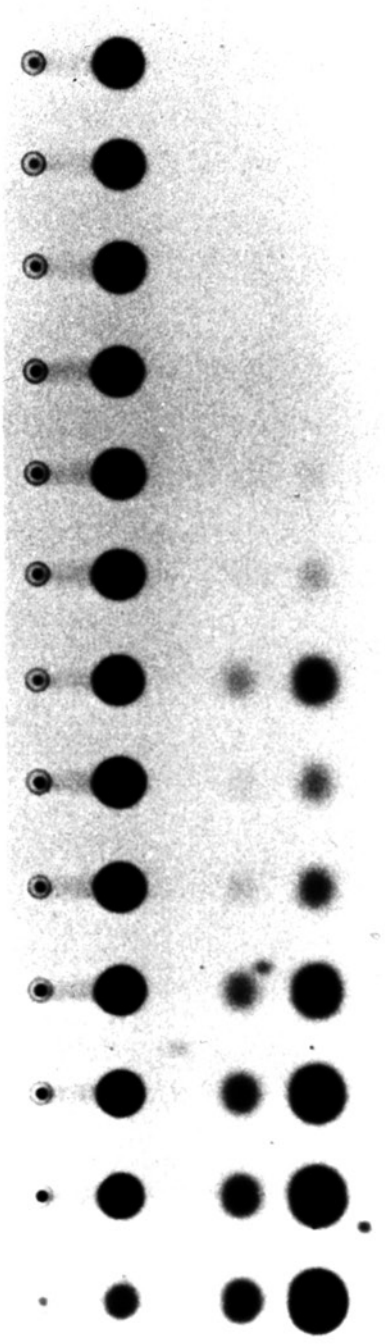

6.1
6
...TACT AATATAAATAACATAA CCAAT
5
4
3.4
3.3
3.2
AGAAA CTT AATA CAGAGTC ACGC CC
3.1
3
CATTAC AACAAGG ATAAAACACGGGAT
2
CATTTTCTTAACAT T GTAGTAGCGCTG
1
AAAAGCGTCCCC...

Figure 17

Activation by VZV ORF62 together with ORF4 of pgpIV-CAT and its deletion plasmids in HFF cells. The pgpIV-CAT plasmid and its deletion plasmids (10 μg) were cotransfected with pCMV62 and pCMV4 (5 μg each) which expressed VZV ORF62 and ORF4, respectively. Cells were harvested 48 hours after DNA transfection and CAT assays were performed. The percentage acetylation is shown on the left column. The pCAT-Control was used as a positive control.

# pgpIV-CAT and its deletion plasmids with pCMV62 and pCMV4 in HFF cells

| % acetylation | plasmid |
|---|---|
| 0.50 | pgpIV1-CAT |
| 0.53 | pgpIV2-CAT |
| 0.51 | pgpIV3-CAT |
| 0.59 | pgpIV3.1-CAT |
| 0.69 | pgpIV3.2-CAT |
| 1.45 | pgpIV3.3-CAT |
| 12.42 | pgpIV3.4-CAT |
| 3.39 | pgpIV4-CAT |
| 5.42 | pgpIV5-CAT |
| 26.69 | pgpIV6-CAT |
| 53.32 | pgpIV6.1-CAT |
| 67.83 | pgpIV-CAT |
| 94.73 | pCAT-Control |

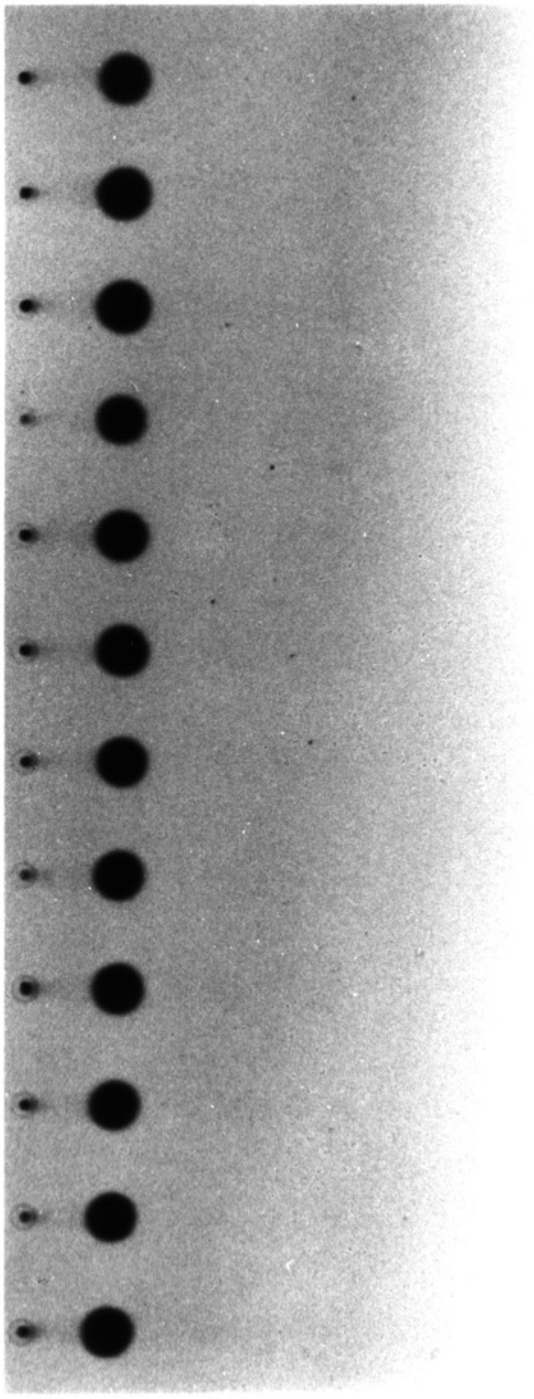


Figures 18-19

Activation by VZV ORF62 together with ORF4 of pgpIV-CAT and its deletion plasmids in T cells A.301. The pgpIV-CAT plasmid and its deletion plasmids (10 µg) were cotransfected with pUC18 (control) (10 µg) (Fig. 18) or pCMV62 and pCMV4 (5 µg each) which expressed VZV ORF62 and ORF4, respectively (Fig. 19). Cells were harvested 48 hours after DNA transfection and CAT assays were performed. The percentage acetylation is shown on the left column. The pCAT-Control was used as a positive control.

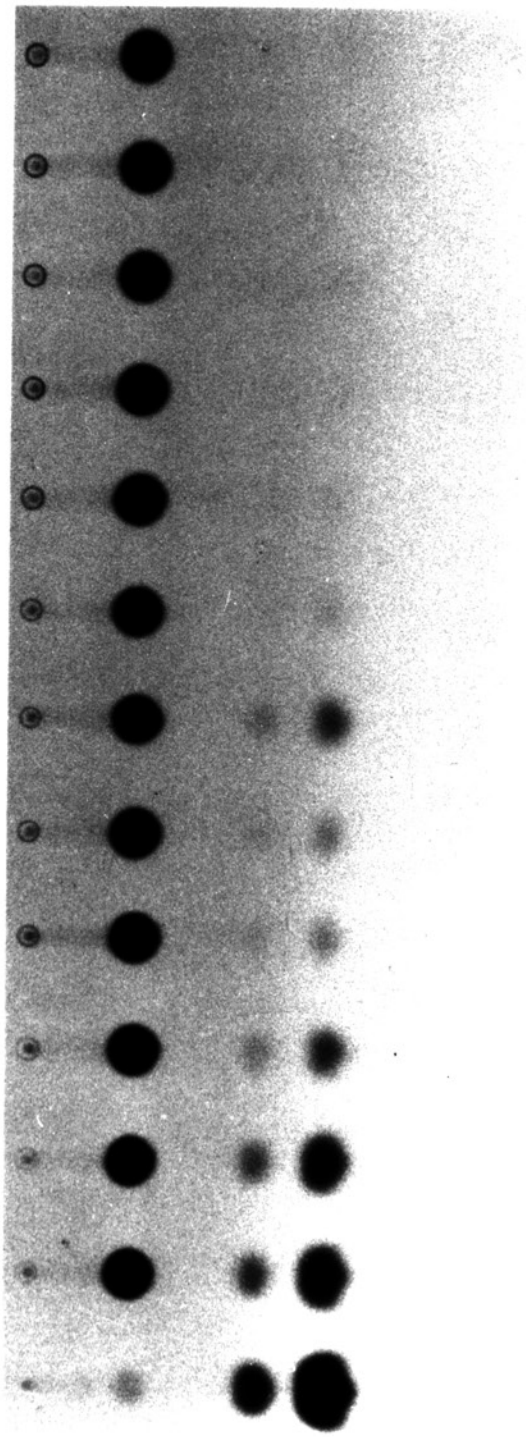
# pgpIV-CAT and its deletion plasmids in T cells A.301(control set)

| % acetylation | plasmid |
|---|---|
| 0.66 | pgpIV1-CAT |
| 0.62 | pgpIV2-CAT |
| 0.54 | pgpIV3-CAT |
| 0.57 | pgpIV3.1-CAT |
| 0.66 | pgpIV3.2-CAT |
| 0.57 | pgpIV3.3-CAT |
| 0.67 | pgpIV3.4-CAT |
| 0.69 | pgpIV4-CAT |
| 0.65 | pgpIV5-CAT |
| 0.55 | pgpIV6-CAT |
| 0.70 | pgpIV6.1-CAT |
| 0.63 | pgpIV-CAT |



# pgpIV-CAT and its deletion plasmids with pCMV62 and pCMV4 in T cells (A.301)

| % acetylation | plasmid |
|---|---|
| 0.33 | pgpIV1-CAT |
| 0.41 | pgpIV2-CAT |
| 0.35 | pgpIV3-CAT |
| 0.36 | pgpIV3.1-CAT |
| 0.53 | pgpIV3.2-CAT |
| 1.22 | pgpIV3.3-CAT |
| 10.08 | pgpIV3.4-CAT |
| 3.14 | pgpIV4-CAT |
| 3.57 | pgpIV5-CAT |
| 10.52 | pgpIV6-CAT |
| 26.62 | pgpIV6.1-CAT |
| 31.15 | pgpIV-CAT |
| 94.36 | pCAT-Control |



CAT activities from the transfections in both HFF cells and A3.01 cells, not surprisingly, did not reach the levels obtained from VZV infection. However, the general pictures in both cell types were similar. As we saw with VZV infected cells, the major rise in promoter activation came with the addition of the ACAGAGTCACGC sequence; however, there was also a second boost in activity when the upstream CCAATAGAAA sequence was present (pgpIV5-CAT - pgpIV6-CAT; e.g. Fig. 17). Clearly, the ORF62 and/or 4 proteins must be able to interact directly or indirectly with these sequences.

**Construction of a fusion plasmid between gpIV activating sequences and the gpV TATA box (pgpV120(3-3.4)-CAT).**

To investigate whether the activating upstream sequence which conferred high expression on the pgpIV3.4-CAT plasmid constituted a movable regulatory element, the VZV gpV promoter in pgpV120-CAT (Fig. 4) was chosen as substrate for construction of a chimera. The rationale for chosing the gpV promoter was (a) that it was another VZV promoter, and (b) it performed at only a low level. We chose to use the sequences between pgpIV3-CAT and pgpIV3.4-CAT (.ACAGAGTCACGCCCCATTAC.) for this test, since they appeared to give maximum stimulation (> 50 fold) in our original assay (Fig. 15). Two synthetic oligonucleotides: "3-3.4L", 5'.GTAATGGGGCGTGACTCTGT .3' and "3-3.4U", 5'.ACAGAGTCACGCCCCATTAC.3' were annealed to form the gpIV activator element and cloned into the *Hind*III

site upstream of the 'TATA' box of pgpV120-CAT, to construct the chimeric pgpV120(3-3.4)-CAT plasmid (Fig. 20).

**Expression from pgpV120(3-3.4)-CAT.**

To compare the CAT gene expresssion of pgpV120(3-3.4)-CAT to that of pgpV120-CAT, each plasmid was transfected into HFF cells, followed by VZV infection on the next day. The results (Fig. 21) show clearly that the CAT activity of pgpV120(3-3.4)-CAT (lane 4) was much higher than that of pgpV120-CAT (lane 2). Indeed, the activity of pgp120(3-3.4)-CAT is analogous to that of the homologous pgpIV3.4-CAT construct (Fig. 15, lane 7). This indicates two important points: (a) that the gpIV 3-3.4 AUS (activating upstream sequences) sequences are able to work outside the gpIV promoter context and (b) that, while the VZV gpV promoter is intrinsically weak, it can be readily activated by appropriate sequences.

**Control of pgpIV3.4-CAT expression by VZV gene product(s) in HFF cells.**

As we showed above, both VZV gene 62 and 4 products can substitute for VZV infection in gpIV promoter activation. In order to complete our knowledge of the VZV proteins which may regulate this promoter, we examined the effects of other known VZV regulatory proteins (ORF62, 4, 61 and 63 gene products) on the expression from the pgpIV3.4-CAT plasmid, which contained the minimum promoter sequences that showed

Figure 20

Construction of the chimeric gpIV/gpV plasmid, pgpV120(3-3.4)-CAT. The double stranded oligonucleotide 3-3.4 (ACAGAGTCACGCCCCATTAC) was cloned into the *Hind*III site located upstream from the gpV promoter in pgpV120-CAT. Nucleotide sequencing was used to confirm the orientation.

