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**Analysis of DNA sequences which regulate the transcription of herpes simplex virus immediate early gene 3: DNA sequences required for enhancer-like activity and response to trans-activation by a virion polypeptide**

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**ABSTRACT**

The far upstream region of herpes simplex virus (HSV) immediate early (IE) gene 3 has previously been shown to increase gene expression in an enhancer-like manner, and to contain sequences which respond to stimulation of transcription by a virion polypeptide, Vmw65. To analyse the specific DNA sequences which mediate these functions, sequential deletions from each end of the far upstream region were made. The effects of the deletions on transcription in the absence or presence of the Vmw65 were measured by use of a transient expression assay. The enhancer-like activity was due to three separable elements, whereas two additional DNA regions were involved in the response to Vmw65. One of the responding elements corresponded to an AT-rich consensus (TAATGARATTC, where R=purine) present in all IE gene far upstream regions, and the other was a GA-rich sequence also present in IE genes 2 and 4/5. The TAATGARATTC element could mediate responsiveness to Vmw65 but it was fully active only in the presence of the GA-rich element. The GA-rich element was unable to confer a strong response alone but could activate an otherwise nonfunctional homologue of TAATGARATTC.

**INTRODUCTION**

Herpes simplex virus (HSV) gene expression is temporally regulated and can be divided into three phases, immediate early (IE), early, and late (1-4). The five IE genes are transcribed shortly after infection, with no requirement for de novo protein synthesis (5,6), and IE gene expression is thought to be repressed at the onset of early gene expression. In situations where repression of IE transcription is prevented, large amounts of IE mRNAs accumulate (3,4,6,7,8), suggesting that IE genes can be transcribed very efficiently under certain circumstances. The efficient transcription of the IE gene family is associated with the presence of strong promoters upstream of their mRNA 5' termini, and in the case of IE gene 3 an enhancer-like element

is located at a far upstream position, between nucleotides -174 and -332 (9-15). Transcription of IE genes is further stimulated by a trans-acting virion component (9,10,11,13,14,16) which is located outside the viral nucleocapsid (17). It has recently been shown that cotransfection of the gene which encodes the virion component can also stimulate IE transcription (16). A virus structural protein, Vmw65, was identified as the trans-acting factor since this was the only polypeptide encoded by the smallest cloned fragment (represented by plasmid pMCl) which was active in the cotransfection assay (16).

The far upstream sequence requirement for stimulation by Vmw65 has been characterized for IE genes 1 and 2 (10,14), 3 (9,10,11,18,19) and 4/5 (19). A consensus sequence, TAATGARATTC (where R represents purine), located at far upstream positions in all HSV IE genes, is a major conserved determinant of the sequence domain which mediates the response to Vmw65 (10,18,20, 21,22). Functional analysis of deletion mutants in the far upstream region of IE gene 4/5 has indicated that the TAATGARATTC-like sequence is an important element in the response (19). A similar conclusion was reached by Kristie and Roizman (14), but both of these functional studies concluded that additional DNA elements are necessary to obtain maximum stimulation.

This paper describes a detailed analysis of the sequence elements of IE gene 3 responsible for the enhancer-like function and for stimulation by Vmw65. Deletions of the far-upstream region were constructed and their activities tested in a transient expression assay, in the presence or absence of a cotransfected plasmid, pMCl, which encodes the trans-acting virion polypeptide Vmw65.

### MATERIALS AND METHODS

#### Plasmids

The basic chimaeric plasmids containing the HSV thymidine kinase (TK) gene used in this study are shown in figures 1 and 2. Plasmid pTKN3, which lacks a far upstream regulatory region, has been described previously (11) and lacks an IE far upstream region. Plasmids pTKN15 and pTKN16 were also described

previously and contain the IE gene 3 far upstream region in normal or opposite orientations (13). Plasmids pS12TKU and pS14TKU are identical to pS12TK and pS14TK (19), except that the HSV and TK sequences are carried in pUC9 rather than pAT153. Plasmid pMC1 (not shown) was used as a source of Vmw65, as described by Campbell *et al.* (16).

