REGULATION OF HSV-1 GENE EXPRESSION BY ICP4

THE FUNCTION OF DNA BINDING IN THE REGULATION OF HSV-1 GENE EXPRESSION BY ICP4

By

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ABSTRACT

Herpes simplex virus is an important model in the study of temporally regulated gene expression in eukaryotic cells. Three classes of genes - immediate early, early, and late - are sequentially expressed during the course of lytic infection. One immediate early gene product, ICP4, is required for transactivation of most early and late genes; it is also implicated in repression of immediate early gene expression. ICP4's mechanism(s) of action is/are not yet understood; although ICP4 binds to specific sequences of DNA, whether this is necessary for transregulation by ICP4 is not clear. To gain a better understanding of how the ability of ICP4 to bind DNA relates to its transregulatory activities, I introduced ICP4 binding sites into a simple model promoter within the viral genome. Two sets of construct were made in which an ICP4 binding site (or mutant site) was placed either downstream or upstream of a TATA box, reproducing the spacing found in (i) the native *ICP4* promoter and (ii) the native *ICP0* promoter (respectively). The promoter of HSV-1 *UL24b* (a nonessential gene in tissue culture) was replaced with these model promoters and levels of transcripts accumulating from these constructs during lytic infection assayed by primer extension. I found that an ICP4 site placed either upstream or downstream of a TATA box shifted kinetics of expression from E/leaky L to true L. Neither the strength of the TATA box nor the helical orientation of the ICP4 binding site with respect to the TATA box affected this result.

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A or dATP	deoxyadenosine 5'-triphosphate
Ad2 MLP	Adenovirus type 2 major late promoter
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
bp	base pair
C or dCTP	deoxycytidine 5'-triphosphate
cm	centimetre
cpm	counts per minute
CTP	cytidine 5'-triphosphate
DNA	deoxyribonucleic acid
E	early
EDTA	ethylenediaminetetraacetate
G or dGTP	deoxyguanosine 5'-triphosphate
gC	glycoprotein C
gD	glycoprotein D
GST	glutathione S-transferase
GTP	guanosine 5'-triphosphate
HSV-1	herpes simplex virus type 1
ICP	infected cell polypeptide
IE	immediate early
kbp	kilobase pair
kD	kiloDaltons

L	late
M	molar
MCS	multiple cloning site
µg	microgram
mg	milligram
µL	microlitre
mL	millilitre
moi	multiplicity of infection
mm	millimetre
mM	millimolar
mRNA	messenger ribonucleic acid
ng	nanogram
nm	nanometre
OD	optical density
PCR (LM-)	polymerase chain reaction (ligation mediated-)
pg	picogram
pmol	picomole
RNA	ribonucleic acid
T or dTTP	deoxythymidine 5'-triphosphate
TF	transcription factor
tk	thymidine kinase
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
ts	temperature sensitive
UL	unique long

I. INTRODUCTION

1.1. Regulation of Expression of Eukaryotic Genes

The coordinated regulation of gene expression is a fundamental process of all cells. In particular, correct regimentation of expression is essential to multicellular organisms for development, maintenance of progenitor cells, and cell differentiation. A primary point of control of gene expression occurs at the level of transcriptional initiation. This is mediated by the interaction of various transcription factors with cis-acting sequences located in a gene's promoter. These factors can be classified as either general (or basal) or gene- (or sequence-) specific transcription factors.

Eukaryotic RNA polymerase II cannot initiate transcription on its own; eight general transcription factors (TFs IIA, IIB, IID, IIE, IIF, IIH, IIJ, and IIS) have been identified whose activities are required to achieve basal levels of transcription (for reviews, see Drapkin et al., 1993; Kadonaga, 1990; Roeder, 1991; Zawel and Reinberg, 1992). In many class II promoters, transcriptional initiation is centred around a cis-acting sequence, termed the TATA box, which is located about 30 nucleotides upstream of the transcriptional start. TFIID, the only general transcription factor known to bind specific sequences of DNA, begins formation of the pre-initiation complex by binding to the TATA element. TFIID is a large multisubunit protein complex which includes the 38 kD TATA binding protein (TBP) and a number of TBP-associated factors (TAFs). Binding of

TFIIA stabilizes the TFIID-DNA complex. TFIIB then binds the complex, providing a scaffolding for attachment of RNA polymerase II. RNA polymerase II is recruited to the forming complex by TFIIF. TFIIE, TFIIH, and TFIIJ then associate with the pre-initiation complex, in that order. The precise mechanisms by which these factors interact to initiate transcription is not yet clear. Transcription factors IIF and IIS are required for efficient of elongation of the transcript.

Transcription by RNA polymerase II can be activated or repressed by a set of sequence-specific DNA binding proteins (for reviews, see Kadonaga, 1990; Mitchell and Tjian, 1989; Morimoto, 1992; Ptashne and Gann, 1990). The cis-acting sequences to which these proteins bind are generally found within several hundred base pairs of a gene's transcriptional start, but may be situated much further away (1-30 kbp upstream) or be located 3' of, or within, the gene's coding sequences. The combination and arrangement of different cis-acting sequences and resultant interactions with positive and negative activators confer on each gene its own unique regulatory scheme. Transcription activators are modular, generally including separable DNA-binding and activation domains (for review, see Mitchell and Tjian, 1989). Four varieties of motifs important to sequence-specific DNA binding have been characterized: zinc fingers (two types), helix-turn-helix (including the homeodomain and POU domain subcategories), leucine zippers, and helix-loop-helix. Three types of regions involved in transactivation have also been identified: glutamine-rich, proline-rich, and acidic domains. The mechanism by which these factors act is unclear; they are thought to interact with the general transcription apparatus via protein - protein

interactions mediated by sequence-specific DNA binding and a third set of regulatory factors known as coactivator molecules (for review, see Gill and Tjian, 1992). A number of TBP-associated factors have been found to have coactivator activity. Not surprisingly, distinct activators appear to recognize different coactivators; it is possible that members of each activator type vary in their affinity for different coactivators. This would introduce another level of control into programmes of gene regulation. Two suggestions have been forwarded regarding how coactivators function: (i) They may serve as bridges between activators and the general transcription machinery. (ii) They may convey the effect of an interaction between an activator and one of the general transcription factors to the rest of the initiation complex. These two possibilities are not mutually exclusive as different coactivators may function by distinct mechanisms.

Much of our current understanding of eukaryotic transcription has been gained from the study of three families of DNA viruses - adenoviridae, papoviridae, and herpesviridae - which utilize host cell RNA polymerase II and other enzymes in the course of their replicative cycle. Specifically, herpes simplex virus, of the herpesviridae family, has provided a relatively simple model of temporally regulated gene expression.