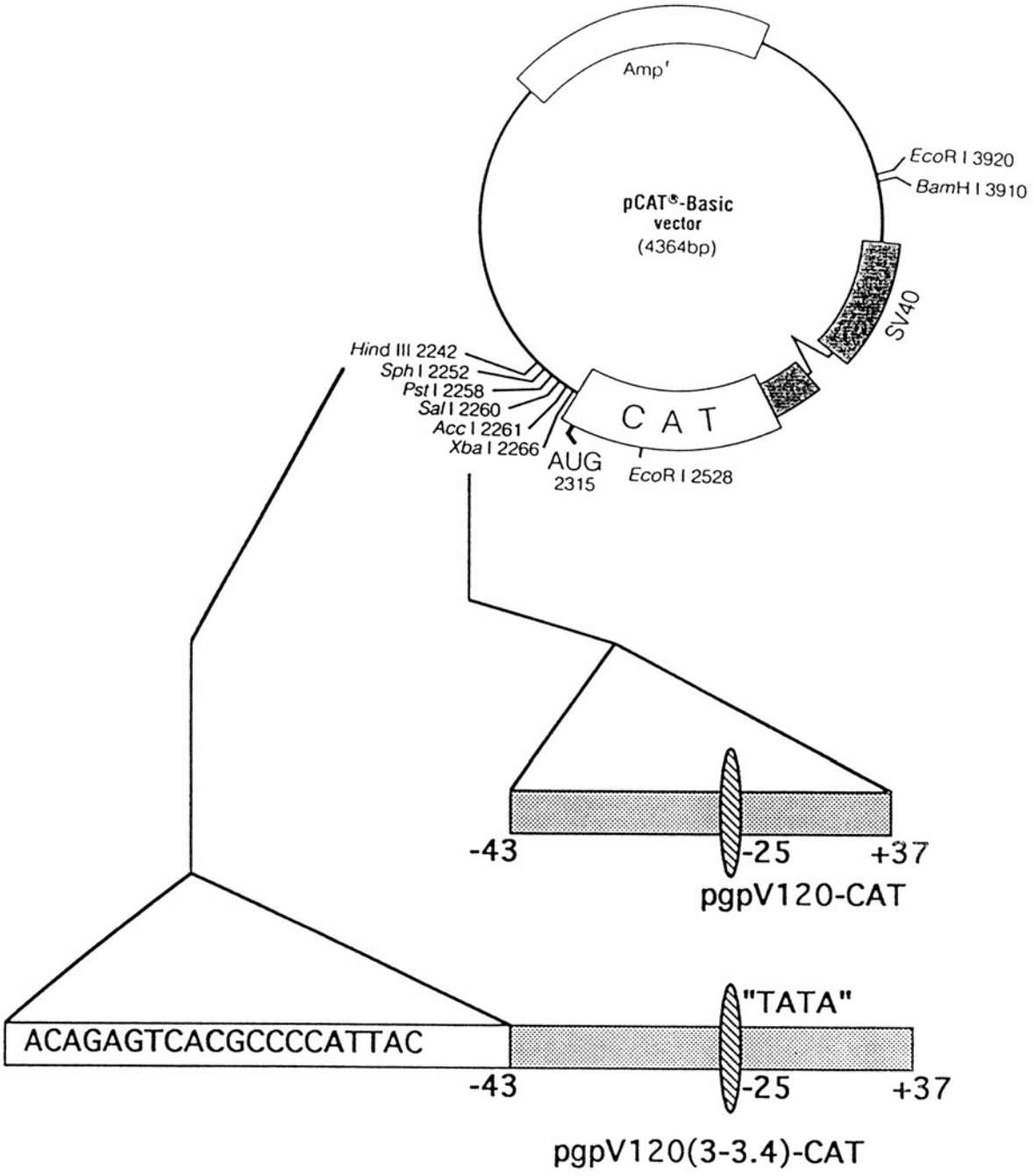
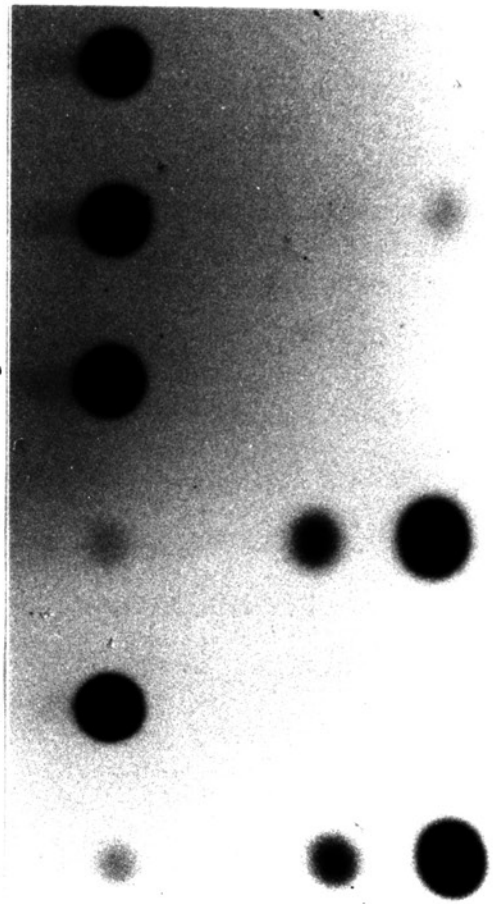

Amp'
EcoR I 3920
BamH I 3910
pCAT®-Basic
vector
(4364bp)
SV40
Hind III 2242
Sph I 2252
Pst I 2258
Sal I 2260
Acc I 2261
Xba I 2266
AUG
2315
C A T
EcoR I 2528
-43
-25
+37
pgpV120-CAT
"TATA"
ACAGAGTCACGCCCCATTAC
-43
-25
+37
pgpV120(3-3.4)-CAT

Figure 21

Expression of pgpV120(3-3.4)CAT compared to that of pgpV120-CAT. HFF cells were transfected with 10 µg of each plasmid by the DEAE-Dextran transfection method. Cells were uninfected or infected with VZV on the following day. At 48 hours post infection, the cells were harvested, extracted and the levels of CAT activity were measured. The percentage acetylation is shown on the left column. The pCAT-Control plasmid was used as a positive control.

# pgpV120-CAT VS pgpV120(3-3.4)-CAT with VZV infection in HFF cells

| % acetylation | plasmid |
|---|---|
| 0.61 | pgpV120-CAT |
| 4.47 | pgpV120-CAT +VZV |
| 0.72 | pgpV120(3-3.4)-CAT |
| 97.58 | pgpV120(3-3.4)-CAT +VZV |
| 0.75 | pCAT-BAsic |
| 98.03 | pCAT-control |



full expression upon VZV infection in HFF cells. PgpIV3.4-CAT was cotransfected with pCMV62, pCMV4, pCMV61 and pCMV63 alone or in combinations, into HFF cells (Fig. 22). As with the gpV promoter (see Fig. 11), only the ORF62 protein alone (lane 2) showed activation; the ORF4, ORF61 and ORF63 proteins alone (lane 3, 4, 5) did not. Also as in the gpV situation, ORF4 had significant synergistic effects with ORF62; however, unlike gpV, the gpIV promoter activity was decreased by the presence of additional genes (e.g. ORF61 or 63). This may indicate that there are different modes of control of these two promoters in the VZV infected cell.

6. Definition of the functional promoter region of the early (E) VZV gene 61.

**Construction of pMluI-CAT and its deletion plasmids.**

Thus far in this study, we have investigated L2 and L1 VZV promoters; the final class of promoter to be studied is that for an early (E) protein, the product of gene61.

Stevenson *et al.*, (1992) mapped the 5' end of gene61 mRNA by primer extension. Two putative 'TATA' boxes at positions -29 and -57, and two putative 'CCAAT' boxes at -117 and -156 were described upstream sequences from the cap site. To determine the cis-acting elements which control the expression from ORF61 in transient assays, the putative promoter and several deletion mutants in the sequence were fused to the CAT gene of pCATBasic (Fig. 23). A 937 bp *MluI* fragment containing the 5' untranscribed and upstream

Figure 22

Effect of VZV gene product(s) on the expression of pgpIV3.4-CAT in HFF cells. The pgpIV3.4-CAT plasmid was cotransfected with effector plasmid(s) (5 μg) expressing VZV ORF 62, 4, 61, and 63 (pCMV62, pCMV4, pCMV61 and pCMV63, respectively) alone or in combinations. In each cotransfection experiment, the total DNA amount was kept constant at 30 μg by adding pUC18 plasmid DNA as a carrier. Cells were harvested 48 hours after DNA transfection and CAT assays were performed. The percentage acetylation is shown on the left column.

# Regulation of pgpIV3.4-CAT expression with VZV gene product(s) in HFF cells

| % acetylation | effector(s) |
|---|---|
| 0.61 | - |
| 16.94 | pCMV62 |
| 0.76 | pCMV4 |
| 0.78 | pCMV61 |
| 0.81 | pCMV63 |
| 57.23 | pCMV62+4 |
| 20.84 | pCMV62+61 |
| 31.66 | pCMV62+63 |
| 30.55 | pCMV62+4+61 |
| 48.17 | pCMV62+4+63 |
| 39.96 | pCMV62+4+61+63 |
| 92.59 | positive control |

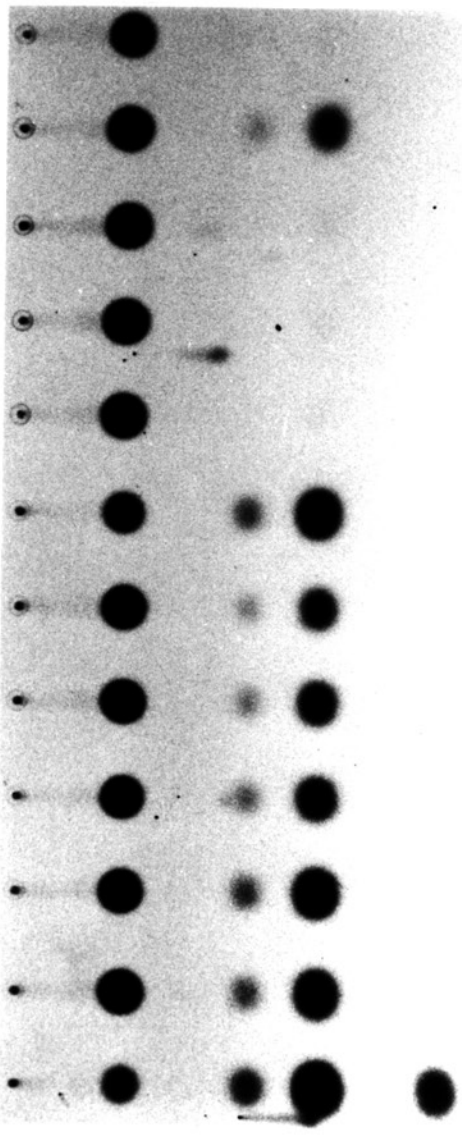
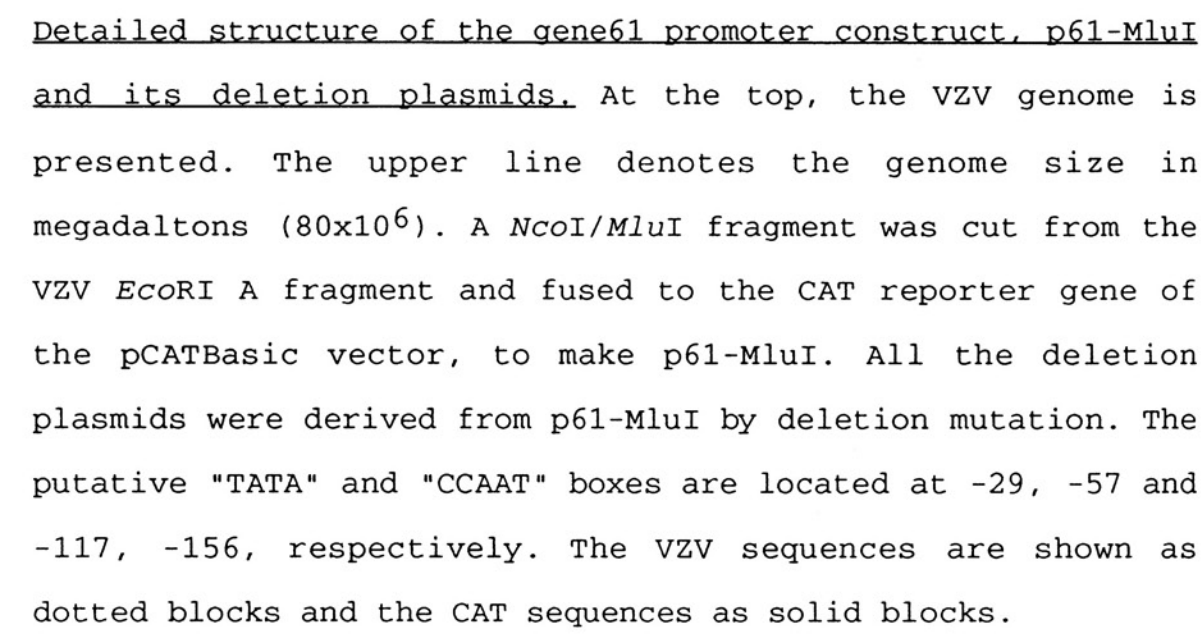
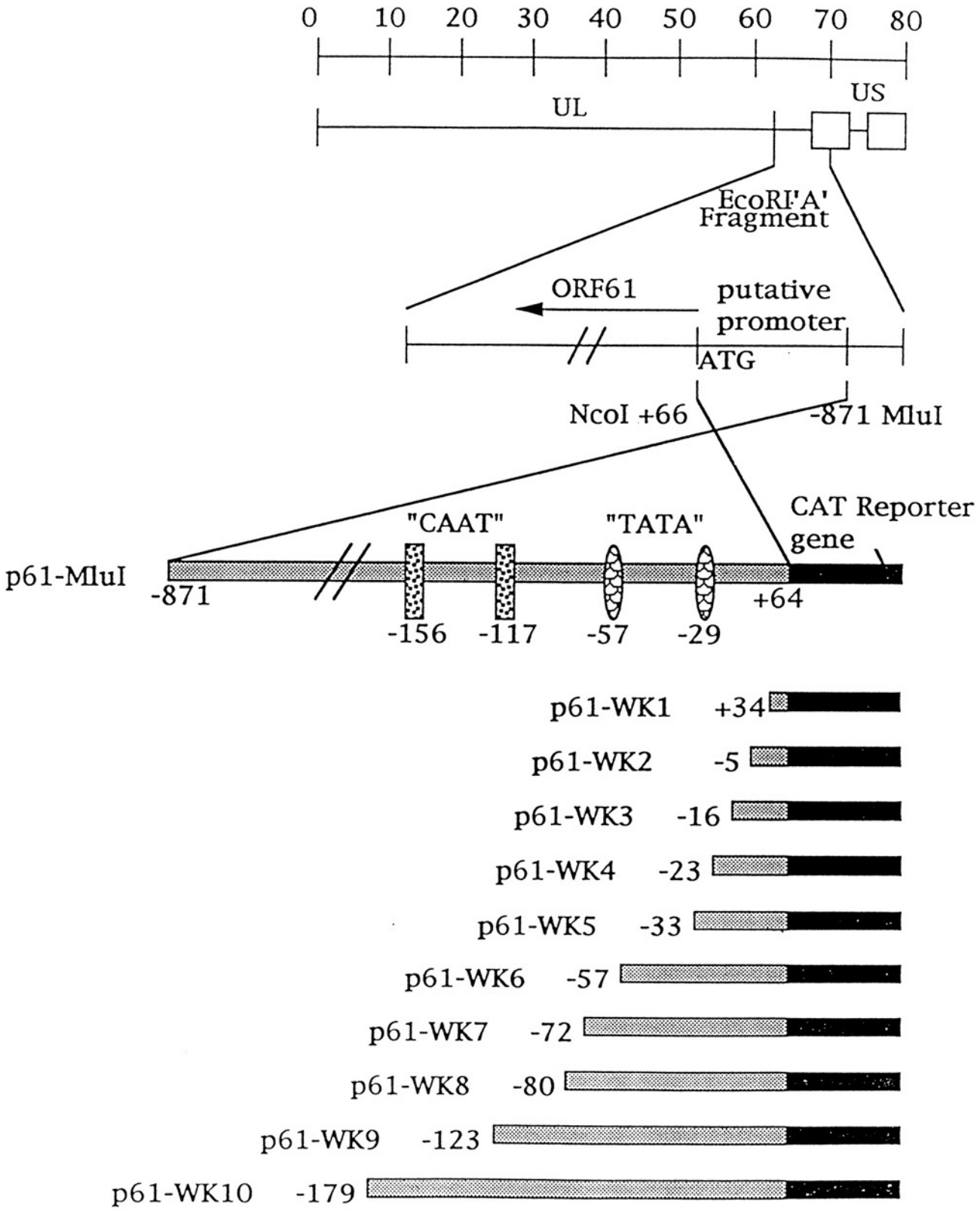


Figure 23



Detailed structure of the gene61 promoter construct, p61-MluI and its deletion plasmids. At the top, the VZV genome is presented. The upper line denotes the genome size in megadaltons ($80x10^6$). A *Nco*I/*Mlu*I fragment was cut from the VZV *Eco*RI A fragment and fused to the CAT reporter gene of the pCATBasic vector, to make p61-MluI. All the deletion plasmids were derived from p61-MluI by deletion mutation. The putative "TATA" and "CCAAT" boxes are located at -29, -57 and -117, -156, respectively. The VZV sequences are shown as dotted blocks and the CAT sequences as solid blocks.