Two sets of plasmid deletions (the pTKN15 and pTKN16 series) were prepared using standard cloning procedures, and their construction is diagrammed in figure 1. After linearization with HindIII, pTKN15 and pTKN16 were digested with Bal 31 nuclease for various times, and self-ligated in the presence of SalI oligonucleotide linkers (GGTCGACC). The extent of deletion and presence or absence of SalI linkers was later confirmed by DNA sequencing. All plasmids constructed in this study were sequenced to ensure the fidelity of the construction. DNA sequencing was performed after cloning appropriate DNA fragments into phage M13mp9, using the dideoxy chain terminator method as previously described (23,24).

Plasmids based on IE gene 4/5 used pS14TKU as a starting point. This contains the IE gene 4/5 promoter sequences fused to the TK structural gene, but it lacks the far upstream region which responds to stimulation by Vmw65 (19). Small DNA fragments from IE gene 3 were cloned into the PstI site (previously converted to XhoI) in the multilinker of pS14TKU, thereby placing them upstream of nucleotide -315 (relative to IE gene 4/5). The fragments selected for insertion were as follows (all numbering relative to IE gene 3): -275 to -332 and -275 to -298 were, respectively, SalI/EcoRI and SalI/SphI fragments from pTKN16-62, (which has a SalI linker at the deletion endpoint, -275), and -275 to -318 was the SalI/Sau3AI fragment from pTKN15-12 (which also has a SalI linker at the deletion endpoint, -318). The nucleotide sequence of these inserted fragments is shown in figure 2.

#### Transfection of cells

Plasmid DNAs were coprecipitated with calcium phosphate and transfected into BHK cells. Precipitates were formed as previously described (25), except that all mixing was performed

by vortexing. For most experiments 1µg of plasmid DNA containing the TK gene under IE control (IE-TK plasmid) was transfected into 10<sup>5</sup> BHK cells in a well (15mm diameter) of a microtitre plate (Nunclon), together with 1µg of plasmid pUC9 or 1µg of plasmid pMC1 (16). After 3h, transfected cells were treated with 25% dimethyl sulfoxide (26), and incubation continued at 38.5° for a further 16h to 18h. Cell extracts were then prepared for TK assays, as described previously (11). Increasing the amount of IE-TK plasmid to 2µg gave a two-fold increase in TK activity, while decreasing the amount of IE-TK plasmid to 0.5µg caused a two-fold decrease in TK activity, demonstrating a linear relationship between IE-TK plasmid DNA and enzyme production. In many experiments, 0.5µg of plasmid pLW2 was cotransfected with the IE-TK plasmid plus pMC1 or pUC9, to standardise the efficiency of transfection and expression. Plasmid pLW2 contains the chloramphenicol acetyl transferase (CAT) gene under the control of promoter sequences of IE gene 5 from HSV-2, as described by Gaffney et al. (27).

### TK Assays

TK assays of cytoplasmic extracts were carried out as described by Cordingley et al. (11). To standardize the results, plasmids pTKN15 or pTKN16 as positive controls, and plasmid pTKN3 as a negative control lacking an IE far upstream region, were included in all transfections. The average values for TK expression from pTKN15 and pTKN16 were determined from many experiments, and the actual value for pTKN15 or pTKN16 and other plasmids tested in a given experiment were corrected to the average by using the same factor. Background TK activity, from cells transfected with pUC9 or pMC1 alone, was subtracted prior to calculating the correction factor.

### CAT assays

CAT assays were carried out on cytoplasmic extracts as described by Gaffney et al. (27). The CAT activity in cell extracts was used as a standard to correct TK values, although in practice this correction did not significantly alter the results.

**RESULTS**

**Boundaries for enhancer and regulatory sequences of IE gene 3**

Plasmid pTKN15 contains the far upstream regulatory element of IE gene 3 (-174 to -332) joined to the promoter of IE gene 3 (+27 to -108) which is linked, via a BglIII site 54bp upstream from the first ATG, to the HSV TK coding sequences (figure 1). Plasmid pTKN16 has an inversion of the IE gene 3 far upstream region with respect to the promoter and TK coding