1.2. HSV-1 - A General Description

Herpes simplex virus type 1 (HSV-1) is a member of the alphavirinae subfamily of the herpesviridae family. Members of this subfamily are characterized by a variable host range, a short reproductive cycle, efficient

destruction of lytically infected cells, and the ability to establish latency primarily in sensory ganglia (reviewed in Roizman, 1990) Viral DNA is found in a toroid-shaped electron dense core (for review, see Roizman, 1990; Roizman and Sears, 1990). This core is contained in an icosahedral capsid consisting of 150 hexameric and 12 pentameric capsomeres; five capsomeres are located on each edge of the icosahedron. Surrounding the capsid is the tegument, which is asymmetrically distributed and varies in quantity. The envelope surrounding the tegument is apparently derived from virus-modified cellular membranes; numerous glycoprotein spikes are embedded in the envelope.

The HSV-1 genome contains 152 kbp of linear, double-stranded DNA, and consists of covalently linked long (L) and short (S) segments (Fig. 1.1; reviewed by Roizman and Sears, 1990). Both the L and S segments are composed of a unique sequence flanked by inverted repeats. The L and S segments both occur in either orientation with respect to the other segment; thus, four isomeric forms of the HSV-1 genome exist, all of which occur at equal frequencies. The HSV-1 genome encodes approximately 70 open reading frames. Transcription of each gene is driven from its own promoter; few of the transcripts are spliced, unlike those of two other DNA virus families, adenoviridae and papoviridae (for reviews, see Acheson, 1981; Flint and Broker, 1981; Wagner, 1985).

Figure 1.1. Arrangement of the HSV-1 Genome and Location of Immediate Early Coding Regions

The arrangement of one isomer of the HSV-1 genome is shown in a schematic diagram. The genome consists of a long (L) and a short (S) segment. In each segment, a length of unique sequence (U_L or U_S) is flanked by inverted repeats (IR_L or IR_S, respectively). The long and short segments occur in either orientation with respect to each other; thus four isomeric forms of the HSV-1 genome exist. The approximate locations of the five HSV-1 IE genes (ICP's *0*, *4*, *22*, *27*, and *47*) are shown; directions of transcription and splice sites are also indicated.

ICP0
ICP27
ICP22
ICP4
IRL
UL
IRL
IRS
US
IRS
ICP0
ICP4
ICP47

1.3. HSV-1 Gene Expression - Overview

HSV-1 genes are coordinately expressed in a three-tiered cascade (Honess and Roizman, 1974; Honess and Roizman, 1975; reviewed by Everett, 1987; Roizman and Sears, 1990). Five immediate-early (IE), or α, proteins are expressed first: ICP4, ICP0, ICP27, ICP22, and ICP47 (or Vmw175, Vmw110, Vmw63, Vmw68, and Vmw12, respectively, in an alternative nomenclature system). IE RNAs can be detected one hour post-infection but reach maximal levels after three or four hours; their expression declines after approximately six hours (Harris-Hamilton and Bachenheimer, 1985; Weinheimer and McKnight, 1987). The IE proteins are expressed without requirement for de novo protein synthesis and depend on a virion component, Vmw65 (or VP16 or αTIF) for activation of their expression. Functional IE gene products are essential for expression of the next class of viral genes, the early (E), or β genes. E gene expression peaks approximately six hours post-infection, but can be detected at three hours post-infection (Harris-Hamilton and Bachenheimer, 1985; Weinheimer and McKnight, 1987). E proteins appear to be involved mainly in nucleic acid metabolism, preparatory to DNA replication. DNA replication is initiated soon after the first E proteins are made, followed by expression of the late (L), or γ, genes. L genes require both functional IE proteins and DNA replication for their expression; they may be further sub classified as either leaky L (γ_1) or true L (γ_2), according to the stringency of their requirement for DNA replication prior to expression. L gene products include many virion components.

1.4. Regulation of HSV-1 Gene Expression

Regulation of HSV-1 gene expression appears to occur primarily at the transcriptional level (Godowski and Knipe, 1986; Post et al., 1981; Silver and Roizman, 1985; Weinheimer and McKnight, 1987). Both viral and cellular factors mediate gene regulation through cis-acting sequences. The number of regulatory signals which are required for efficient induction of expression appears to decrease according to the time at which a gene is expressed. Thus, IE promoters are most complex; in addition to a TATA box and cap site, they contain recognition sites for cellular factors (SP1 and/or the CCAAT binding protein) and one to three copies of the TAATGARAT motif (Cordingly et al., 1983; Gaffney et al., 1985; Jones and Tjian, 1985; Mackem and Roizman, 1982a,b; Preston et al., 1984). The virion component Vmw65 induces IE gene expression by formation of a complex with Oct-1 and other cellular factors; activation is mediated through binding of this complex to TAATGARAT (Batterson and Roizman, 1983; Baumruker et al., 1988; Campbell et al., 1984; Cordingly et al., 1983; Gerster and Roeder, 1988; Kristie and Roizman, 1987; McKnight et al., 1987; O'Hare and Goding, 1988; Post et al., 1981; Preston et al., 1988). Four of the five IE gene products appear to be involved in subsequent E and L gene regulation, as well as repression of IE gene expression (see below).

Like IE promoters, E gene promoters contain CCAAT-boxes and/or SP1 binding sites in addition to a TATA box and cap site (Costa et al., 1985; Eisenberg et al., 1985; Everett, 1983, 1984; Jones et al., 1985; McKnight and Kingsbury, 1982; Su and Knipe, 1987). In contrast, a TATA box, cap site,

and origin of replication appear to be the only sequences required for maximal expression of L genes (Everett, 1986; Flanagan et al., 1991; Homa et al., 1986, 1988; Johnson and Everett, 1986; Shapira et al., 1987). One interpretation of this is that L gene expression may be a default state conferred by a minimal promoter whereas additional upstream elements are required to induce E gene activation. This possibility is supported by results of Johnson and Everett (1986) demonstrating that deletion of sequences upstream of the *gD* TATA box in a transfected construct result in an increased dependence on DNA replication for expression. Furthermore, in a recombinant virus in which the sequences 5' of the *tk* (an E gene) TATA box are linked to the *gC* (a true L gene) TATA box, expression of the chimeric gene is shifted to E kinetics (Homa et al., 1988). However, when Imbalzano et al. (1991) deleted the SP1 and CCAAT binding protein recognition sites from the E *tk* promoter, no alteration in the kinetics of *tk* expression was observed, implying that L regulation requires some specific signal, rather than merely existing as a default state. Conversely, when SP1 binding sites were added to a minimal promoter consisting of only a TATA element, expression driven by this promoter was increased but not altered temporally (Kibler et al., 1991). Experiments in which different TATA boxes have been substituted within the same flanking sequences indicate that while these sequences can determine level of expression, they do not affect kinetics of expression (Kibler et al., 1991; Imbalzano and Deluca, 1992). However, Mavromara-Nazos and Roizman (1989) found that sequences downstream of -12 (relative to the transcriptional start) in the promoter of true L gene *UL49.5* impose an increased dependency on DNA

replication for expression of a chimeric gene in a viral recombinant. Furthermore, Kibler et al. (1991) found that the cap/leader of true L gene *US11* confers a strict requirement for DNA replication on expression from a minimal promoter within the viral genome. The specific sequences responsible for imposition of true late kinetics and the mechanisms involved are not yet understood.