0
10
20
30
40
50
60
70
80
UL
US
EcoRI'A'
Fragment
ORF61
putative
promoter
ATG
NcoI +66
-871 MluI
"CAAT"
"TATA"
CAT Reporter
gene
p61-MluI
-871
+64
-156
-117
-57
-29
p61-WK1
+34
p61-WK2
-5
p61-WK3
-16
p61-WK4
-23
p61-WK5
-33
p61-WK6
-57
p61-WK7
-72
p61-WK8
-80
p61-WK9
-123
p61-WK10
-179

sequences of gene61 was used to construct the plasmid p61-MluI. By removing 696 bp from upstream sequences with *AccI*, the p61-WK10 plasmid was constructed. By deletion procedures, the *MluI* fragment of p61-MluI was mutated from position -869 downstream, to construct 7 more plasmids. To construct p61-WK6 and p61-WK7 a PCR approach was used. The mutant plasmids are as follows: p61-WK1(+34) [no mRNA start site], p61-WK2(-5) [with the mRNA start site], p61-WK3(-16), p61-WK4(-23), p61-WK5(-33) [with one putative 'TATA' box], p61-WK6(-57) [with one putative 'TATA' box + upstream sequences], p61-WK7(-72) [with two putative 'TATA' boxes], p61-WK8(-80) [with two putative 'TATA' boxes + upstream sequences], p61-WK9(-123) [with two putative 'TATA' + one putative 'CCAAT' box], p61-WK10(-179) [with two putative 'TATA'+ two putative 'CCAAT' boxes].

**Expression from p61-MluI and its deletion plasmids.**

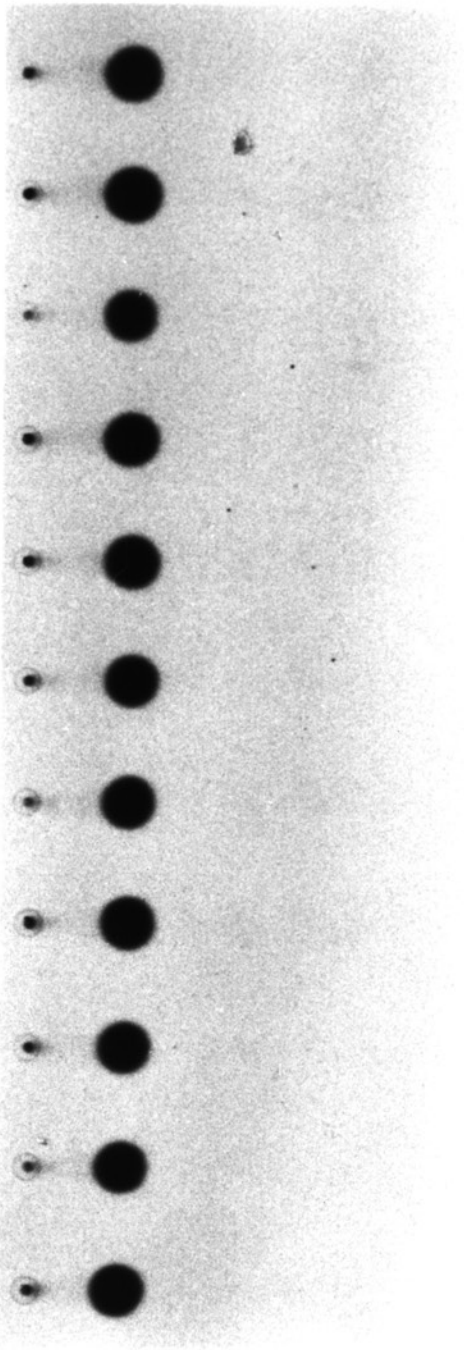
To examine the expression of CAT gene, p61-MluI and each of its deletion plasmids was transfected into HFF cells, followed by VZV infection on the next day (Fig. 24, 25). The activation of the CAT gene started with the p61-WK5 mutant, which contained the first putative 'TATA' box. Activity was raised fifteen-fold from the p61-WK4 mutant when the TTATAAAAAT sequence was added and then another four-fold rise was seen from the next mutant in the series (p61-WK6), when the GGGGTGTGTCTTCGTTGGTACCAA sequence was added. However, the activity then dropped down with p61-WK7, before attaining

Figures 24-25

Expression from p61-MluI and its deletion plasmids. HFF cells were transfected with 10 µg of plasmid by the DEAE-Dextran transfection method. Cells were uninfected or infected with VZV on the following day. At 48 hours post infection, the cells were harvested, extracted and the levels of CAT activity measured. The percentage acetylations of mock-infection and VZV infection are shown on the left column of Fig. 24 and Fig. 25, respectively. The pCAT-Control plasmid was used as a positive control.

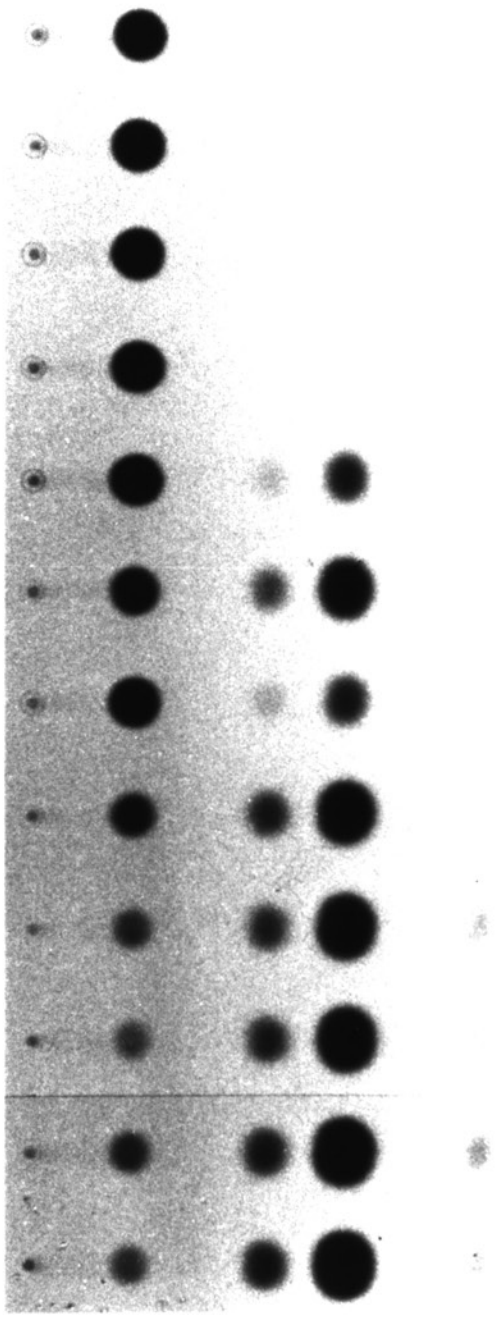
# p61-MluI and its deletion plasmids in HFF cells (control set)

| % acetylation | plasmid |
|---|---|
| 0.57 | p61-WK1 |
| 0.62 | p61-WK2 |
| 0.65 | p61-WK3 |
| 0.58 | p61-WK4 |
| 0.78 | p61-WK5 |
| 0.67 | p61-WK6 |
| 0.71 | p61-WK7 |
| 0.73 | p61-WK8 |
| 0.69 | p61-WK9 |
| 0.77 | p61-WK10 |
| 0.56 | p61-MluI |



# p61-MluI and its deletion plasmids with VZV infection in HFF cells

| % acetylation | plasmid |
|---|---|
| 0.76 | p61-WK1 |
| 0.90 | p61-WK2 |
| 0.81 | p61-WK3 |
| 0.93 | p61-WK4 |
| 15.23 | p61-WK5 |
| 60.89 | p61-WK6 |
| 17.43 | p61-WK7 |
| 85.65 | p61-WK8 |
| 94.26 | p61-WK9 |
| 96.21 | p61-WK10 |
| 94.07 | p61-MluI |
| 95.11 | pCAT-Control |



essentially the maximum level with p61-WK8.

Thus, as we have described in the other VZV promoters, a 'TATA' box is necessary for promoter activity; in this case the first box at position -29. However, to achieve full activity, two additional sequences appear to be necessary. One is 5'..GGGGTGTGTCTTCGTTGGTACCAATTATAAAAAT..3' in the p61-WK6 plasmid and the other is 5'..AAAGAAAT..3' in the p61-WK8 plasmid (Fig. 26).

7. Identification of VZV proteins involved in expression of gene 61 in different cell types.

**Expression from p61-MluI and its deletion plasmids by ORF62 and ORF4 gene products in HFF cells and T cells.**

It has been shown (Perera *et al.*, 1992a) that the ORF4 protein synergizes the ORF62 protein in transactivation of the putative gene61 promoter. The ORF4 protein alone was shown to have no effect on the expression from the promoter in Vero cells (Defechereux *et al.*, 1993). To investigate whether these two gene products are responsible for the promoter activation seen within the above assays with VZV infection, each of the gene61 plasmids was cotransfected with pCMV62 and pCMV4 into HFF cells using the DEAE-Dextran method (Fig. 27) or into T cells (A.301) by electroporation (Fig. 28, 29). We found that, in HFF cells, the patterns of transactivation by VZV and 'ORF62+ORF4' were essentially the same (Fig. 25 and Fig. 27), although there was less difference between WK5, WK6 and WK7. Unlike the gpIV

Figure 26

Detailed map of upstream sequences in the putative promoter of gene61. The numbers above the arrows were correlated to the gene61 deletion clones shown in Fig. 23. 'ATTATA' & 'ATATA' are the putative "TATA" boxes and 'CCAAT' sites are putative "CCAAT" boxes. 'A' is the mRNA start site. 'TAAAAATATTAACCATGGTTGCTTCTGTGTGGGG', 'AAAGAAAT', 'ATTCAGGGTGGGTGATTTTGCACCCATATTTTACACATAACCC' are the additional upstream sequences which confer marked upregulatory effects in the p61-WK6, p61-WK8, p61-WK9 (only in T cell A.301) plasmids, respectively.