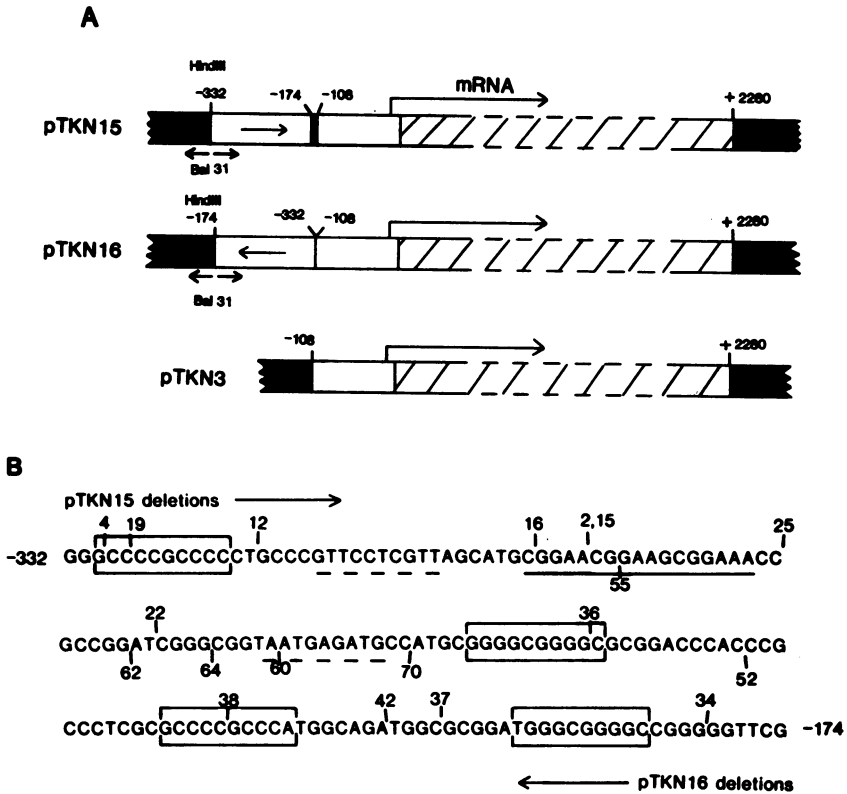


Figure 1. The pTKN15 and pTKN16 deletion series. A: Vector (pAT153) DNA is represented by filled areas, the TK coding region by hatched areas, and IE gene 3 upstream sequences by clear areas. The HindIII site from which Bal31 deletions commenced is marked for the two plasmids, and pTKN3, which lacks the far upstream region, is also shown. B: The DNA sequence of the far upstream region, with deletion endpoints for the pTKN15 (above) and pTKN16 (below) series indicated. The GA-rich region is underlined, and the sequence elements which are related to the TAATGARATTC consensus are underscored with broken lines. Sequences which direct the binding of Sp1 (28) are boxed.

sequences. Two groups of plasmids based on deletions were constructed from pTKN15 and pTKN16 (figure 1). The pTKN15 series sequentially removes DNA sequences from -332 to -174 in relation to the mRNA 5' terminus, while the pTKN16 deletion series removes the DNA sequences in the inverted order, -174 to -332. Therefore, the two groups of deletions should not reduce the normal promoter function (-108 to +27), but sequentially delete DNA sequences required for enhancer-like activity and response to the trans-acting virion polypeptide, Vmw65 (12,13). The properties of the deleted plasmids were measured by transient expression of TK activity following transfection of chimaeric plasmids into BHK cells. For each plasmid tested, monolayers of BHK cells were transfected with the chimaeric plasmid only, giving a value named here 'promoter' activity, or with the chimaeric plasmid plus pMCl (16) to test the stimulated level of expression due to Vmw65. After incubation at 38.5° for 16h to 18h, the TK activity of cell lysates was determined. Plasmid pTKN3, which lacks the far upstream region, was included in these experiments to determine the level of TK activity due to the near promoter sequences of IE gene 3.

The presence of DNA sequences representing the far upstream region of IE gene 3 (-174 to -332) in both pTKN15 and pTKN16 increased the apparent promoter activity 10-fold compared to the absence of these sequences (pTKN3) (tables 1 and 2). This result is in agreement with previous reports in which the increase in promoter activity was characterised as an enhancer-like activity. For the pTKN15 series (table 1), promoter activity was reduced by deletion from -327 (pTKN15-19) to -318 (pTKN15-12). Promoter activity was unchanged for deletions between -318 (pTKN15-12) and -293 (pTKN15-2, or -15), but was reduced approximately 2-fold by deletion of sequences between -293 (pTKN15-2, or -15) and -279 (pTKN15-25) or -272 (pTKN15-22). No further reduction in promoter activity occurred as the deletion extended to -198 (pTKN15-37), but after removal of sequences between -198 and -179 (pTKN15-34), no effect of the remaining portion of the far-upstream region could be detected.