1.5. The Immediate Early Proteins

As stated above, four of the five IE proteins appear to involved (albeit to varying degrees) in the regulation of the cascade of HSV-1 gene expression. Four of the five IE genes are associated with the repeat regions of the genome (Fig. 1.1). ICP4 and ICP0 are diploid while ICP22, ICP27, and ICP47 are present in a single copy. Each of the IE proteins are discussed below.

1.5.1. ICP47

ICP47 is a cytoplasmic protein with an apparent molecular weight of 12 kD on a denaturing polyacrylamide gel (Marsden et al., 1982; Preston, 1979b). A virus mutant deleting the entire ICP47 gene has been constructed (Longnecker and Roizman, 1986; Mavromara-Nazos et al., 1986a). This mutant grows as well as its wild-type parent in Vero, BHK, and Rat-1 cell lines. Thus ICP47 is considered dispensable for growth in tissue culture. ICP47's function is uncertain; however, recent results of I. A. York and D.

C. Johnson indicate that it may be involved in evasion of cytotoxic T-lymphocytes (personal communication).

1.5.2. ICP22

ICP22 is a nuclear phosphoprotein with an apparent molecular weight of 68 kD on a denaturing polyacrylamide gel (Honess and Roizman, 1974; Wilcox et al., 1980). A recombinant virus mutant has been constructed bearing a deletion of ca. 70% of the carboxy terminal of ICP22 (Post and Roizman, 1981). This recombinant grows as well as its parent virus in HEp-2 and Vero cell lines (Post and Roizman, 1981; Sears et al., 1985), but grows poorly in rat skin cells, BHK, and Rat-1 cell lines (Sears et al., 1985). Infection at low moi of Rat-1 or HEL cells results in poor yields of progeny. In HEL cells, infection with the ICP22 mutant results in delayed shutoff of early gene expression and extended duration of late gene synthesis, as well as reduced expression of at least one late gene. In addition, the ICP22 mutant shows greatly reduced neurovirulence but retains the ability to establish latency in mice (Sears et al., 1985). A loss of repression of cellular gene transcription is observed during infection with the same ICP22 mutant (Kemp and Latchman, 1988). Thus, ICP22 appears to be required for efficient induction of late gene expression and repression of cellular gene expression. Perhaps once a mutant deleting the entire ICP22 gene is available, ICP22's role in the regulation of gene expression may become more clear.

1.5.3. ICP27

ICP27 is a nuclear phosphoprotein with an apparent molecular weight of 63 kD (Ackermann et al., 1984; Honess and Roizman, 1974; Knipe et al., 1987; Pereira et al., 1977; Wilcox et al., 1980). ICP27 was first demonstrated to be an essential regulatory protein in studies of temperature sensitive (ts) mutants. In these mutants, levels of some IE proteins are elevated and expression of some L genes is greatly reduced at the nonpermissive temperature (Sacks et al., 1985). It was later observed that levels of viral DNA replication in ICP27 deletion mutants are diminished , and that while E genes are overexpressed, levels of leaky L proteins are reduced and true L gene products are undetectable (McCarthy et al., 1989). Transient expression assays have shown that ICP27 can repress induction of some genes (generally E genes) by ICP4 and ICP0 and enhance the activation of others (generally L genes; Everett, 1986; Sekulovich et al., 1988; Su and Knipe, 1989). Together, these results suggest that ICP27 is involved in regulating the switch from E to L gene expression.

The regions of ICP27 necessary for the positive and negative regulatory activities observed in transient expression assays and during lytic infection have been mapped to two separate regions within the carboxy terminal half of the protein (Hardwicke et al., 1989; McMahan and Schaffer, 1990; Rice et al., 1989; Rice and Knipe, 1990). However, recently Rice et al. (1993) have isolated a virus in which the highly acidic region near the amino terminal was deleted. This virus is defective in DNA replication, repression of E genes, and induction of L genes, although none

of these defects are as severe those observed in the carboxy terminal mutants. It is of interest to note that ICP27 binds to single stranded DNA and that its carboxy terminal 105 amino acids (ICP27 is composed of 512 aa) encode a putative metal binding domain that binds zinc in vitro (Hardwicke et al., 1989; Vaughan et al., 1992); it is not known if these activities are required for ICP27's regulatory abilities.

ICP27 may regulate gene expression by multiple mechanisms. There is evidence that it acts post-transcriptionally; it has been found to enhance gene expression in a promoter-independent fashion (Chapman et al., 1992). Furthermore, studies of an ICP27 ts mutant showed no correlation between levels of RNA synthesis and accumulation of mRNA and protein synthesis (Smith et al, 1992). Thus, transcription of *ICP4* and *ICP27* was reduced whereas accumulation of their mRNAs and protein products was elevated. In contrast, transcription of *gC* (a true L gene) was unaffected but levels of accumulated gC transcripts and protein were greatly reduced. Sandri-Goldin and Mendoza (1992) have correlated the repressor function of ICP27 with the presence of introns in the target gene and its activation function with different polyadenylation sites, suggesting that ICP27 acts at the level of mRNA processing. However, other modes of action by ICP27 cannot be ruled out. Nuclear run-off assays using viruses bearing mutations in the ICP27 gene demonstrate reduced transcription of IE and some L genes (McCarthy et al., 1989; Smith et al., 1992). In transient expression assays, ICP27 represses activation by ICP4 and ICP0 but not that by E1A (Su and Knipe, 1989), which suggests interactions between ICP27 and some transcriptional activators. Of possible relevance to this, the electrophoretic

mobility of ICP4 is reduced in ICP27 mutants (McMahan and Schaffer, 1990; Rice et al., 1989; Su and Knipe, 1989); this indicates that ICP27 may be involved in post-translational modification of ICP4 (e.g. phosphorylation). It is unclear whether ICP27 is involved directly in enhancing the efficiency of DNA replication or whether incorrect regulation of expression of one or more other proteins in ICP27 mutants results in the defective DNA replication observed (McCarthy et al., 1989; Rice and Knipe, 1990; Rice et al., 1993). Future studies of ICP27 will need to clarify which of the many defects observed in ICP27 mutants are directly attributable to the mutation in ICP27 and which are indirect results of the mutation. In particular, it has been shown in ICP27 ts mutants that *ICP4* and *ICP27* (and possibly other IE genes) are overexpressed (Sacks et al., 1985; Smith et al., 1992); thus, some of the aberrant regulation observed may arise from elevated levels of other IE proteins.