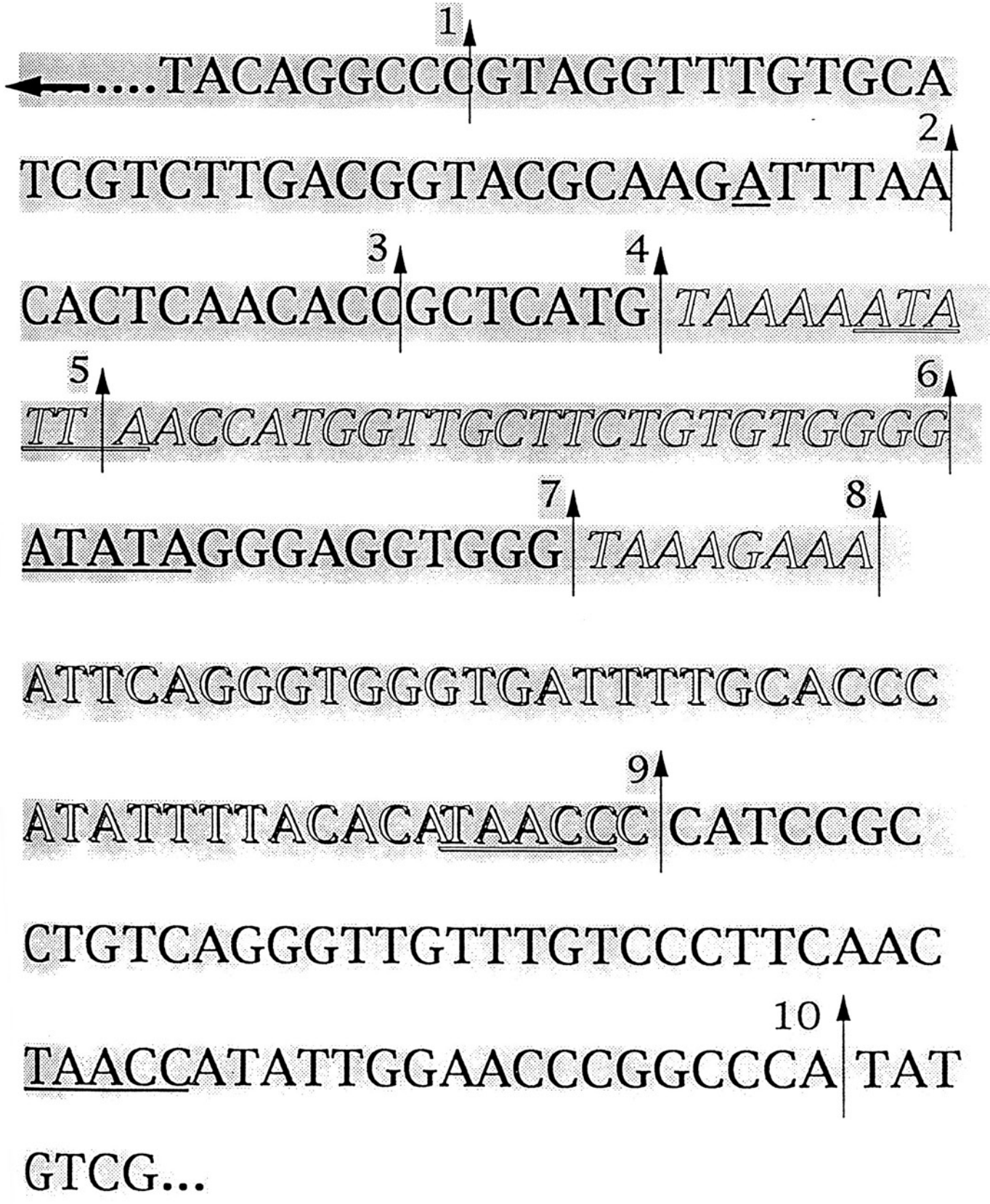
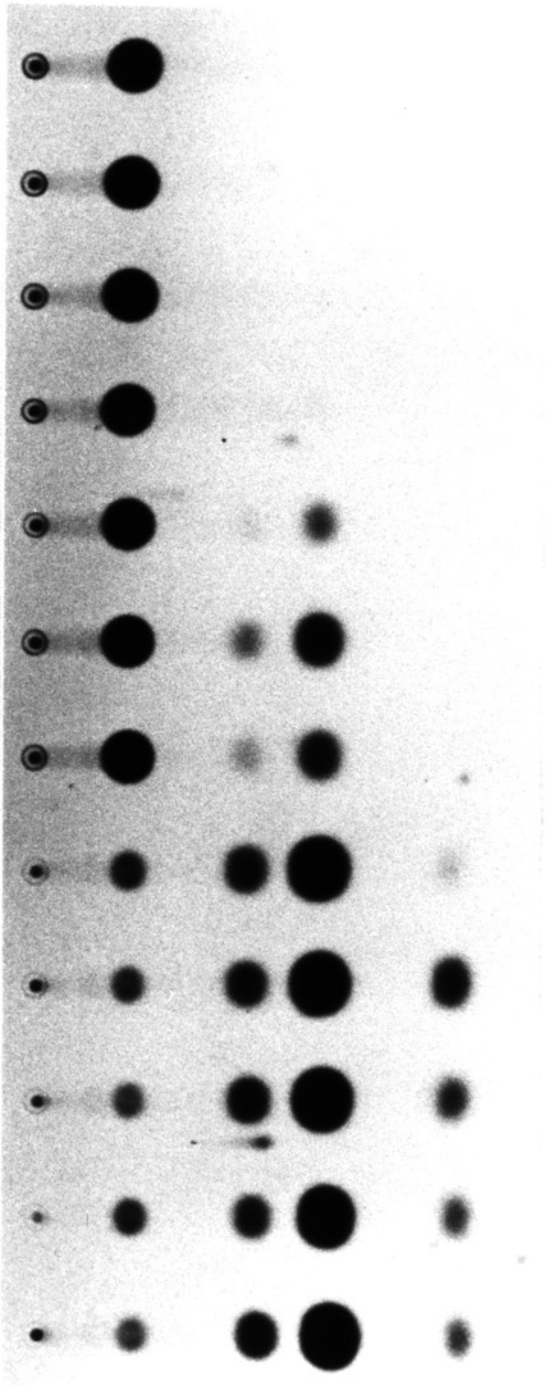

1
←....TACAGGCCCGTAGGTTTGTGCA
2
TCGTCTTGACGGTACGCAAGATTTAA
3
4
CACTCAACACCGCTCATG|TAAAAATA
5
6
TT|AACCATGGTTGCTTCTGTGTGGGG
7
8
ATATAGGGAGGTGGG|TAAAGAAA
ATTCAGGGTGGGTGATTTTGCACCC
9
ATATTTTACACATAACCC|CATCCGC
CTGTCAGGGTTGTTTGTCCCTTCAAC
10
TAACCATATTGGAACCCGGCCCA|TAT
GTCG...

Figure 27

Activation by VZV ORF62 together with ORF4 of p61-MluI and its deletion plasmids in HFF cells. The p61-MluI plasmid and its deletion plasmids (10 μg) were cotransfected with pCMV62 and pCMV4 (5 μg each) which expressed VZV ORF62 and ORF4, respectively. Cells were harvested 48 hours after DNA transfection and CAT assays were performed. The percentage acetylation is shown on the left column. The pCAT-Control was used as a positive control.

# p61-MluI and its deletion plasmids with pCMV62 and pCMV4 in HFF cells

| % acetylation | plasmid |
|---|---|
| 0.81 | p61-WK1 |
| 0.93 | p61-WK2 |
| 0.85 | p61-WK3 |
| 0.97 | p61-WK4 |
| 6.69 | p61-WK5 |
| 19.98 | p61-WK6 |
| 11.25 | p61-WK7 |
| 88.67 | p61-WK8 |
| 95.67 | p61-WK9 |
| 94.48 | p61-WK10 |
| 94.71 | p61-MluI |
| 97.17 | pCAT-Control |

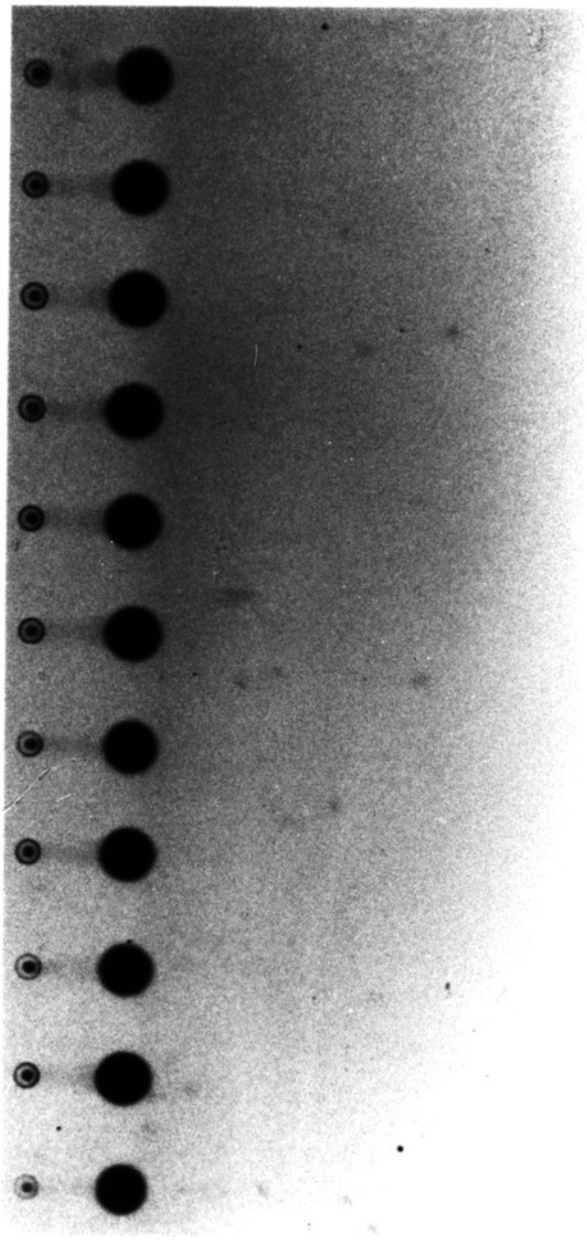


Figures 28-29

Activation by VZV ORF62 together with ORF4 of p61-MluI and its deletion plasmids in T cells (A.301). The p61-MluI and its deletion plasmids (10 μg) were cotransfected with pUC18 (control) (10 μg) (Fig. 28) or pCMV62 and pCMV4 (5 μg each) which expressed VZV ORF62 and ORF4, respectively (Fig. 29). Cells were harvested 48 hours after DNA transfection and CAT assays were performed. The percentage acetylation is shown on the left column. The pCAT-Control was used as a positive control.

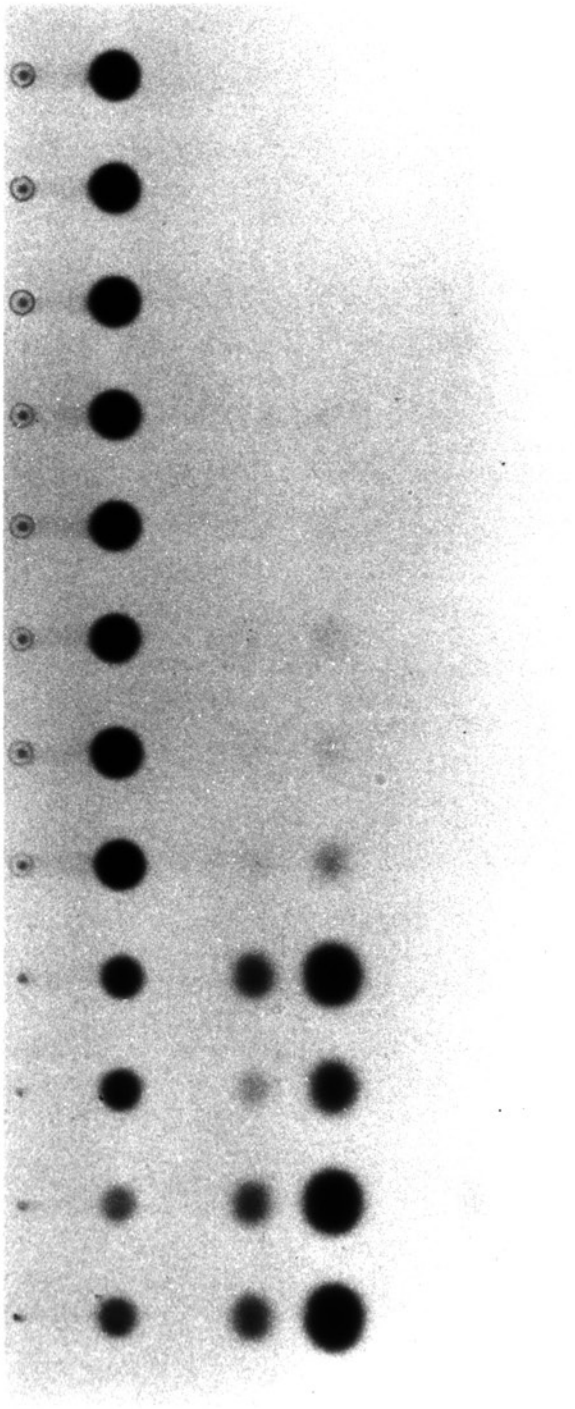
# p61-MluI and its deletion plasmids in T cells A.301 (control set)

| % acetylation | plasmid |
|---|---|
| 0.52 | p61-WK1 |
| 0.65 | p61-WK2 |
| 0.57 | p61-WK3 |
| 0.67 | p61-WK4 |
| 0.63 | p61-WK5 |
| 0.59 | p61-WK6 |
| 0.66 | p61-WK7 |
| 0.65 | p61-WK8 |
| 0.70 | p61-WK9 |
| 0.67 | p61-WK10 |
| 0.64 | p61-MluI |



# p61-MluI and its deletion plasmids with pCMV62 and pCMV4 in T cells (A.301)

| % acetylation | plasmid |
|---|---|
| 0.50 | p61-WK1 |
| 0.48 | p61-WK2 |
| 0.78 | p61-WK3 |
| 0.87 | p61-WK4 |
| 1.15 | p61-WK5 |
| 2.07 | p61-WK6 |
| 2.96 | p61-WK7 |
| 4.67 | p61-WK8 |
| 80.47 | p61-WK9 |
| 60.12 | p61-WK10 |
| 94.51 | p61-MluI |
| 91.17 | pCAT-Control |



promoter, 'ORF62+ORF4' seemed as capable as VZV infection in allowing the gene61 promoter to reach full activity. On the other hand, the patterns of transactivation by 'ORF62 + ORF4' in T cells (Fig. 29) were quite different from that in HFF cells (Fig. 25); indeed, only with the 5'..CCCAATACACATTTTATACCCACGTTTTAGTGGGTGGGACTTA..3' sequences included in the WK9 plasmid was any substantial activity seen. These data illustrate several points: (a) that a 'TATA' box is needed for promoter activity; (b) that upstream element(s), different from those required for the gpIV promoter are essential for full activity and (c) that different cell types appear to require different promoter sequences for full activity.

**Comparison of the expression from gene61 promoter plasmids with 'ORF62' VS 'ORF62+ORF4' gene products in HFF cells**

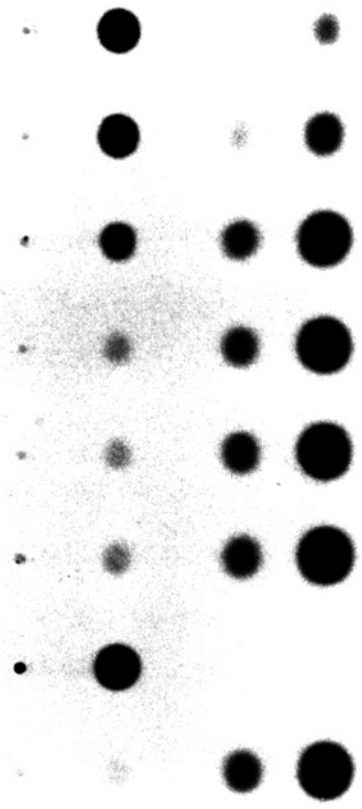
The gene product of ORF62 is probably the major regulatory protein in VZV (Inchauspe *et al.*, 1989a, 1989b; Cabirac *et al.*, 1990; Perera *et al.*, 1992a, 1992b). Results above (Fig. 25 and Fig. 27) showed that activation of p61-WK8 by VZV infection was the same as that by 'ORF62+ORF4'. To examine whether ORF62 protein alone could also give this full expression, p61-WK6, p61-WK8 or p61-MluI was cotransfected with pCMV62+pUC18 or with pCMV62+pCMV4 into HFF cells (Fig. 30). The results show that, while ORF62 alone can confer the bulk of the activation on all the plasmids used, ORF4 was

Figure 30

Activation by VZV ORF62 compared to that of VZV ORF62 together with VZV ORF4 on p61-WK6, p61-WK8, p61-MluI in HFF cells. The p61-WK6 or p61-WK8 or p61-MluI plasmids (10 μg) were cotransfected with pCMV62* (expressed VZV ORF62) alone or pCMV62* together with pCMV4# (expressed VZV ORF4) {5 μg each}. In each cotransfection experiment, the total DNA amount was kept constant at 20 μg by adding pUC18 plasmid DNA as a carrier. Cells were harvested 48 hours after DNA transfection and CAT assays were performed. The percentage acetylation is shown on the left column. The pCAT-Control was used as positive control.