Deletion of DNA sequences in the opposite order, -174 to -332, in the pTKN16 series also caused incremental reductions in

**Table 1. Transient expression of TK by pTKN15 plasmids**

Plasmid	Endpoint	TK Activity <sup>a</sup>		
		Promoter <sup>b</sup>	Total <sup>c</sup>	Stimulation <sup>d</sup>
pTKN15	-332	37	201	5.4 (7) <sup>e</sup>
pTKN15-4	-329	31	191	6.2 (2)
pTKN15-19	-327	33	192	5.8 (2)
pTKN15-12	-318	22	143	6.5 (4)
pTKN15-16	-297	23	118	5.1 (4)
pTKN15-2	-293	25	93	3.7 (4)
pTKN15-15	-293	27	100	3.7 (4)
pTKN15-25	-279	10	33	3.3 (4)
pTKN15-22	-272	15	52	3.5 (4)
pTKN15-36	-240	14	15	1.1 (2)
pTKN15-38	-214	11	11	1.0 (2)
pTKN15-32	-202	10	10	1.0 (2)
pTKN15-37	-198	10	10	1.0 (2)
pTKN15-34	-179	5	5	1.0 (2)
pTKN3	-174	4	4	1.0 (7)

- a - TK activities are expressed as cts/min per min of assay per  $\mu\text{g}$  protein per  $\mu\text{g}$  plasmid  
b - Cells were transfected with  $1\mu\text{g}$  of plasmid plus  $1\mu\text{g}$  of pUC9  
c - Cells were transfected with  $1\mu\text{g}$  of plasmid plus  $1\mu\text{g}$  of pMC1  
d - Stimulation represent the increase in expression relative to the pTKN3 value  
e - The number of separate determinations is shown in brackets

the promoter activity (table 2). It decreased 2-fold after deletion of DNA sequences between -174 (pTKN16) and -230 (pTKN16-52), but was unaffected by extension of the endpoint to -275 (pTKN16-62). Further deletion to -292 (pTKN16-55) caused a

**Table 2. Transient expression of TK by pTKN16 plasmids**

Plasmid	Endpoint	TK Activity <sup>a</sup>		
		Promoter	Total	Stimulation
pTKN16	-174	40	136	3.4 (4)
pTKN16-52	-230	23	97	4.2 (3)
pTKN16-70	-254	22	83	3.8 (4)
pTKN16-60	-264	23	66	2.9 (4)
pTKN16-64	-269	23	54	2.4 (3)
pTKN16-62	-275	20	50	2.5 (4)
pTKN16-55	-292	14	14	1.0 (4)
pTKN3		4	4	1.0 (7)

- a - Presentation of data as described in the legend to table 1

small reduction in promoter activity, and the remaining enhancer-like activity was lost upon deletion of sequences between -292 (pTKN16-55) and -332 (pTKN3).

Therefore, from the two sets of deletions, no single DNA element responsible for enhancer activity was detected in the far upstream region. It appeared that the 10-fold increase in promoter activity was due to the cumulative effect of three separate elements, each giving a stimulation of approximately 2-fold. The pTKN15 and pTKN16 deletion series were consistent in the location of these elements, which are contained within nucleotides -327 to -292, -293 to -275 and -198 to -174.

The level of stimulated expression of TK activity, measured by co-transfection with plasmid pMCl, was unchanged in the pTKN15 series as DNA was deleted from -332 (pTKN15, 5.4-fold) to -297 (pTKN15-16, 5.1-fold) (table 1). Deletion of DNA sequences between -297 (pTKN15-16) and -293 (pTKN15-2, or -15) resulted in a decrease of stimulation from 5.1-fold to 3.7-fold, and further removal of sequences to -272 (pTKN15-22, 3.5-fold) had no extra effect on the stimulated level of TK activity. Deletion of sequences to -240 (pTKN15-36) abolished stimulation of TK activity, thereby localizing an upstream boundary between -272 and -240 for DNA essential for the response. The sequence TAATGAGATGC (-265 to -255) in this region strongly resembles the consensus, TAATGARATTC, that occurs in the regulatory region of all HSV IE genes.