1.5.4. ICP0

ICP0 is a 775 amino acid nuclear phosphoprotein with an apparent molecular weight between 110 and 124 kD on a denaturing polyacrylamide gel, depending on the crosslinking agent used and the percent acrylamide content of the gel (Ackermann et al., 1984; Honess and Roizman, 1974; Pereira et al., 1977; Perry et al., 1986). ICP0 activates expression from IE, E, and L promoters in transient assays (Everett, 1986; Gelman and Silverstein, 1985, 1986; Mavromara-Nazos et al., 1986b; O'Hare and Hayward, 1985a,b; Quinlan and Knipe, 1985); activation is enhanced considerably when both

ICP0 and ICP4 are present (Everett, 1984b, 1986; O'Hare and Hayward, 1985; Quinlan and Knipe, 1985). Results of mutational analysis suggest that synergism between ICP0 and ICP4 may be separable from ICP0's ability to transactivate on its own (Everett, 1988a) and that ICP0 may transactivate different promoters by different mechanisms (Cai and Schaffer, 1989). Yao and Schaffer (1993) have recently reported physical interactions between ICP0 and both ICP4 and TFIID; these findings may prove relevant to the ability of ICP0 to activate gene expression on its own or synergistically with ICP4. ICP0 binds DNA in crude cell extracts (Hay and Hay, 1980), but no specific binding site has been identified, nor has it been shown whether this ability is required for induction of gene expression. ICP0 contains a putative zinc binding (cysteine rich) domain (Perry et al., 1986) and binds zinc in vitro (Vaughan et al., 1992). Mutational analysis indicates that diverse regions of ICP0 are required for transactivation, but that a region including this cysteine rich domain appears to be particularly important (Cai and Schaffer, 1989; Chen et al., 1991; Everett, 1987b, 1988a).

Although ICP0 transactivates gene expression in the absence of ICP4 in transient expression assays, it is not clear whether it does this during lytic infection. Many ICP4 mutants fail to produce E and L gene products (see below), despite the presence of functional ICP0. Perhaps the mutant ICP4 polypeptide represses ICP0. Alternatively, since some ICP4 mutants are defective for transport of ICP0 from the cytoplasm to the nucleus (Knipe and Smith, 1986; Preston, 1979b), ICP0 may be physically separated from its site of action. Consistent with either of these possibilities, interactions

between ICP0 and ICP4 have recently been reported (Mullen and Hayward, 1993; Yao and Schaffer, 1993)

Viral mutants in ICP0 grow poorly, particularly at low multiplicities of infection, producing reduced levels of infectious progeny with high particle-to-pfu ratios (Sacks and Schaffer, 1987; Stow and Stow, 1986). Thus, although ICP0 is not strictly essential for productive infection in tissue culture, it does play an important role in viral replication. Mutational analysis has indicated that the putative zinc binding domain is essential for ICP0's function in lytic infection (Cai and Schaffer, 1989; Everett, 1989). Despite its inability to functionally substitute for ICP4, ICP0 appears to function as a transactivator during productive infection; infection at low moi. with ICP0 mutants results in reduced levels of E and L proteins (Cai and Schaffer, 1992; Everett, 1989). However, although ICP0 mutants accumulate reduced levels of mRNA from IE gene *ICP27*, levels of mRNA and polypeptides from IE gene *ICP4* are similar to those of the wild type virus, or slightly elevated very early (1.5-2 hours post-infection) in infection (Cai and Schaffer, 1992; Chen and Silverstein, 1992). Reasoning that transactivation by the IE gene transducer, Vmw65, might eclipse any detectable effect of ICP0 during productive infection, Cai and Schaffer (1989, 1992) transfected cells with infectious DNA (thus lacking the virion component, Vmw65) from ICP0 mutants. Both expression of *ICP4* and production of progeny virus were reduced; this deficiency was relieved by cotransfection with a plasmid expressing wild type ICP0. Based on these results, Cai and Schaffer proposed that ICP0 may function in the induction of IE gene expression during reactivation from latency. Supporting this

hypothesis, superinfection with an adenovirus vector encoding ICP0 reactivates latent HSV type 2 in an in vitro latency system, whereas HSV-1 or adenovirus encoding mutant ICP0 fails to result in reactivation (Russell et al., 1987; Zhu et al., 1990). Furthermore, ICP0 mutants are defective in their ability to reactivate from the latent state in mouse ganglia (Clements and Stow, 1989; Leib et al., 1989). Results of mutational analysis of ICP0 indicate that the cysteine rich transactivation domain is necessary for reactivation but other domains previously shown to be important for virus growth and plaque formation are not required (Zhu et al., 1990). Although ICP0 appears to function in reactivation from the latent state, since the ICP0 open reading frame is contained entirely within the latency transcripts' coding sequences (Zwaagstra et al., 1990), results of mutational analysis must be interpreted with caution.

Study of the function of ICP0, whether by transient expression assays or during productive or latent infection, has been rendered more difficult due to much variation in results depending on the cell line, or in some cases, the virus strain, used in the study (Chen et al., 1991; Chen and Silverstein, 1992; Everett, 1988a,b; Leib et al., 1989). Cai and Schaffer (1991) have found that a cell-cycle dependent cellular function can complement ICP0 mutants. One possible explanation for the range of effects observed with ICP0 mutants in different cell lines may be variation in levels of this cellular function.

In summary, ICP0 appears to function as a transactivator both in productive infection, and in reactivation from the latent state, although the mechanism by which it acts remains unclear. Since it cannot substitute for

the function of transcriptional regulatory protein ICP4 (DeLuca et al., 1985; Dixon and Schaffer, 1980; Preston, 1979a; Watson and Clements, 1980), it is apparent that ICP0 must operate by a distinct mechanism. Given the ability of ICP0 to induce expression from a variety of diverse promoters (including non-HSV-1 promoters; Everett, 1984a, 1985; O'Hare and Hayward, 1985) and its apparently nonspecific binding to DNA, it appears to act as a general transcriptional activator, possibly operating by facilitating the function of other transcription factors.