# p61-WK6, p61-WK8 and p61-MluI with pCMV62* VS pCMV62*+pCMV4# in HFF cells

| % acetylation | plasmid(s) |
|---|---|
| 10.85 | p61-WK6 * |
| 21.87 | p61-WK6 *# |
| 82.34 | p61-WK8 * |
| 90.73 | p61-WK8 *# |
| 96.61 | p61-MluI * |
| 96.54 | p61-MluI *# |
| 0.71 | p61-MluI |
| 98.34 | pCAT-Control |



capable of providing a boost in activity which varied somewhat with the plasmid used. These data are supported by the titration experiments shown in Fig. 31.

**Regulation of p61-WK8 expression by VZV gene product(s) in HFF cells.**

As we have done with the other promoters studied, we wanted at this point to check the known VZV gene regulatory proteins (in addition to ORF62 and ORF4) in combinations to assess their ability to control gene61 expression.

Thus, we examined the effects of the VZV ORF62, 4, 61, and 63 gene products on the expression of the p61-WK8 plasmid which contained the minimum promoter sequences allowing full expression by VZV infection in HFF cells. P61-WK8 was cotransfected with pCMV62, pCMV4, pCMV61 and pCMV63 alone or in combinations into the HFF cells (Fig. 32). Only ORF62 protein alone (lane 2) showed activation of the expression of the CAT gene; ORF4 or ORF61 or ORF63 proteins alone (lane 3, 4, 5) did not. However, each of them showed synergism with ORF62 protein to activate CAT expression (lanes 6-8). Moreover, ORF61 together with ORF4 synergized ORF62 (lane 9) more effectively than ORF4 alone (lane 6). On the other hand, ORF63 repressed the activation of 'ORF62+ORF4' (lane 10 VS lane 6) and of 'ORF62+ORF4+ORF61' (lane 11 VS lane 9).

Figure 31

Titration of the effect of pCMV62 and pCMV4 (expressed VZV ORF62 and ORF4, respectively) on the expression of p61-WK8. The p61-WK8 plasmid (10 μg) was cotransfected with pCMV62 alone, or pCMV62 together with pCMV4. The amounts of pCMV62 in lanes 1-4 were 0.1 μg; lanes 5-8, 0.5 μg. The amount of pCMV4 in lane 2 was 0.1 μg; lanes 4 & 8, 5 μg; lane 6, 0.5 μg. The total amount of DNA was kept constant at 20 μg by adding pUC18 plasmid DNA as a carrier. Cells were harvested 48 hours after DNA transfection and CAT assays were performed. The percentage acetylation is shown on left column. The pCAT-Control was used as positive control.

# Titration of pCMV62 and pCMV4 on the expression of p61-WK8 in HFF cells

| % acetylation | pCMV62 | pCMV4 |
|---|---|---|
| 16.62 | 0.1 (μg) | - |
| 24.93 | 0.1 | 0.1 |
| 16.03 | 0.1 | - |
| 24.53 | 0.1 | 5 |
| 60.93 | 0.5 | - |
| 72.05 | 0.5 | 0.5 |
| 62.49 | 0.5 | - |
| 77.89 | 0.5 | 5 |
| 97.01 | positive control | |

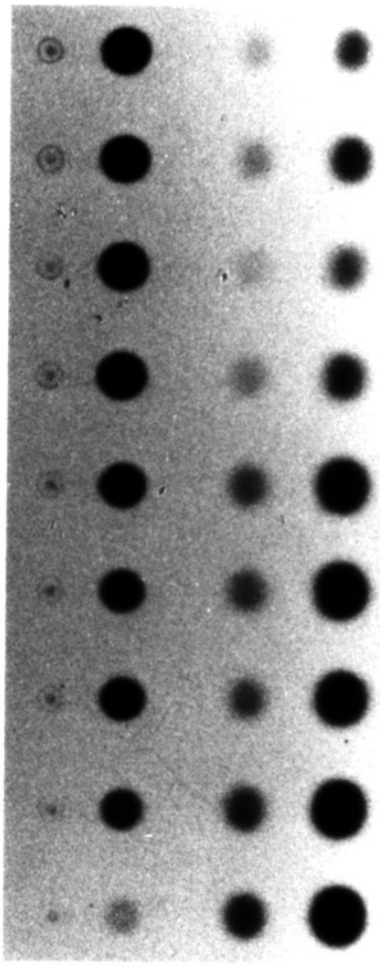
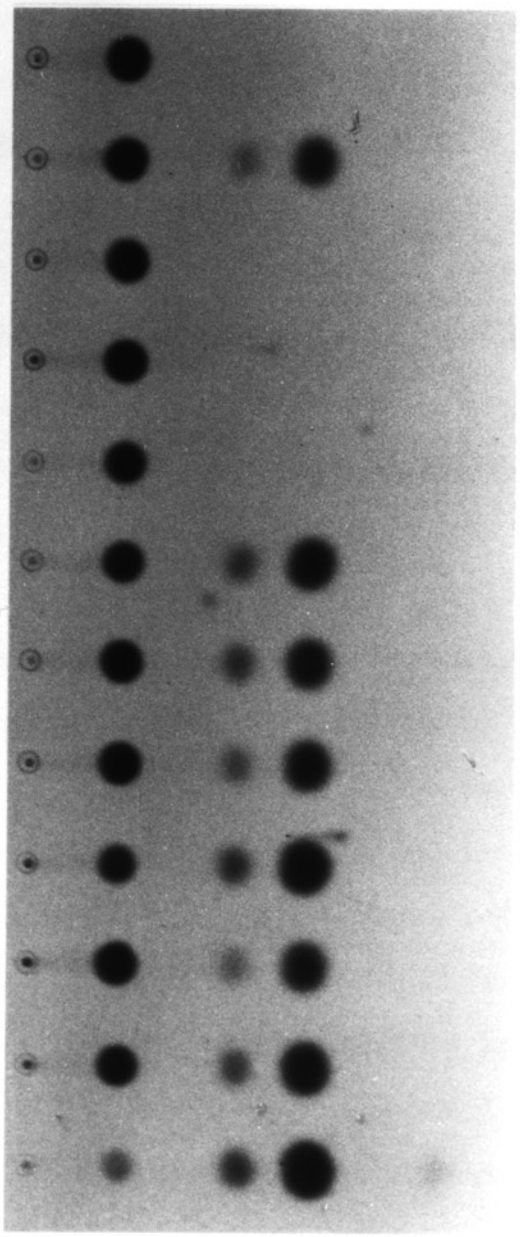


Figure 32

Effect of VZV gene product(s) on the expression of p61-WK8 in HFF cells. The p61-WK8 plasmid was cotransfected with effector plasmid(s) (0.2 μg) expressing ORF 62, 4, 61 and 63 (pCMV62, pCMV4, pCMV61 and pCMV63, respectively) alone or in combinations. In each cotransfection experiment, the total DNA amount was kept constant at 12 μg by adding pUC18 plasmid DNA as a carrier. Cells were harvested 48 hours after DNA transfection and CAT assays were performed. The percentage acetylation is shown on the left column.

# Regulation of p61-WK8 expression with VZV gene product(s) in HFF cells

| % acetylation | effector(s) |
|---|---|
| 0.84 | - |
| 28.37 | pCMV62 |
| 0.66 | pCMV4 |
| 0.78 | pCMV61 |
| 0.81 | pCMV63 |
| 55.83 | pCMV62+4 |
| 42.13 | pCMV62+61 |
| 40.96 | pCMV62+63 |
| 77.42 | pCMV62+4+61 |
| 42.16 | pCMV62+4+63 |
| 62.07 | pCMV62+4+61+63 |
| 95.59 | positive control |



## Discussion

Definition of promoter elements for viral genes depends, as with other genes, upon the description of the transcripts produced from these genes.

Thus far, there have been reports of the fine mapping of the encoded mRNAs from seven of the 68 VZV unique ORFs, viz: ORFs 36 (pyrimidine deoxynucleoside kinase; dPyK), 62 (IE62), 14 (gpV), 67 (gpIV) and 61, 4 and 63 (that encode three early gene regulatory proteins) (Davison and Scott, 1986; McKee *et al.*, 1990; Ling *et al.*, 1991; 1992; Stevenson *et al.*, 1992; Kinchington *et al.*, 1994). Examination of putative promoter sequences upstream from the start sites for these mRNAs reveals some general features, such as potential 'TATA' boxes, usually around position -25. However, many atypical and canonical 'TATA' sequences, as well as 'CCAAT' sequences are located in various places upstream of the transcriptional start sites; this is a feature of relatively low GC viral DNA which might be expected on random grounds. For example, the VZV pyrimidine deoxynucleoside kinase (dPyK) mRNA has been studied in some detail (Davison and Scott, 1986). These authors described a putative 'TATA' box (TATTAA) at -25 bp upstream of the transcriptional start site. However, there is a second consensus 'TATA' element at -57 bp, and whether this or the first 'TATA' box is used is not yet clear. VZV ORF62 promoter localization is another example; in 1988, Felser *et al.* (1988) predicted the 'TATA' box and further upstream

regulatory sequences around 374 bp from the first AUG by simple inspection of the nucleotide sequences. However, McKee *et al.* (1990) experimentally identified the 5' end of transcripts of ORF62 71 bp upstream from the first AUG and predicted the putative 'TATA' box (TTTAAA) at -25 to -30 upstream from the transcriptional start site. They also showed that a minimum region 131 bp upstream from the transcriptional start site functioned as a promoter, using deletion analysis. Interestingly, within this minimal region, there are three addition 'TATA' elements at -56 bp (AAATAT), -83 bp (TATTTA), -92 bp (ATTAA), none of which seem likely to be used.

Recently, Kinchington *et al.* (1994) reported multiple transcriptional start sites for VZV ORF63. These are at position 87 to 95 (proximal site), 151 to 153 (distal site) and (tentatively) 238 to 243 bp upstream of the ORF start codon. They also defined the sequences surrounding these initiation sites. Upstream of the site proximal to the ORF 63 AUG is a poor-consensus 'TATA' box at position -25, and two possible 'CCAAT' boxes at -47 and -78. A good-consensus 'TATA' box lies at 31 bp upstream of the major site distal to the AUG (located 65 bp upstream of the proximal site), but there is no consensus 'CCAAT' box. A similar pattern is shown in the tentative third site. Three possible SP1-binding sites are also identified by scanning the sequence. To answer whether each start site represents the initiation of only one of the two ORF63 transcripts (1.3 and 1.9 Kb poly(A)$^+$ RNAs)

or each site reflects the initiation of both ORF63 transcripts, the authors used oligonucleotides derived from sequences between the two major initiation sites in Northern blots. These results show that the major distal site is used for both ORF63 transcripts, while the most proximal site is also used for one ORF63 transcript. It will now be interesting to examine which of the promoter elements upstream from each of the transcriptional start sites is functional.

The recognition and activation of promoter sequences upstream of genes encoding products of each temporal class is the major regulatory mechanism controlling VZV gene expression. As seen from above, prediction of the true regulatory sequences for transcriptional initiation is generally not possible from examining sequences visually, so they must be experimentally determined.

Previous work in our laboratory had identified ORF14, encoding the true late gene for glycoprotein V (gpV), mapping to the *Eco*RI P fragment in the $U_L$ segment of VZV (Kinchington *et al.*, 1986) and also described the transcriptional start site of its mRNAs (Ling *et al.*, 1991). Another glycoprotein, gpIV (an early-late gene) encoded in ORF67 (Davison *et al.*, 1985) was also transcript-mapped by our laboratory (Ling *et al.*, 1992). Finally, a third gene VZV gene has been transcript-mapped, gene 61, and we are considering it here as an early gene, as previously mentioned. Its transcripts were mapped by Stevenson *et al.*, (1992). In this study, we have

experimentally identified the promoter regions which are the cis-acting sequences that control the expression of these genes (ORF14, 67, 61). We have also examined the functional relationship of the VZV encoded regulatory proteins from ORF4, 61, 62 and 63 on the promoter region of these three genes as well as that of HSV glycoprotein C.

A true late promoter (for gpV)

Initial work on transcription regulation in our laboratory had examined the putative promoter sequence of gpV by cloning a fragment of approximately 580 bp in front of the Chloramphenicol acetyltransferase (CAT) reporter gene of pCAT3M. Interestingly, the gpV promoter was 40 to 50 fold less active than that for another glycoprotein (gpIV), although *in vivo*, similar levels of gpV and gpIV transcripts were detected (unpublished observations). There may be several reasons for this finding, and we have been able to address some of them here. For example, although the CAT reporter gene is widely used, in many cases it has been shown to generate a highly unstable transcript, which may be poorly translated in the context of certain regulatory sequences in eukaryotic cells. To find out if this is a problem with gpV, we cloned the 580 bp promoter fragment upstream of the firefly luciferase (luc) reporter gene of the pGL2-Basic Vector (PROMEGA), and tested it with VZV infection in HFF cells in transient assays. No significant increase in activity was found (data not shown), showing that whatever

the reason for poor gpV promoter activity, it is not likely to be an artifact of the reporter system.

Another possibility was that the 580 bp gpV promoter fragment may be too large for promoter function. Certain (inhibitory) sequences may be needed to be deleted before full expression could occur. Putative 'TATA' and 'CCAAT' boxes appeared at -25 bp and -55 bp upstream from the gene14 mRNA start site, consistent with other eukaryotic promoters. However, there are several other consensus 'TATA' sequences lying upstream from this gene: e.g. TTATAATT at -145 bp and TTATAATT at -371 bp. We thus tested pCAT-Basic gpV deletion plasmids in transient assays with VZV infection. The CAT activity of the shorter fragment (80 bp) from pgpV120-CAT was approximately the same as that of the largest one (580 bp) from pgpV-CAT (Fig. 4), and both were still less active than the gpIV promoter which was also recloned into pCAT-Basic (Fig. 15). What was most important, though, was that this experiment showed that only the 'TATA' sequences located at -25 bp from the transcriptional start site were needed for expression of gpV. Even the putative 'CCAAT' sequences which lay at -55 bp were not necessary. This result correlates well with work on the promoter region of the gpV homologue, glycoprotein C (gC) of HSV which also needs only a 'TATA' box for expression in transient assays (Homa *et al.*, 1986a; 1988).

Yet another formal possibility to explain the low gpV promoter activity is that this 580 bp fragment may be too

small to be the gpV promoter. A larger fragment can be created by adding the sequences either upstream or down stream of this 580 bp fragment. A 1.32 Kb fragment upstream from the AUG for gpV was cloned upstream to the luciferase reporter gene of pGL2-Basic and tested in transient assays, but no increase in luciferase activity was detected (data not shown). Thus, additional upstream sequences do not seem to help.