In the pTKN16 series, the level of stimulation of TK activity was not affected as DNA was deleted from -174 (pTKN16) to -254 (pTKN16-70), but a decrease in stimulation resulted from deletion of sequences between -254 (pTKN16-70) and -264 (pTKN16-60) (table 2). The degree of stimulation by pMCl remained unchanged by further removal of nucleotides to -275 (pTKN16-62), but no response was observed when the deletion extended to -292 (pTKN16-55). Thus, in the inverted orientation, DNA sequences with an endpoint between -275 and -292 were essential for stimulation, and the TAATGAGATGC element only added to the magnitude of the effect.

Further analysis of IE gene 3 sequences between -275 and -332.

The properties of plasmids pTKN16-60, -62 and -64 were



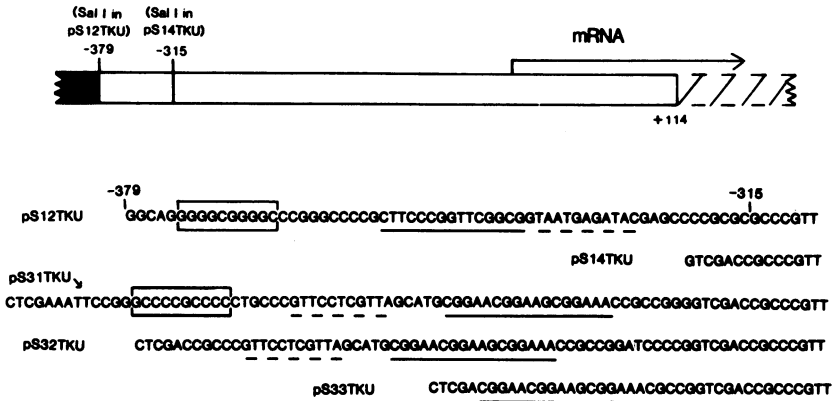


Figure 2. Plasmids derived from IE gene 4/5. The basic plasmid design is shown, using the same format as in figure 1. The sequences upstream of -315 in the chimaeric plasmids are also presented, annotated as in figure 1.

unexpected, since the residual nucleotides from the far upstream region (-275 to -332) do not contain an obvious TAATGARATTC consensus sequence. Previous workers have noted the related element TAACGAGGAAC, which is present in an inverted form (relative to IE gene 3) between nucleotides -303 to -313 (18), but this was found not to be active in functional assays (19). Various regions of the DNA between -275 and -332 were therefore cloned into plasmid pS14TKU, in which the TK structural gene is under the control of the HSV-1 IE gene 4/5 promoter (figure 2). The plasmid constructions shown in figure 2 contain isolated elements from IE gene 3 in a position equivalent to the natural far upstream element which mediates stimulation of IE gene 4/5 transcription by Vmw65 (Preston *et al.*, 1984). As shown in table 3, pS14TKU failed to respond detectably to pMC1, whereas pS12TKU, which contains all the far upstream region of IE gene 4/5, was stimulated 5.8-fold. Plasmid pS31TKU, which contains IE gene 3 sequences from -275 to -332 cloned at position -315 relative to IE gene 4/5, was stimulated 4.3-fold, as expected from the properties of pTKN16-60, -62 and -64. The element between -275 and -318 (with respect to IE gene 3) was also able to mediate stimulation, as shown by the properties of pS32TKU (4.8-fold stimulation). In contrast, the region between -275 and

Table 3. Transient expression of TK by IE gene 4/5-TK plasmids

Plasmid	TK Activity <sup>a</sup>		
	Promoter	Total	Stimulation
pS12TKU	46	266	5.8 (4)
pS14TKU	48	46	1.0 (4)
pS31TKU	59	254	4.3 (4)
pS32TKU	61	294	4.8 (4)
pS33TKU	60	96	1.6 (4)

a - Presentation of data as described in the legend to table 1

-298, which lacks the TAACGAGGAAC element, conferred only a low level of stimulation, generally less than 2-fold, as shown by the poor response of pS33TKU. Taking these results together with the properties of the plasmids derived from pTKN16 (table 2), it appears that nucleotides between -275 and -318 can confer a significant response to Vmw65, but that subdivision of this region abolishes activity.