1.5.5. ICP4

ICP4 is a 1298 amino acid nuclear phosphoprotein which is required for induction of HSV-1 E and L gene expression as well as down-regulation of IE genes (Ackermann et al., 1984; DeLuca et al., 1985; Dixon and Schaffer, 1980; McGeoch et al., 1986; Pereira et al., 1977; Preston, 1979a; Watson and Clements, 1980; Wilcox et al., 1980). Its apparent weight on a denaturing polyacrylamide gel is approximately 175 kD (Honess and Roizman, 1974), however, several closely migrating species are observed (Ackermann et al., 1984; Pereira et al., 1977; Wilcox et al., 1980); this is thought to be due to the presence of different forms of the highly modified ICP4. ICP4 is phosphorylated at both serine and threonine residues (Faber and Wilcox, 1986b), using both ATP and GTP as sources of phosphate (Blaho et al., 1993). Phosphorylation sites occur in a serine rich tract located in the amino terminus of ICP4 but also at other locations within the protein (DeLuca and Schaffer, 1988). Phosphate cycles on and off of ICP4 during the

infectious cycle (Wilcox et al., 1980). ICP4 is also guanylylated, adenylylated, and poly(ADP-ribosyl)ated (Blaho et al., 1991, 1992, 1993; Preston and Notarianni, 1983). The regions of ICP4 required which accept these modifications have not been identified, nor are their functions known. Preston (1979b) has observed that in the ICP4 mutant tsK, at the nonpermissive temperature, forms of ICP4 with lower electrophoretic mobility are less abundant, suggesting that at least some post-translational modifications of ICP4 are required for ICP4 function. Furthermore, ICP27 appears to be involved in some post-translational modifications of ICP4 (McMahan and Schaffer, 1990; Rice et al., 1989; Su and Knipe, 1989); thus, some of the defects observed in ICP27 mutants may be due to nonfunctional ICP4. Physical studies indicate that native ICP4 exists as a highly elongated homodimer, and possibly forms dimer oligomers (Metzler and Wilcox, 1985). ICP4 is distributed throughout the nucleus early in infection (Knipe and Smith, 1986). However, once replication commences, ICP4 is localized into 'replication compartments' which also contain the major DNA binding protein, DNA polymerase, and viral DNA. The significance of this relocalization is not clear. In some ts mutants, the mutant ICP4 polypeptide accumulates in the cytoplasm at nonpermissive temperature (Cabral et al., 1980; Preston, 1979b); upon shift to the permissive temperature, ICP4 is localized to the nucleus without requirement for de novo protein synthesis (Preston, 1979b).

One remarkable feature of ICP4 is the high GC content (81.5%) of its coding sequence (McGeoch et al., 1986). This high GC content is reflected in the amino acid content of the protein; the four most abundant amino acids

in ICP4 are alanine, proline, glycine, and arginine, all of which possess codons containing only G and C residues. ICP4 can be divided into five regions based on protein sequence comparison with homologous proteins in other alphaviruses: varicella zoster virus (VZV), pseudorabies virus (PRV), and equine herpesvirus type 1 (EHV-1) (Cheung, 1989; Grundy et al., 1989; McGeoch et al., 1986; Vlcek et al., 1988). Region 1 encompasses amino acid residues 1 to 314 in ICP4. Limited homology is found in this region among the alphaviruses except for a serine rich tract (extending from residues 186 to 204 in ICP4) which is followed by acidic residues. The greatest degree of conservation between these homologues occurs in regions 2 and 4 (corresponding to residues 315 to 484 and 797 to 1224 (respectively) of ICP4). Little homology is found in regions 3 and 5, which encompass amino acid residues 485 to 796 and 1225 to 1298, respectively, in ICP4. The nonconserved regions, 1, 3, and 5, of the ICP4 homologues are heterogeneous in size whereas the conserved regions are of similar lengths. Many mutations and deletions which result in loss of ICP4 function map to regions 2 and 4 (Davison et al., 1984; Deluca et al., 1988; Paterson and Everett, 1988a,b; Paterson et al., 1990; Preston, 1981; Shepard et al., 1989). However, the conserved serine-rich region in region 1 is not essential for growth in tissue culture (Paterson and Everett, 1990). As might be predicted, a higher concentration of the amino acids with codons containing only G and C occurs in the nonconserved regions of ICP4 (McGeoch et al., 1986).

An important property of ICP4 is its ability to bind to specific sequences in DNA (Kattar-Cooley and Wilcox, 1989; Michael et al., 1988). ICP4 binds the consensus sequence 5'-ATCGTCnnnnYCGRC-3' (Faber and

Wilcox, 1986a, 1988; Muller, 1987), although binding to nonconsensus sites also occurs (Imbalzano et al., 1990; Kristie and Roizman, 1986a; Michael et al., 1988; Tedder et al., 1989). Although ICP4 binds DNA as a dimer, the lack of symmetry in its binding site suggests that either only one unit of the dimer actually participates in protein-DNA interactions (dimerization perhaps inducing a conformational change required for this) or that the DNA recognition domain is formed through association of two ICP4 monomers (Kattar-Cooley and Wilcox, 1989; Michael and Roizman, 1989; Wu and Wilcox, 1990). Supporting the former possibility, a heterodimer formed between two nonfunctional ICP4 mutants, one defective in DNA binding, the other transcriptionally inactive but retaining DNA binding ability, is able to bind DNA and regains partial transactivating ability (Shepard and DeLuca, 1991a). Amino acids 262-490 of ICP4 (encompassing region 2 and the carboxy portion of region 1) have been shown to be sufficient for sequence-specific DNA binding (Everett et al., 1990; Wu and Wilcox, 1990). Many mutations and deletions which result in severely defective phenotypes map to this region (Deluca and Schaffer, 1987, 1988; Paterson and Everett, 1988a,b; Shepard et al., 1989). The predicted secondary structure of this region in ICP4 and of the homologous region in VZV resembles a helix-turn-helix (Shepard et al., 1989). Michael et al. (1988) have demonstrated that electrophoretic isoforms of ICP4 differ in their DNA binding affinity. This suggests that binding to DNA by ICP4 (and thus, its subsequent functions) is regulated by post-translational modifications.

ICP4 was first shown to be essential for induction of HSV-1 E and L gene expression in experiments using ts mutants; many of these mutants overproduce IE gene products but fail to express most E and L genes (Courtney et al., 1976; Dixon and Schaffer, 1980; Knipe et al., 1978; Preston, 1979a,b; Preston, 1981; Watson and Clements, 1978). Other mutants are only partially defective in E gene expression and synthesize appreciable levels of viral DNA at the nonpermissive temperature, but produce greatly reduced or undetectable levels of L gene products (DeLuca et al., 1984; Paterson et al., 1990). This suggests firstly that the observed defect in L gene expression is not due to the absence of an E gene product or of viral DNA replication, but is directly attributable to the loss of ICP4 function. Secondly, this implies that ICP4 induces E and L gene expression by separate mechanisms. In general, mutations resulting in more severely affected phenotypes map to region 2 or to parts of region 1 immediately adjacent to region 2 (i.e. within the DNA binding domain) whereas lesions in the more permissive mutants map to the carboxy terminal third of ICP4 (DeLuca et al., 1984; Dixon and Schaffer, 1980; Paterson et al., 1990; Davison et al., 1984). This generalization also holds true for many insertion and deletion mutants (DeLuca and Schaffer, 1987, 1988; Paterson and Everett, 1988a,b; Shepard et al., 1989).