As mentioned earlier, there are 7.76 copies of a 42 bp repeated sequence located almost at the beginning of ORF14. These elements had already been tested for enhancer activity in our laboratory under a variety of conditions, using a conventional enhancer test vector. We could not detect classical enhancer activity from these elements (unpublished observations). However, we now considered the possibility that they may act as enhancers only in the gpV promoter context. We carried out the experiment shown in Fig. 6 and found no effect of the presence of the repeated elements. It now seems clear that although we had seen a difference between VZV Scott and VZV Oka in transcription from ORF14 (20 -fold more in Scott), this is not likely to be due to the differences in their 42 bp repeat elements (Scott has 7.76, while Oka has 3.76 copies; Ling *et al.*, 1991). At this point we have no reasonable explanation for this Scott/Oka difference; it may simply be the result of different transactivating factor(s) in the Oka strain. In this same context, we have also tested the level of gpV from several

VZV isolates from Japan that display a wide range of size heterogeneity in the number of these 42 repeats. All of them produce wild-type levels of gpV, confirming that the repeats probably do not function in regulating expression of gpV (unpublished observations).

One of the more surprising results we have with gpV is that VZV infection induced the HSV gC promoter (Fig. 8 and lane 11, Fig. 10) better than HSV infection did (lane 7, Fig. 10) and much better than (its homologous) gpV promoter (Fig. 4 and lane 12, Fig. 10). On the other hand, HSV infection could barely activate the expression of pgpV-CAT (lane 8, Fig. 10). This indicates that VZV transactivation factors fit very well with the HSV gC promoter to give full expression in the transient assays, but the opposite is not true. Interestingly, this result correlates with the report from Inchauspe and Ostrove (1989b), when they showed that VZV ORF4 and ORF62 stimulate the HSV-1 thymidine kinase (*tk*) gene while HSV-1 infection can not stimulate the VZV *tk* promoter in transient assays in Vero cells.

A possible explanation has come from Everett group (Everett 1984; Tyler and Everett, 1993), who showed that plasmids containing VZV ORF62 can transcriptionally activate the HSV-1 gD promoter after co-transfection and that the ORF62 protein DNA binding domain was less sequence-specific than its homologue (HSV Vmw 175) in its interactions with DNA for autoregulation. This low specificity of DNA binding may explain the promiscuous transactivation by this VZV major

transactivator relative to that of HSV.

Another puzzle with gpV is that, while we identified its minimal promoter at the 'TATA' box (5'.TCATTTAAATTCCGC.3'), and then changed it to reflect the HSV gC 'TATA' box sequence (lane 9, 10, 12, Fig. 10), we could not develop as much promoter activity as seen for gC. We propose that the transcriptional factors of VZV (and HSV) are able to react through these 'TATA' sequences to activate the HSV gC promoter only if the 'TATA' sequences lie in their own genomic environment. The sequences around this HSV gC sequence in the promoter may be necessary for forming the right conformation of promoter to be recognized by transactivating factors. The 'TATA' sequence of each gene may need its own surrounding region for proper expression. For example, Homa *et al.* (1988) showed that the HSV *tk* 'TATA' sequences can not substitute for the HSV gC 'TATA' sequences. They constructed a chimeric *tk*-gC gene in which sequences of the promoter for the HSV-1 *tk* gene between base -37 and +52 (a 'TATA' box located at -27 to -21 which has been shown to be essential for efficient expression of *tk* during infection by Coen *et al.*, 1986) replaced those of the gC promoter between bases -146 and +124. When cells were infected with this virus carrying this chimera, no gC-specific mRNA could be detected on a Northern blot.

In similar experiments, Arsenakis *et al.* (1986) took the entire gC gene, including several Kb of 5' flanking sequence, and integrated it into a mouse cell genome. Cells that

contained the gC gene did not constitutively produce gC; however, infection with HSV lacking a functional gC gene was able to induce gC expression. Addition of PAA (to inhibit viral DNA replication) to the culture media did not affect the induction. Hence, how the gC gene is regulated depends on the environment of the genome it lies in; the mechanism behind this remains obscure.

The detailed mapping of the cis-acting promoter sequences of the HSV-1 gC gene was carried out by Homa *et al*; (1986a, 1986b, 1988; Shapira *et al*; 1987). They chose gC because it is a model for a true late gene which needs DNA replication for its expression, and which is dispensable for viral growth in tissue culture, so that whole virus mutants could be used in their studies. While we belive that VZV gpV is also not essential for growth in cell culture (unpublished observations) it has only very recently become possible to consider construction of VZV gpV mutants using sets of overlapping cosmids. We will now be able to carry out mutation analysis of this promoter in the (proper) context of the whole virus. Some potential experiments might include cloning one of the VZV ori sequences (origin of replication) into the pgpV-CAT plasmid (pgpV-CAT-Ori) and then confirming its replication ability by transfecting into cell cultures with or without VZV infection, extracting the plasmids after two days and measuring CAT activity. If DNA replication is needed for full gpV promoter activity, we should see significantly more activity than that from mock-infection.

If the CAT activity from pgpV-CAT-Ori is the same as that of pgpV-CAT, the other explanation for the lower expression from the gpV promoter is that it is inherently weak. Indeed, since VZV grows only very poorly in cell culture, one of its contributing defects may well be that it does not make enough gpV to be effectively viable.

An early/late promoter (for gpIV).

Previous work in our laboratory had already mapped transcripts for gene 67, encoding glycoprotein IV (gpIV) (Ling *et al.*, 1992). We had also shown that 430 bp upstream from the translational start site was sufficient for promoter activity. As for gpV, we set about identifying the minimal cis-acting sequences which would define the gpIV promoter. What we found was that, while the 'TATA' box was sufficient for (a low level) activity (not much different from that for gpV), for this promoter, unlike the gpV element, additional upstream sequences were required for maximum activity (Fig. 15). The more upstream sequences were added, the more CAT expression was shown (lanes 4-7, Fig. 15). In particular, inclusion of the sequence 5'...CAGAGTCACGC...3', which we named 'AUS' (activating upstream sequence), allowed upregulation of CAT activity from 4% in pgpIV3.2-CAT to full expression (>90%) in pgpIV3.4-CAT. To investigate whether this increased activity came from the sequences themselves or from the promoter configuration changing when the sequences were added, we cloned the upstream sequences

5'...ACAGAGTCACGCCCCATT...3', including the 'AUS', upstream from the gpV promoter in pgpV120-CAT, naming the construct pgpV120(3-3.4)-CAT, then tested it in transient assays. Interestingly, the CAT activity rose from 5% acetylation in pgpV-CAT (lane 2, Fig. 21) to full expression (>90% acetylation) in pgpV120(3-3.4)-CAT (lane 4, Fig. 21), which, interestingly, is the same level as that of pgpIV3.4-CAT (lane 7, Fig 15). This result not only confirmed that this gpIV 'AUS' was in itself a significant upregulator for gpIV gene expression, but also showed that it is movable; i.e. could function with a promoter other than that of gpIV. These data also demonstrated that, in the transient assay, the gpV promoter could be simply stimulated to act as a strong promoter. Thus, as we suspected, the gpV promoter is potentially fully functional, but, in its native form, is merely weak. Future work should test whether the gpIV 'AUS' is capable of functioning with other VZV and non-VZV promoters, and whether it is a classic enhancer, by cloning it into different sites (e.g. downstream from the gpIV gene) or in different orientations in the pgpIV-CAT system.

It is worth mentioning here that, although the HSV gC (VZV gpV homologue) promoter has been extensively studied, the HSV glycoprotein I, (gI, the VZV gpIV homologue) promoter has yet to be mapped in any detail. It would be very interesting to examine the HSV gI promoter, to see whether it has a similar pattern to that of VZV gpIV, and whether their specific sequences have similarities or are interchangable.

A close examination of Fig. 15 shows that the CAT activity in pgpIV4-CAT was decreased from 92% acetylation (in pgpIV3.4-CAT) to 52%, before starting to increase again in pgpIV5-CAT (Fig. 15). This inhibition is apparently due to only 4 base pairs 5'...AATA...3', which may be too short to act as a repressor sequence, and which may bring about a change in the configuration of the promoter DNA. To address this problem, the 4 bp sequence will have to be cloned upstream from another strong promoter and then tested for decreased activity.

As discussed in the introduction to this dissertation, we and others have described gene regulatory proteins in VZV, four of which are thought to be major influences (e.g., ORF4, 61, 62 and 63). We have examined the ability of all four in combination to regulate the expression from the gpIV promoter and compared the activity with that after VZV infection (Figs. 22 and 15). Clearly, the ORF62 product, like its homologue HSV Vmw175, is the major transactivator, and its activity can be substantially augmented by the ORF4 product. So far, from our studies and those of others (Inschuaspe *et al.*, 1989a; Perera *et al.*, 1992a), VZV ORF4 always synergizes ORF62 for VZV gene transactivation in transient assays. However, HSV ICP27, which is VZV ORF4 homologue, has been reported to act as a trans-repressor or a trans-activator in combination with ICP4 (VZV ORF62 homologue) and ICP0 (VZV ORF61 homologue) depending on the target gene (Everett, 1986; Block and Jordan, 1988; Sekulovich *et al.*, 1988; Hardwicke *et*

*al.*, 1989; Rice *et al.*, 1988, 1989). In the case of VZV ORF61 and 63, their products have more minor influences on the expression of these promoters. The HSV homologue of VZV ORF61, ICP0, is known to be a promiscuous and potent transactivator. The ORF63 homologue, HSV ICP22, has not yet been well characterized, thus our results with regard to ORF63 present novel findings. Of course, we cannot supply these proteins in transient assays in the same quantities in which they are available in the VZV infected cell, but we believe that, for expression of gpIV, the ORF62 and ORF4 proteins have the bulk of the responsibility.

In this study, we also asked whether cellular factors had any effect on the expression from the gpIV promoter. We chose T cells (A3.01) to compare with HFF cells in our experiments because there is good evidence that VZV grows in circulating T lymphocytes during the viremic phase of human infection (Asano *et al.*, 1989; Gilden *et al.*, 1987; Koropchak *et al.*, 1989, 1991; Ozaki *et al.*, 1986; Vonsover *et al.*, 1987), and, thus, that these cells are permissive for VZV. HFF cells, of course, represent some of the skin cells in which VZV grows during the appearance of its characteristic rash. Using these cells in transient assays, we felt, might reveal interesting features of authentic regulation control for VZV genes. Being uncertain of the ability to infect T cells *in vitro* with VZV virus and having shown (above) that the ORF62 and 4 products were essentially all that was needed to gain maximum activity from the gpIV promoter, we chose

ORF62 and ORF4 plasmids to turn on the gpIV plasmids in both T-cells and HFF cells. Interestingly, the level of activation in T-cells (Fig. 19) was much lower than in HFF cells (cf. Fig 15.), but the patterns (i.e. important regulatory sequences) was the same. This suggests that there is no significant difference in cellular factors involved with ORF62 and ORF4 in expression from the gpIV promoter, at least in T-cells and HFF cells. As we will show in the following section, however, this is not the case for the gene61 promoter.

Finally, we have been interested in the possibility that the gpIV 'AUS' is similar to another known eukaryotic gene regulatory sequence. As far as we can tell, this VZV sequence is unique. Whether it may be present in the regulatory element(s) of other VZV genes is hard to tell, since so very few VZV promoters have been mapped. Certainly, among those known so far, the gpIV 'AUS' is unique.

An early VZV gene promoter (for gene61)

To analyse the VZV gene61 promoter, we have used the same deletion mutation strategy as for gpIV and V, and have attempted to analyse the data comparatively. The transcriptional map of this gene was completed by Stevenson *et al*, (1992). Examining sequences upstream from the transcriptional start site shows that there are two putative 'TATA' boxes and two putative 'CCAAT' boxes. In HSV, the ß genes (in the same putative kinetic class as VZV gene61; for

example the *tk* gene) require a 'TATA' element and additional distal signals such as a 'CCAAT' box or SP1-binding sites for maximum transcriptional activity (McKnight *et al.*, 1981; 1982a; 1984). To investigate whether this VZV early gene promoter fitted the same pattern as in HSV, we cloned a fragment upstream from the translational start site into pCATBasic and performed deletion analyses. As for the previous two VZV genes studied (gp IV and V), we found that only a 'TATA' box (in this case the first (most 3') one) is needed for expression of this gene. While we again saw that the 'TATA' box alone allowed only minimal promoter activity, the data suggested that the transcriptional factors from VZV worked slightly better on the 'TATA' box of gene61 (lane 5, Fig. 26) than on that gpIV (lane 3, Fig. 15).

Also as we had seen with gpIV, addition of upstream sequences markedly upregulated promoter activity but not in a similar fashion to gpIV. For one thing, the important upstream regions in the gene61 promoter were different from those in gpIV and the sequences involved were quite different from any seen before. The gene61 promoter seems more complex than that of gpIV, which, in turn is more complex than that of gpV. Two separate upstream promoter elements (3'...TAAAAATATTAACCATGGTTTGCTTCTGTGTGGGG...5') from p61-WK4 to p61-WK6 and (3'...TAAAGAAA...5') from p61-WK7 to p61WK8, respectively; (Fig. 26), allowed significant increases in CAT activity (lanes 5, 6, 7; Fig. 26). We will test whether these sequences are able to act as movable elements (see the gpIV

'AUS' above) by cloning them upstream to other promoters and testing them in transient assays. If they act in the same way as in the gpIV promoter (as we predict), we will construct additional deletions to determine the minimal sequences which still have an upregulatory character.

As we pointed out in the gpIV analysis, in addition to upregulatory sequences, we also found downregulatory elements. The situation for gene61 appears similar. Examination of the deletion mutations (Fig. 25) shows that between p61WK6 and p61WK7 there is a four-fold inhibition of promoter activity; the sequence involved is 3'...ATATAGGGAGGTGGG...5'. It may be important that this sequence contains a short AT stretch (compare the gpIV 3'...ATAA...5' sequence), and that this might act as a secondary 'TATA' box, confusing the ability of the original (most proximal) 'TATA' box to be used. We would need to carry out a 5' terminal analysis of the mRNAs resulting from these assays in order to confirm (or rule out) this hypothesis; these experiments have not yet been done.