#### DISCUSSION

The far-upstream region of IE gene 3 (-174 to -332) subjected to deletion analysis in this study was previously shown to increase the TK activity of a linked TK gene and to contain sequences which respond to stimulation of expression by a HSV virion polypeptide, Vmw65. Both effects were shown to be independent of orientation, and to act at the transcriptional level. The DNA sequences which increase promoter activity were designated enhancer-like because the effect was not dependent upon orientation, it acted at a distance, and showed host cell specificity (12,13).

Consistent with previous results, the enhancer-like activity of the IE gene 3 far-upstream regulatory region caused a 10-fold increase in TK activity in each orientation. From the combined data derived from the pTKN15 and pTKN16 deletion series, we assign enhancer-like activity to three separate elements of the IE gene 3 far upstream region, contained within nucleotides -327 to -292, -293 to -275, and -198 to -174. Each enhancer element imparts approximately a 2 to 3-fold increase in

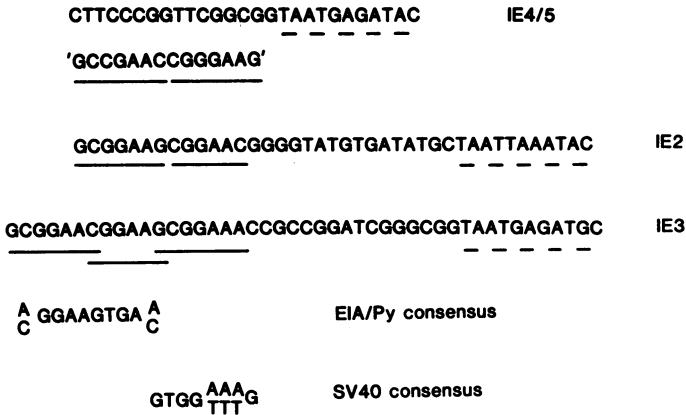


Figure 3. Comparison of IE gene 3 responder elements with other genes. Sequences in functionally important far upstream regions of IE genes 2, 3 and 4/5 are shown, with maximum alignment for homology to the GA-rich region of IE gene 3. Note that the proposed homology in IE gene 4/5 is present in the inverted orientation, drawn under the actual orientation. Short sequences related to the motif GCGGAAG are underlined, and the TAATGARATTC consensus is underscored with broken lines. Sequences thought to be important in the enhancers of the adenovirus EA, polyoma virus and SV40 early genes are aligned with the best matches in IE gene 3.

promoter activity, and the effect of the separate elements was, to a first approximation, additive. The element between -198 and -174 is almost certainly a subset of a sequence, located between -197 and -135, which has previously been shown to augment expression from the IE gene 3 promoter (14).

The two functional regions at the extremities of the far upstream region of IE gene 3 have recently been shown to contain binding sites for a cellular transcription factor known as Spl (28). This factor stimulates transcription by binding to specific sequences whose core element is CCGCCC or its complement GGGCGG (29). The hexanucleotide functions in the HSV TK and SV40 early promoters, and it can act in a manner which is independent of orientation or distance (30-34). It is likely, therefore, that Spl binding is at least partially responsible for the effects of the sequences between -327 to -292 and -198 to -174. The role of Spl in the HSV IE gene 3 far upstream region must be more complicated, because it can bind at two

other locations (-252 to -235 and -224 to -204 [28]) which did not contribute significantly to promoter activity in the experiments described here. The third component with enhancer-like activity, located between -293 and -275, contains the sequence GCGGAAAC, which resembles the SV40 enhancer 'core' GTGGAAAAG (35), and an element CGGAAGCGGAA which is homologous to an important motif in the adenovirus E1A and polyoma virus enhancers (36,37) (figure 3). The finding that enhancer function is mediated by multiple small units has also been made in polyoma virus, SV40, human cytomegalovirus and bovine papilloma virus (36-41).