Further evidence regarding ICP4's transregulatory role comes from transient expression assays. ICP4 has been found to induce expression of reporter genes driven by E or L promoters (DeLuca and Schaffer, 1985; Everett, 1984; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a; Quinlan and Knipe, 1985) and to repress expression from constructs

bearing IE promoters (Deluca and Schaffer, 1985; Gelman and Silverstein, 1987a, b; O'Hare and Hayward, 1985b; Resnick et al., 1989; Roberts et al., 1988), as measured both by activity of reporter gene products and by accumulation of mRNA. As noted above, transactivation by ICP4 is augmented in the presence of ICP0 (Everett, 1984, 1986; Quinlan and Knipe, 1985; O'Hare and Hayward, 1985). These experiments, together with studies of ts mutants and mutational analysis of ICP4, show that ICP4 is a transcriptional activator of some genes and a repressor of others.

How is transregulation by ICP4 accomplished? The ability of ICP4 to bind specific sequences of DNA invites the hypothesis that transregulation by ICP4 is mediated by DNA binding, as is the case with other sequence-specific transcription factors (see above). However, whether this is so is unclear. Results of extensive mutational analysis indicate that ICP4's DNA binding domain coincides with regions required for transactivation and transrepression (Deluca and Schaffer, 1987, 1988; Paterson and Everett, 1988a,b; Shepard et al., 1989). This region is particularly rich in proline residues (McGeoch et al., 1986); proline-rich transactivating domains are found in some other transcription factors (for review, see Mitchell and Tjian, 1989). Although several mutants have been isolated which are defective in DNA binding in vitro but retain wild type or only partially reduced transactivational activity (Paterson et al., 1990; Shepard and Deluca, 1991b), this cannot be considered definitive evidence that DNA binding is not required as these mutants may bind in vivo in the presence of other factors. Consistent with this, Smith et al. (1993) have recently shown that ICP4 forms a tripartite complex with TFIIB and TFIID and that the

presence of these factors increases ICP4's affinity for its binding site. Thus, mutants previously demonstrated not to bind DNA in vitro may, in fact, bind if TFIIB and TFIID are included in the assay. A role for DNA binding in transactivation by ICP4 is supported by the finding that fewer transcripts accumulate from a *UL49.5* (*ICP42*) promoter after a mutation is introduced into the ICP4 binding site present in that promoter (Romanelli et al., 1992). Furthermore, ICP4 binding sites present in the *gD* promoter increase ICP4-induced expression in vitro and multimerisation of one of these sites enhances activation by ICP4 in a transient expression assay (Beard et al., 1986; Tedder et al., 1989; Tedder and Pizer, 1988). However, when three binding sites in the *gD* promoter were deleted or mutated to eliminate binding, no change in gD expression was observed in the course of viral infection (Smiley et al., 1992). Furthermore, a virus encoding a mutant ICP4 which fails to bind the weak nonconsensus binding site present in the *tk* promoter produces wild type levels of tk (Imbalzano et al., 1990). Again, caution must be exercised in the interpretation of these data as binding ability measured in vitro may not reflect events within the cell. An additional problem with the model of transactivation mediated by DNA binding is that many E and L genes do not contain detectable binding sites within their promoters. Furthermore, expression of several cellular genes is induced when those genes are introduced into the viral genome under the control of their own promoters whereas expression of the endogenous genes is suppressed during HSV-1 infection (Panning and Smiley, 1989; Smibert and Smiley, 1990; Smiley et al., 1987). This activation requires functional ICP4 (Smiley and Duncan, 1992); no ICP4 binding sites have

been identified within the cellular genes' promoters, nor is induction dependent on flanking sequences (Panning and Smiley, 1989). However, ICP4 binding sites appear to occur at a high frequency throughout the viral genome; DiDonato et al. (1991) have predicted the occurrence of up to 500 ICP4 binding sites within the HSV-1 genome, based on statistical analysis of known binding sites. It is possible that ICP4 can function by binding to any one of a number of sites located either proximally or further removed from a gene's transcriptional start; this would account for results described above in which elimination of binding to an individual site does not affect expression. This would then suggest that rather than acting as a gene-specific transactivator, ICP4 targets all genes within the viral genome for induction.

There is stronger evidence supporting a role for individual binding sites in transrepression of IE gene expression by ICP4. High affinity binding sites occur across the *ICP4* transcriptional start and immediately upstream of the *ICP0* TATA element (Faber and Wilcox, 1988; Kristie and Roizman, 1986b; Muller, 1987). Although binding of ICP4 to a region in the *ICP27* promoter has been reported (Kristie and Roizman, 1986a), Faber and Wilcox (1988) could not detect binding when attempting to further characterize this site; no ICP4 binding site has been observed in the *ICP22/ICP47* promoters (which are identical, see Fig. 1.1). The binding sites located in the *ICP4* and *ICP0* promoters result in reduced expression of reporter genes in transient expression assays (Deluca and Schaffer, 1985; Gelman and Silverstein, 1987a,b; O'Hare and Hayward, 1985b; Resnick et al., 1989; Roberts et al., 1988); this repression is relieved by mutation of

these sites. In keeping with these results, Michael and Roizman (1993) have recently found that the ICP4 binding site located at the *ICP4* transcriptional start mediates negative regulation during viral infection. Contrary to this, however, Everett and Orr (1991) found that a mutation of the site in the *ICP0* promoter which removed negative regulation in transient expression assays did not affect expression of ICP0 once transferred into the viral genome. Thus, the role of DNA binding in both transactivation and transrepression by ICP4 remains unclear.

1.6. Experimental Purpose and Design

The purpose of this study was to gain an improved understanding regarding the function of ICP4's ability to bind specific sites in DNA. Reasoning that effects of mutations in ICP4 binding sites in native HSV-1 promoters may be obscured due to the effects of other regulatory elements, I introduced ICP4 binding sites into a simple model promoter within the viral genome. Two sets of construct were made in which an ICP4 binding site (or mutant site) was placed either downstream or upstream of a TATA box, reproducing the spacing found in (i) the native *ICP4* promoter and (ii) the native *ICP0* promoter (respectively). The promoter of HSV-1 *UL24b* (a nonessential gene in tissue culture) was replaced with these model promoters and levels of transcripts accumulating from these constructs during lytic infection assayed by primer extension. I found that an ICP4 site placed either upstream or downstream of a TATA box shifted kinetics of expression from E/leaky L to true L. Neither the strength of the TATA box

nor the helical orientation of the ICP4 binding site with respect to the TATA box affected this result. An ICP4-GST fusion protein bearing the DNA binding domain of ICP4 was used to confirm that the ICP4 binding site used in these constructs was indeed bound by ICP4 whereas the mutant binding site was not.