We showed that the ORF62 and ORF4 products were sufficient for essentially maximal activity of the gpIV promoter (see above) and the same appears to be true for the gene61 promoter (Fig. 27). Again, it is not clear what role if any the additional regulatory proteins play in controlling this early gene expression, and it's not even clear that our assays are capable of such fine analysis. We further examined whether the ORF62 product alone was enough for strong

transactivation of the gene61 promoter. We picked some of the deletion plasmids to test, and p61-WK8 and p61-MluI showed CAT expression at a similar level with pCMV62 or pCMV62+pCMV4 (Fig. 30). We also titrated pCMV62 using expression from p61-WK8, and the data showed that even in very small quantities (0.1 μg), pCMV62 still could transactivate this construct to allow up to 16 % acetylatiion (Fig. 31). These results may suggest that the mechanism for the switch-on of the gene61 (early) promoter is more ORF62-dependent than the other promoters. Previous studies in our laboratory showed that the VZV particle contains significant amounts of the ORF62 transactivator protein, IE 62 (Kinchington *et al.*, 1992). Other previous studies in our laboratory showed that IE62 can positively autoregulate its cognate promoter in transient assay (Perera *et al.*, 1992b). Thus, the gene61 product may be able to act as an early (perhaps very early) protein because its promoter is so well-activated by IE62 and was easy to turn on with only a small amount of transactivator (IE62) already present in the tegument of viral particle.

As in the previous section, we carried out the same study with the set of gene61 promoter deletion mutants in T cells as we had done with HFF cells, in order to examine the possible effect of cellular factors on transcriptional control. As is clear from the data, the pattern of regulation of gene61 constructs in T cells (Fig. 29) is quite different from that in HFF cells (Fig. 27). As with gpIV, we used transactivation by ORF62 and ORF4 in the comparative tests in

the cell lines. Clearly, there is a T-cell-specific sequence (3'..ATTCAGGGTGGGTGATTTTGCACCCATATTTTACACATAACAC..5'), while the T-cells are incapable of using the HFF cell sequences we have defined above. Again, as with gpIV, levels of activation in T cells seem lower than in HFF cells. These data imply that there are differences in cellular factors and/or transcriptional controlling mechanisms between these two cell lines which affect the expression from gene61 deletion mutant plasmids. Whether this translates into different modes of replication of virus in these two cell types remains to be seen, but is an intriguing possibility.

Promoter comparisons

In these studies we have defined the promoters for VZV early (E), early/late (L1) and true late (L2) genes; Everett and his colleagues (McKee *et al*, 1990) have defined the immediate early (IE62) promoter. It is obvious from our data that the cis-acting sequences that control transcription of IE, E, L1 and L2 genes of HSV and VZV are very different.

The VZV IE gene (ORF62) requires a 'TATA' box, an upstream octamer/TAATGARAT-like motif (ATGTAAATGAAAT) and a simple octamer motif in inverse orientation, but no 'CCAAT' box or SP1-binding sites (McKee *et al.*, 1990) while the HSV IE gene (Vmw175, the VZV ORF62 homologue) requires a 'TATA' box, distal signals (such as a 'CCAAT' box or SP-1 binding sites), and an upstream TAATGARAT motif (Mackem and Roizman, 1982a; Preston *et al.*, 1984). The TAATGARAT motif is the

element that mediates stimulation of IE transcription by the virion protein Vmw65 (or VP16) in HSV and the ORF10 protein in VZV. Although both of them can interchangably transactivate each IE gene, i.e. Vmw65 and ORF10 can transactivate VZV ORF62 and HSV Vmw175 in HeLa cells (McKee *et al.*, 1990) and Vero cells (Moriuchi *et al.*, 1993a), the mechanisms may be quite different. Vmw65 does not bind directly to DNA but forms a complex with cellular proteins such as Oct-1, then binds to the TAATGARAT element of both Vmw175 and ORF62. On the other hand, VZV ORF10, which lacks the acidic carboxy terminal activation region of Vmw65, cannot form a complex with TAATGARAT. Hence, VZV ORF10 must use other pathways to transactivate VZV ORF62 and HSV Vmw175.

The HSV E gene promoter which has been most extensively studied is the *tk* gene (McKnight *et al.*, 1981; 1982a; 1984). This gene requires a 'TATA' box and distal signals: a 'CCAAT' box and SP-1 binding sites. However, the transcriptional control region of HSV Vmw110 (ICP0, the homologue to VZV ORF61) has not been studied in detail yet. We were able to show that a VZV E gene (gene61 in this case) did not require the upstream 'CCAAT' element, unlike the HSV *tk* gene. However, for full expression, it required specific distal elements, but nothing similar to those seen in HSV.

In both the glycoprotein promoters (VZV gpIV and V), we were able to define sequences needed for activity; these were also novel and different from those seen in HSV. What was

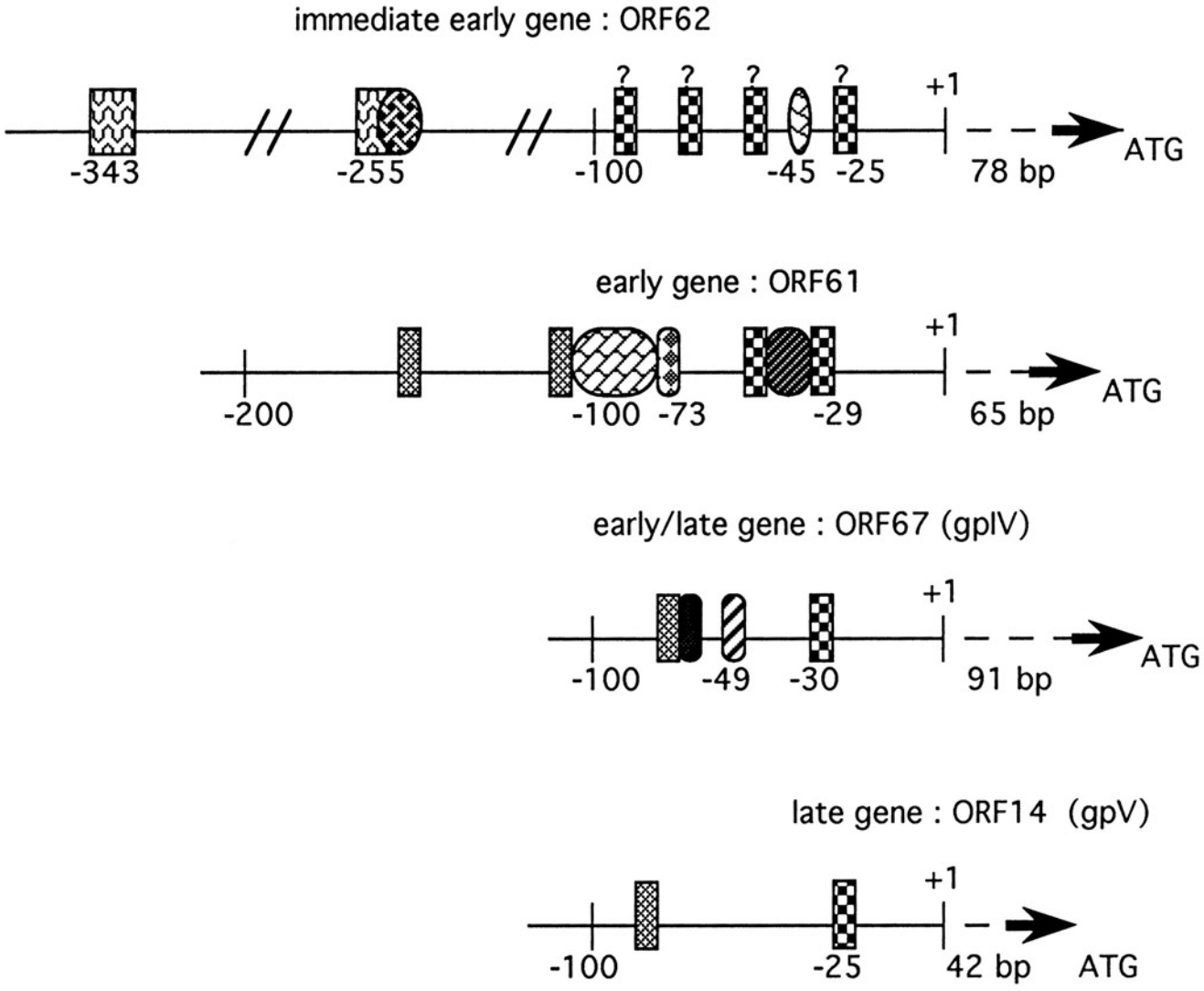
similar to HSV, however, was the progression of decreasing complexity of promoter as the gene class became progressively later. The L2 class is the simplest promoter, while the IE class is most complex (Figs. 2 and 33).

As we have mentioned elsewhere, the promoter sequence, cell type, and transfection method all affect the results of promoter regulation experiments, and our assays may not always represent the *in vivo* situation. Also, the maximum expression of each promoter (regardless of the number of combinations of cotransfected plasmids) still did not quite reach the level of expression with actual VZV infection. This indicates that there may be other viral gene products involved in the expression from these promoters.

It will be very important to continue to examine the 'AUS' upstream sequences of both the gpIV and gene61 promoters. To address the many questions about which VZV gene products and/or cellular factors are involved in this transcriptional regulation, gel retardation assays and DNaseI footprinting assays will be performed. In the gel retardation assay, VZV infected or transfected nuclear extracts are incubated with end-labeled DNA fragments which include the 'AUS' sequence of interest. Interactive proteins are detected by their ability to retard mobility of this labeled DNA fragment through a nondenaturing gel. We have been able, in preliminary experiments, to see "gel shifts" with both gpIV and gene61 sequences, as well as with VZV proteins or IE62-transfected cells. Several bands on these gels are

Figure 33

Diagram of examples of different kinetic classes of VZV gene promoters. The translational start site is at codon "ATG", the transcriptional cap site is at +1 and the leader sequence is shown in base pairs between them. Varieties of sequence elements identified as being important for each promoter are shown in different shaded boxes. The distance from the cap site is shown as a negative number of base pairs. TFIID, C/EBP (CCAAT box binding protein), Oct1 and Oct2 are cellular transcription factors; ORF10 and ORF62 are VZV regulatory proteins. The G-String is an ORF62 promoter sequence confering constitutive expression in neural cells (Perera, Ruyechan, Hay, unpublished observation).




| | responsive to |
|---|---|
| TATAA | TFIID factor |
| TAATGARAT-like | ?ORF10, ?Cellular factor, ?ORF62 |
| CCAAT | C/EBP |

| | responsive to |
|---|---|
| octamer (ATGCAAAT) | Oct1 and Oct2 |
| G-String | ORF62 |
| ORF67 AUS | ??? |
| ORF61 AUS | ??? |

selectively competed out with excess cold probe, showing specificity (data not shown).

In conclusion, we have defined, for the first time, the promoter elements for three of the four kinetic classes of VZV genes. They are all clearly different, and different from those in HSV, and these differences probably form the basis for the complex cascade controls which characterize VZV protein synthesis and regulation of the viral life cycle.

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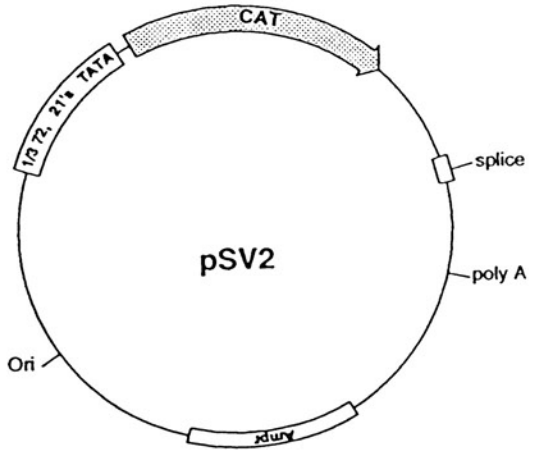
# Appendix

The description of some of the plasmid constructions used in this work.

Index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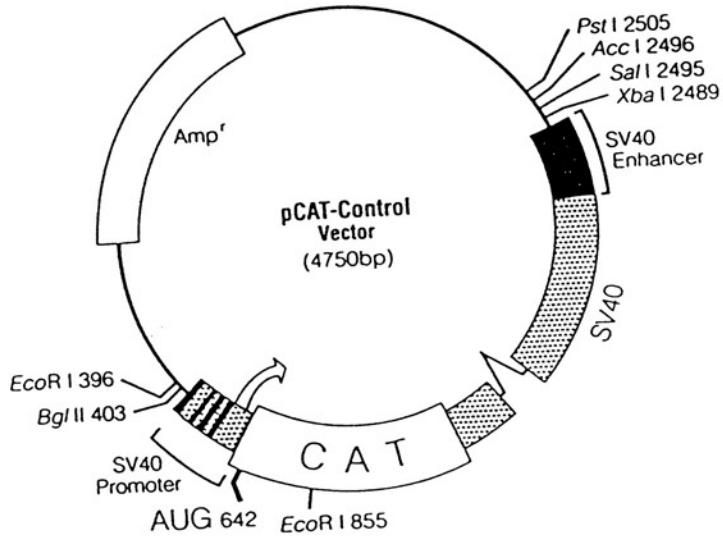
Ori
Ampr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Bgl II 403
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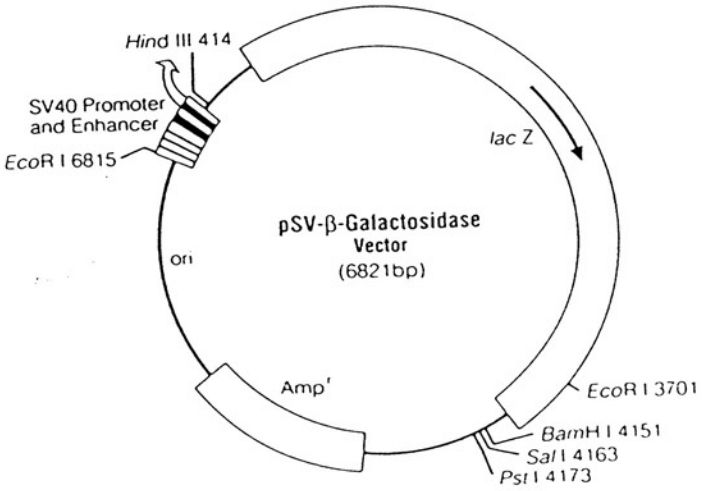
C A T
AUG 642
EcoR I 855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lac Z
pSV-β-Galactosidase Vector (6821bp)
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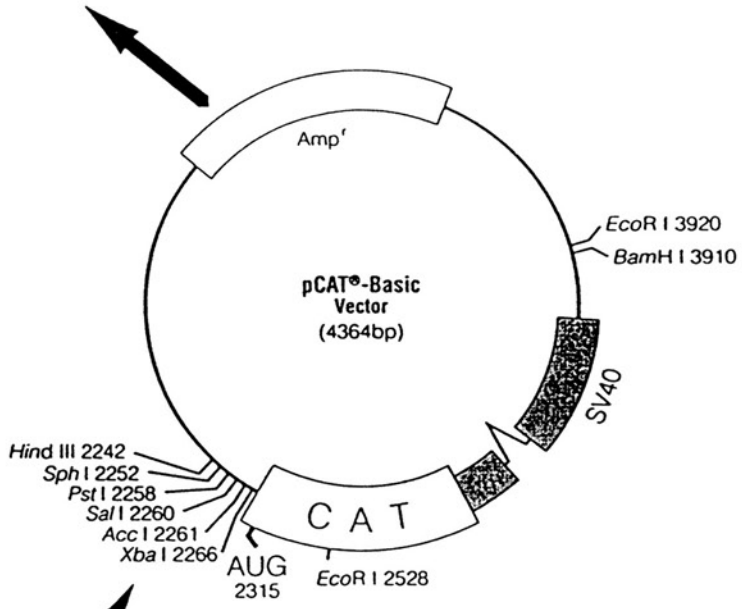
Sal I 4163
Pst I 4173