The DNA regions involved in the stimulation of expression by Vmw65 were distinct from those responsible for enhancer activity. It appears, therefore, that these two responses are mediated by different mechanisms, ruling out the hypothesis that Vmw65 acts by exaggerating the enhancer effect. The far upstream regulatory domain is thus composed of many individual units which augment transcription from the IE gene 3 promoter by different and, presumably, additive mechanisms.

The deletion mapping approach designed to locate precisely the DNA sequences which respond to Vmw65 gave unexpectedly complex results. Deletions carried out on the normal orientation of the far upstream region suggested that the TAATGAGATGC sequence was active alone but that upstream flanking sequences, especially the GA-rich element between -297 and -272, was necessary for the full effect. This conclusion agrees with a similar functional analysis of IE gene 4/5 (19) although the important flanking sequences were previously thought to be GC-rich regions. Studies by Kristie and Roizman (14) also suggested the importance of sequences close to the TAATGARATTC consensus in IE gene 2. As shown in figure 3, similar GA-rich elements are found upstream from the TAATGARATTC consensus in functionally active regions of IE genes 2, 3 and 4/5, although the IE 4/5 copy is in the inverted orientation.

When deletions were made in the opposite orientation of the IE gene 3 far upstream region (the pTKN16 series), the data apparently suggested an independent, additive role for the TAATGAGATGC and GA-rich elements. Further examination, however,








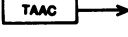
<u>Plasmid</u>	<u>Structure</u>	<u>Response to V<sub>mw</sub>65</u>
pS12TKU		5.8
pTKN15		5.4
pS31TKU		4.3
pTKN15-16		5.1
pS32TKU		4.8
pTKN15-22		3.5
pS33TKU		1.6
pTKN16-52		1.0

Figure 4. Diagrammatic summary of the effects of IE gene 3 far upstream sequence elements on the response to V<sub>mw</sub>65 (fold stimulation). The combinations of the Spl-binding region (Sp1), the GA-rich region (GA), TAATGAGATAC or TAATGAGATGC (TAAT) and TAACGAGGAAC (TAAC), described in this paper, are shown.

revealed that the GA-rich element functioned poorly alone, and required the presence of the weak homologue of TAATGARATTC, TAACGAGGAAC, for full activity. In turn, TAACGAGGAAC had no activity alone, as shown by the properties of pTKN16-55, and did not contribute to the effect of the complete far upstream region, since pTKN15-16 was stimulated normally. The failure of the TAACGAGGAAC element to affect the response to V<sub>mw</sub>65 was also found in the analysis of IE gene 4/5 (19).

The activities of the various elements between -254 and -332 in IE gene 3 are summarised in figure 4. For this IE gene, and probably also 2 and 4/5, the TAATGARATTC consensus is the most important unit necessary for response to the virion component, but full activity requires the proximity of a GA-rich region. In IE gene 3, TAACGAGGAAC is normally unimportant but can be functional in the absence of TAATGAGATGC if flanked by the GA-rich region. The mechanism by which V<sub>mw</sub>65 acts to stimulate IE transcription is therefore likely to be more complex than previously suspected and may involve the

interaction of this polypeptide with many cellular factors.

A more extensive analysis will be necessary to determine the important nucleotides of the GA-rich element. The motif GCGGAAG, or sequences similar to it, is repeated two or three times in HSV IE genes 2, 3 and 4/5 (figure 3). The longer sequence CGGAAGCGGAA in IE gene 3 is similar to the consensus which is functionally important in adenovirus E1A and polyoma virus enhancers (36,37). The way in which the GA-rich element exerts its effects is unclear at present. A direct alteration of the structure of TAATGARATTC elements by modification of DNA topology seems unlikely, since no extreme structural features, for example a propensity to form Z-DNA or the presence of inverted repeats, are evident. It is more probable that this element forms a binding site for one or more cellular polypeptides, and that these influence the activity of the TAATGARATTC consensus sequence.

The studies presented here show that many different sequence elements contribute to the positive effects of the far upstream region on transcription of IE gene 3. Some of these interact solely with cellular components, whilst others are active only in the presence of Vmw65. Whatever their individual characteristics, the overall effect of these elements appears to be the rapid and efficient production of IE mRNA 3.

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