A second approach used to study the role of DNA binding by ICP4 involved in vivo footprinting using ligation mediated PCR. The goal of this study was to examine protein-DNA interactions in regions of the viral genome containing previously characterized ICP4 binding sites (such as those in the *ICP4* and *gD* promoters) during viral infection. The site located in the ICP4 promoter is widely believed to mediate autoregulation; whether the binding sites located in the *gD* promoter are involved in activation of *gD* expression is a matter of some contention (Beard et al., 1986; Smiley et al., 1992; Tedder and Pizer, 1988; Tedder et al., 1989). I intended to correlate the presence (or absence) of a footprint in vivo with gene expression in wild type virus and in mutants in which binding of ICP4 to ICP4 binding sites had been eliminated. Viral mutants bearing either altered ICP4 binding sites or mutant ICP4 may have been studied. For example, one ICP4 mutant which I intended to study was pri12, an ICP4 revertant defective in ICP4 binding but having wild type transregulatory activity (Shepard and DeLuca, 1991b). If no binding could be detected in vivo, this would have suggested that DNA binding truly is not required for transregulation by ICP4 during lytic infection. Unfortunately, due to technical difficulties in the optimization of conditions for LM-PCR, this approach had to be discontinued.

II. MATERIALS AND METHODS

2.1. Bacterial Culture Conditions

E. coli strain HB101 was used in the propagation of all plasmids with the exception of plasmids pJB39 and pgD123mut, which were grown in *E. coli* strain JM109. Bacterial cultures were grown at 37°C under continuous agitation in suspension in Terrific Broth (TB), which was composed of 1.2% bactotryptone, 2.4% yeast extract, 0.4% glycerol, and 88.7 mM potassium phosphate (pH 7.4). Where appropriate, ampicillin was added to a final concentration of 20 μg/mL. Growth of large volumes (1 L) of culture was initiated by the addition of a 5 mL culture grown 4-6 hours from a stab of a bacterial colony or a frozen culture. Alternatively, cultures were initiated by the addition of 1 mL of stock bacterial culture which had been frozen at -70°C in the presence of 7% dimethyl sulphoxide (DMSO). Cultures were allowed to grow 12-16 hours. To isolate individual colonies, bacteria were spread thinly over Luria agar, which was composed of 1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, 10 mM Tris-HCl (pH 7.6), 1.5% bactoagar, and 0.4% glucose. Where appropriate, ampicillin was added to a final concentration of 40 μg/mL. Bacterial plates were incubated at 37°C overnight or until colonies were visible.

2.2. Cloning Techniques and Manipulations of Plasmid DNA

The procedures for the cloning techniques utilized are described in Sambrook et al. (1989). The majority of the restriction enzymes used were supplied by New England Biolabs (NEB). Digests were performed using the buffers supplied by the manufacturer following their recommended conditions.

In cases where oligonucleotide duplexes were to be inserted into a plasmid, the duplexes were prepared as follows. 60 ng of each complementary oligonucleotide were mixed in a total volume of 10 μL that was 1x in the ligation buffer supplied with the T4 DNA ligase (NEB). This mixture was incubated at 37°C for one hour and then mixed with 10 μL of 1x ligation buffer containing 10 U T4 DNA ligase and 0.5-10 μg of plasmid cut with a restriction enzyme at the insertion site. This mixture was incubated overnight at 14°C and then used for transformation of competent bacteria. Oligonucleotides were designed with 5' protruding ends complementary to the 5' protruding ends of the restriction enzyme cut plasmid. Oligonucleotide duplexes were purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University.

2.3. Transformation of Bacteria

Bacteria were made competent for transformation by the following method. 100 mL of TB was inoculated with 1 mL of an overnight culture and

allowed to grow for 2.5 hours. Bacteria were then harvested by centrifugation at 1300 g for 10 minutes at 4°C and the bacterial pellet resuspended in 20 mL of MOPS I buffer (50 mM 3-[N-Morpholino] propane-sulphonic acid (MOPS; pH 7.0), 10 mM RbCl). Bacteria were collected again by centrifugation and resuspended in 20 mL of MOPS II buffer (100 mM MOPS (pH 6.5), 70 mM $CaCl_2$, 10 mM RbCl). After a final collection by centrifugation, the bacterial pellet was resuspended in 2 mL MOPS II buffer. The bacteria were now competent for transformation.

1 ng of plasmid DNA or 0.5 μg of DNA from a ligation were mixed with 100 μL of competent bacteria and incubated for 30 minutes at 4°C. The mixture was then incubated at 42°C for 2 minutes. 900 μL of TB were added and 100 mL and 400 μL of the transformed bacteria spread evenly over 10 cm Luria agar plates. Plates were incubated at 37°C overnight to allow growth of colonies.

2.4. Colony Hybridisation

Colony hybridisation was used in some cases to select positive plasmid clones. Once bacterial colonies had grown on a Luria agar plate, a nitrocellulose circle with two or three notches cut into its edge was placed onto the growth surface. The pattern of the notches was traced onto the plate to allow for subsequent realignment of the plate and the circle. The nitrocellulose circle was then carefully peeled away from the agar and placed, colony side up, on two sheets of Whatman 3MM blotting paper which had been soaked in 0.5 M freshly made NaOH. After incubation for 1

hour, the nitrocellulose circle was submitted to four sequential 10 minute incubations on two sheets of blotting paper soaked in the following solutions: 2 incubations with 1 M Tris-HCl (pH 7.5), 1 incubation with 1 M Tris-HCl (pH 7.5) containing 1.5 M NaCl, and a final incubation with 20x SSC (3 M NaCl, 0.3 M Na citrate). The lysed bacterial DNA was then crosslinked to the nitrocellulose using a Stratagene UV crosslinker, following the manufacturer's directions. The nitrocellulose circle was now ready for hybridisation.

Hybridisation were performed in a Techne HB-1 hybridisation oven, according to the directions of the manufacturer. The nitrocellulose circle was first incubated for one hour at 60°C in Hybridisation buffer (5x SSC, 0.5 mg/mL sonicated denatured salmon sperm DNA, 10 mM Tris-HCl (pH 7.5), 2x Denhardt's solution (50x Denhardt's solution is composed of 1% polyvinylpyrrolidine, 1% bovine serum albumin (BSA), and 1% ficoll)). After this preincubation, the hybridisation solution was drained and fresh Hybridisation buffer containing an appropriate ^{32}P-labelled oligonucleotide was added. Hybridisation was allowed to proceed overnight at 60°C. The nitrocellulose circle was then subjected to four 5 minute washes with 500 mL of Wash solution (2x SSC, 0.1% sodium dodecyl sulphate (SDS); 60°C). A sheet of Kodak XAR-5 film was then exposed to the nitrocellulose circle for approximately 2 hours. Positive clones were identified by aligning the developed film, the nitrocellulose circle, and the notch-marked Luria agar plate.