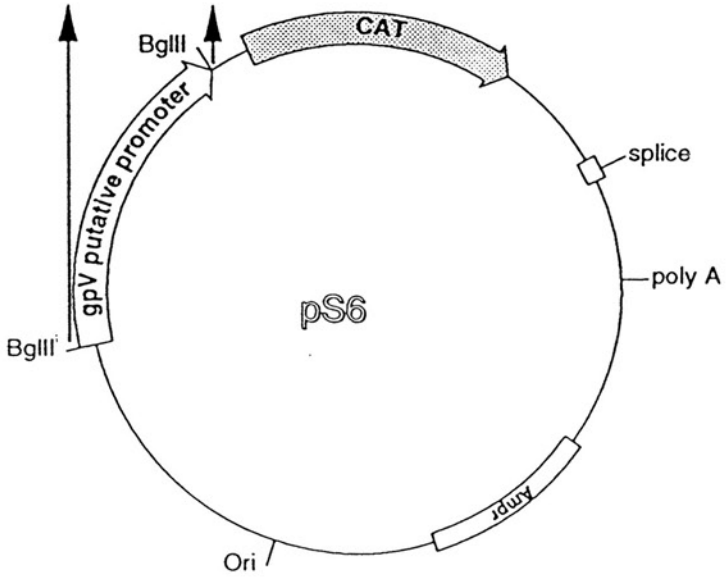
pgpV-CAT




-cut with Bgl II

-Klenow 'fill-in'

-ligate into Xba I site (already blunt-ended) of pCAT-Basic



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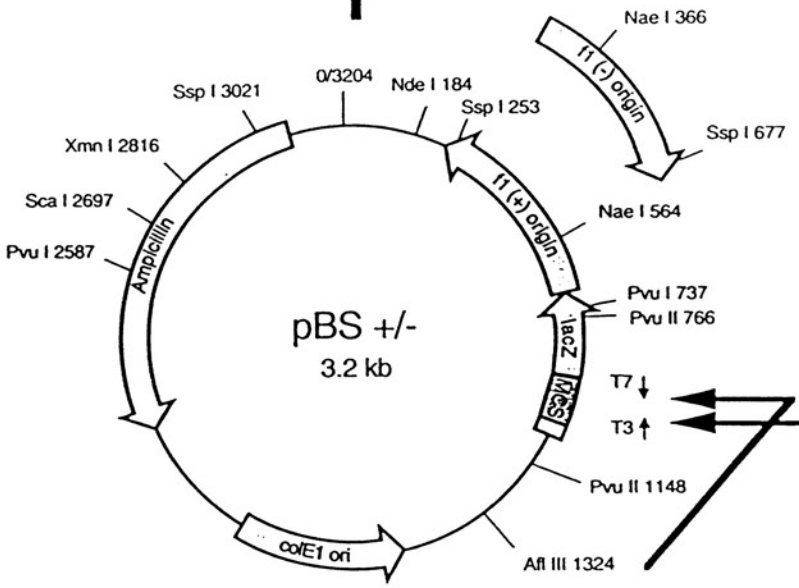
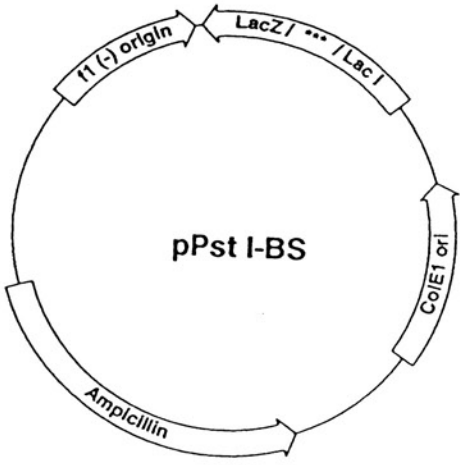



: cut with Nde I / EcoR I
: Klenow 'fill in'
: ligate into Sma I site
of pBS(-)



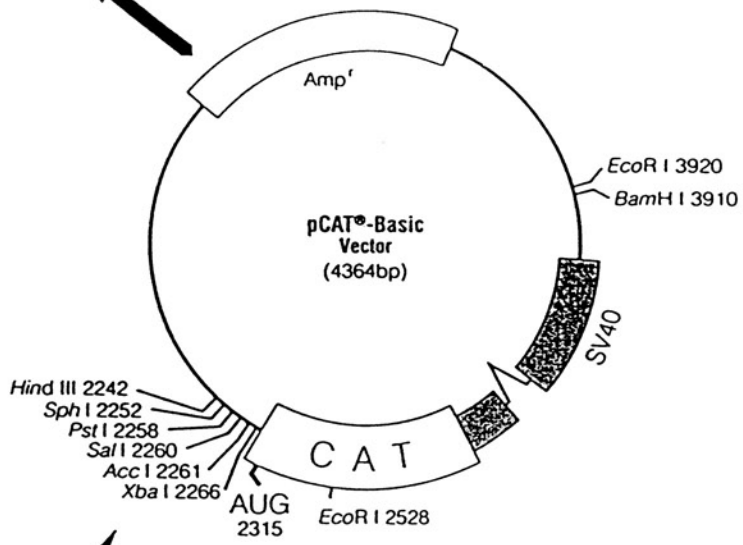

*** Pst I fragment of
VZV Kpn I 'A' fragment

: cut with BamH I
: ligate into BamH I site of pBS(-)

pDp503

contained 3.08 Kb BamH I
fragment of β-galactosidase

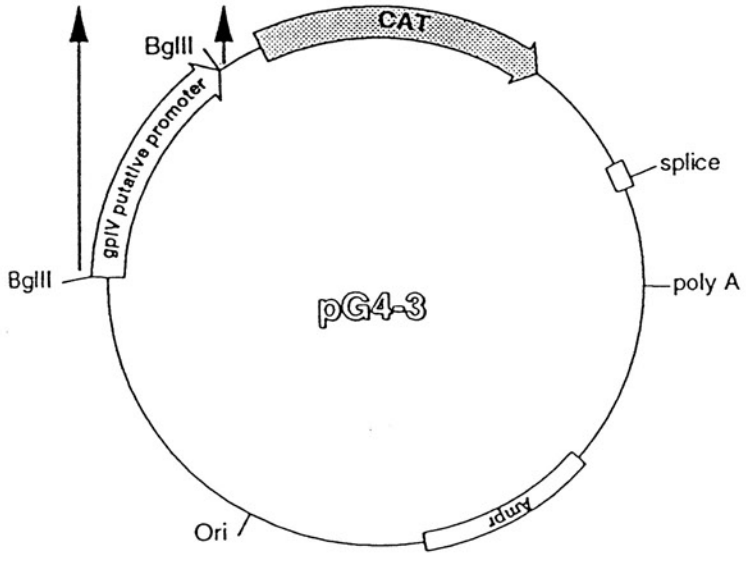
pgpIV-CAT




-cut with Bgl II

-Klenow 'fill-in'

-ligate into Xba I site (already blunt-ended) of pCAT-Basic



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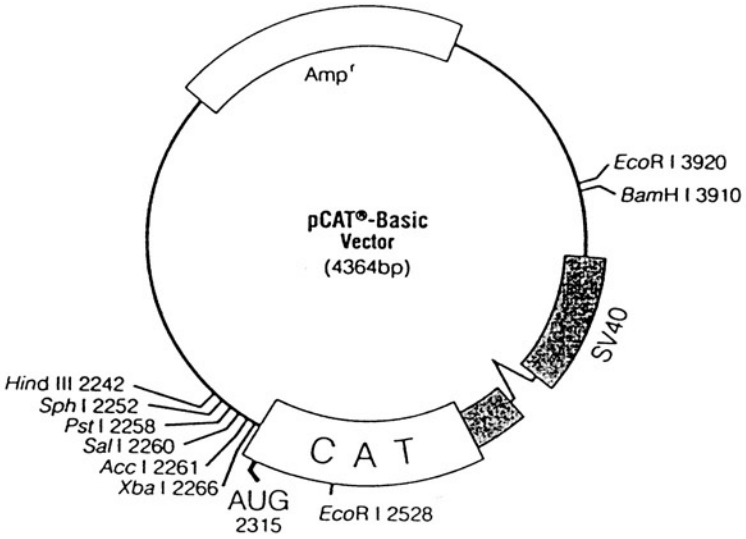
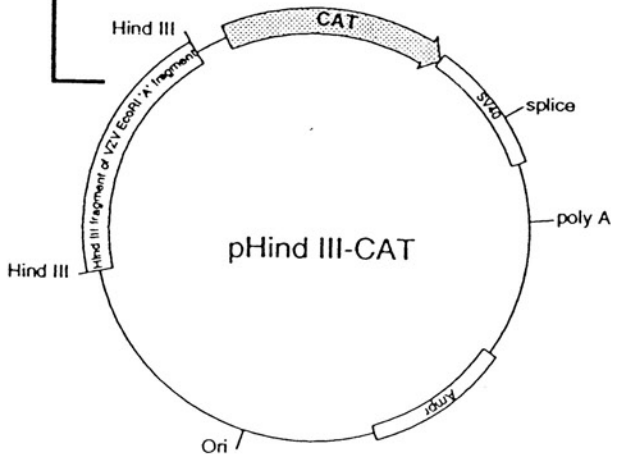
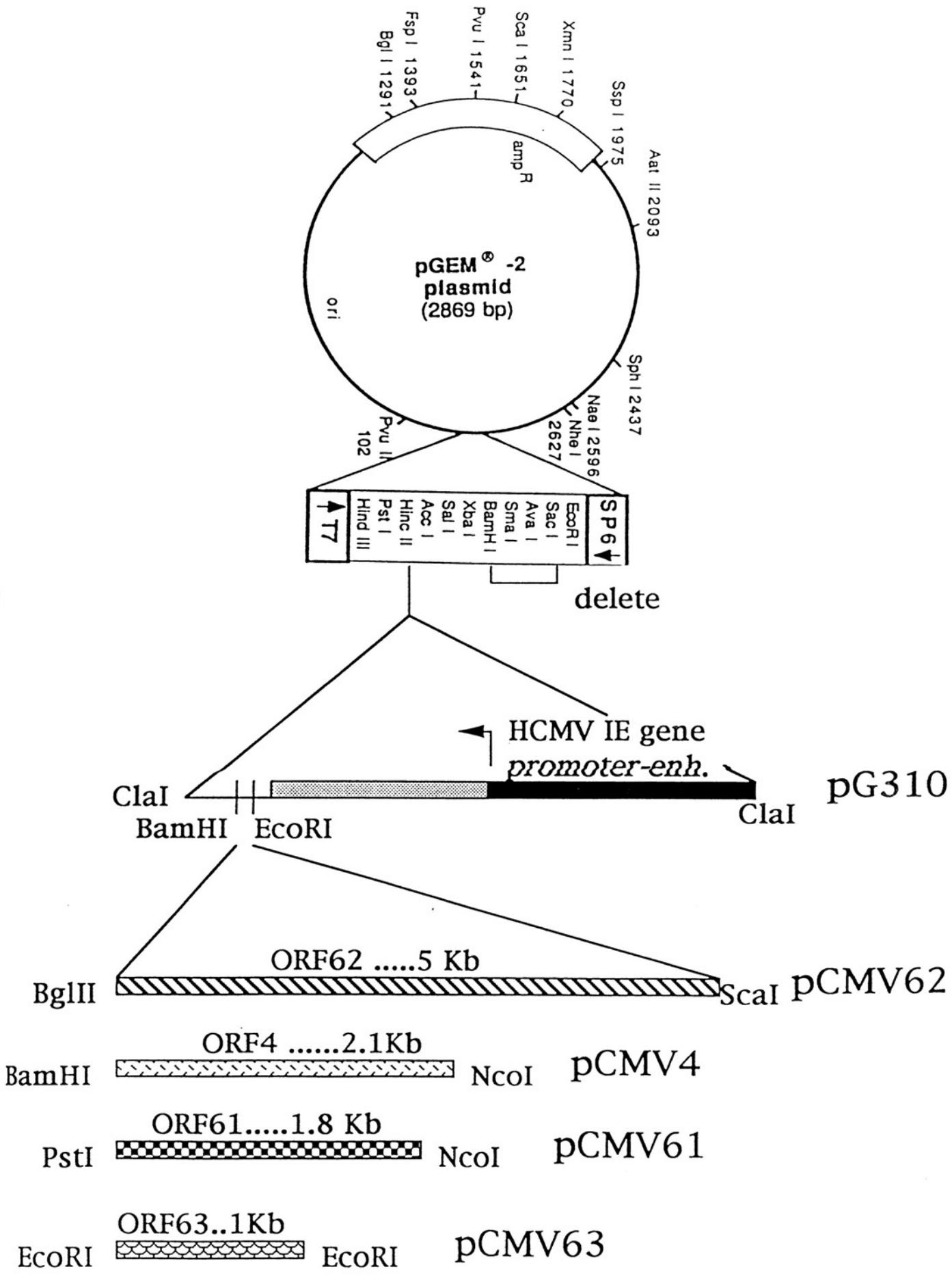



: cut with Mlu I
: blunt end with T4 polymerase and 4dNTP
: ligate into Xba I site (already blunt-ended) of pCAT-Basic

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T7
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Pst I
Hinc II
Acc I
Sal I
Xba I
BamH I
Sma I
Ava I
Sac I
EcoR I
SP6
delete
HCMV IE gene
promoter-enh.
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BamHI
EcoRI
ClaI
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BglII
ScaI
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ORF4 ......2.1Kb
BamHI
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EcoRI
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