2.5. Purification of Plasmids

2.5.1. Small-Scale Preparations

Small-scale purifications, prepared using a modified version of the method of Birnboim and Doly (1979), were used for screening recombinant plasmids. Colonies were picked from Luria agar plates and grown in 5 mL of TB (plus appropriate antibiotic) overnight. A 1.5 mL microfuge tube was filled with each culture and centrifuged for 1 or 2 minutes at 14000 g. The supernatant was removed and the pellet resuspended in 100 μL of Solution 1 (25 mM Tris-HCl (pH 8.0), 50 mM glucose, 10 mM EDTA) containing 2 mg/mL lysozyme and incubated for 15 minutes on ice. After addition of 200 μL of Solution 2 (0.2 N NaOH, 1% SDS, freshly prepared), tubes were vortexed and incubated an additional 5 minutes on ice. 150 μL of 3 M potassium acetate (pH 4.8) was added, and the tubes gently mixed, incubated on ice for 30 minutes, and centrifuged at 14000 g for 15 minutes at 4°C. Nucleic acids were ethanol precipitated twice, and dried samples resuspended in 50 μL of distilled water containing 5 μg RNase A and incubated at 37°C for 30 minutes. The DNA was now ready for restriction enzyme analysis (using 5 μL per digest) or sequencing by the chain termination method (using 8 μL per set of four reactions).

2.5.2. Large-Scale Preparation by LiCl and Polyethyleneglycol

A 1 L culture was grown overnight and then harvested by centrifugation at 4400 g for 20 minutes at 4°C. The bacterial pellet was resuspended in 20 mL of Solution 1 containing 5 mg/mL lysozyme and incubated for 5 minutes at room temperature. 40 mL of Solution 2 was added and the lysate incubated on ice for 10 minutes after gentle mixing. Next, 30 mL of 3 M potassium acetate (pH 4.8) was added, gently mixed, and the mixture incubated on ice for 10 minutes. The lysate was centrifuged at 27000 g for 15 minutes at 4°C, the supernatant transferred to a fresh tube, and centrifuged at 27000 g for an additional 15 minutes. The supernatant was warmed to room temperature, mixed with 0.6 volumes of isopropanol, and incubated for 15 minutes at room temperature. After centrifugation at 12000 g for 30 minutes at room temperature, the supernatant was discarded and the pellet washed first with 70% ethanol, and then with 95% ethanol. The pellet was then dissolved in 4 mL TE (10 mM Tris-HCl, 1 mM EDTA; pH 7.4), mixed with an equal volume of LiCl, and incubated on ice for 15 minutes. After centrifugation at 12000 g for 15 minutes at 4°C, 2.5 volumes of ethanol were added to the supernatant and this was incubated at -20°C for a minimum of 30 minutes. The precipitate was collected by centrifugation at 12000 g for 30 minutes at 4°C and then resuspended in 1 mL of TE. RNase A was added to a final concentration of 40 μg/mL and this was incubated for 15 minutes at 37°C. Next, 1 mL of 13% PEG (polyethyleneglycol) containing 1.6 M NaCl was added and the reaction allowed to incubate on ice for 30 minutes. After centrifugation at 14000 g for

15 minutes at 4°C, the pellet was resuspended in 500 µL of TE. This was then extracted against equal volumes of 1:1 phenol:chloroform until no interface was visible and then one additional time. The DNA was ethanol precipitated twice, dissolved in distilled water, and quantified by measurement of its optical density at 260 nm, using a UV spectrophotometer (LKB Biochem Ultrospec II). Yields ranging from 2 to 5 mg of DNA were routinely obtained.

2.5.3. Large-Scale Preparation using Qiagen Columns

Plasmid DNA was purified from bacteria using Qiagen columns according to the manufacturer's instructions with the following modifications. 250 mL of culture (grown in TB) was used per column. RNase A was not included in the P1 buffer. Instead, an additional step was performed following centrifugation of the sample after addition of P3 buffer. 1 mg of RNase A was added to the clear lysate and incubated for 20 minutes at 37°C. Following centrifugation at 4°C for 15 minutes at 30000 g, the manufacturer's protocol was followed until the final step; at this point the redissolved DNA was centrifuged at 4°C for 15 minutes at 14000 g to eliminate insoluble material. Yields ranging from 100 to 500 µg of DNA were obtained.

2.6. Gel electrophoresis

A variety of gel electrophoresis techniques were employed in the course of the work described in this thesis. All acrylamide gels used for the separation of DNA fragments were made with TBE buffer (10x TBE contains 892 mM Tris base, 890 mM boric acid, 25 mM EDTA). Agarose gels were made with either TBE buffer or TAE buffer (10x TAE contains 40 mM Tris base, 198 mM acetic acid, 10 mM EDTA (pH 8.0)). Electrophoresis was performed using 1x TBE or 1x TAE as running buffer, as appropriate. Once gel electrophoresis was complete, unlabelled DNA fragments were visualized by examination under UV light after soaking the gel for 10 to 20 minutes in ethidium bromide solution (made by addition of 100 mL of 3 mg/mL stock ethidium bromide to 500 mL of 1x TBE or TAE).

Standard polyacrylamide gels were used for examination of restriction enzyme digests, isolation of DNA fragments for use in cloning, and isolation of labelled DNA fragments to be used as probes. Stock standard acrylamide solution contained 40% acrylamide and 1.37% bis-acrylamide (N,N'-Methylenebisacrylamide). Standard acrylamide stock solution was mixed with 0.01 g/mL mixed bed resin (Bio-Rad AG 501-X8 Resin, 20-50 mesh) for one hour, filtered, and stored at 4°C in a light-protected bottle. Gels containing 3.5-15% acrylamide were used, depending on the degree of resolution required; 5% acrylamide was the concentration employed for most purposes. Gels were made with a final concentration of 1x TBE, 0.04% APS (ammonium persulphate), and 1μL/mL N,N,N',N',-Tetramethylethylenediamine (TEMED). Samples for gel electrophoresis

were mixed with 10x standard loading buffer (50% glycerol, 0.4% bromophenol blue, and 0.4% xylene cyanol). Gels were run with an applied voltage between 150 and 250 V.

Urea-polyacrylamide gels were used for purification of ^{32}P-labelled oligonucleotides to be used in primer extension assays, and for resolution of DNA fragments from sequencing reactions (and ligation mediated PCR amplified sequencing reactions), DNAse I footprinting assays, and DNA capture assays. Stock acrylamide contained 38% acrylamide and 2% bis-acrylamide. The stock was mixed with 0.01 g/mL mixed bed resin for one hour, filtered, and stored at 4^{o}C in a light-protected bottle. Urea-acrylamide gels contained 7.6% acrylamide, 7 M urea, 0.04% APS, 1μL/mL TEMED, and a final concentration of 1x TBE. Gels were run with an applied voltage between 1600 and 2000 V (100 W) and were prerun for one hour before loading samples. Samples for gel electrophoresis were resuspended in sequencing gel loading dye (80% deionized and recrystallised formamide, 1x TBE, 0.1% bromophenol blue, 0.1% xylene cyanol).

Agarose gels were used for examination of DNA fragments from restriction enzyme digests yielding large DNA fragments (>1 kbp), for Southern blots, and for isolation of large fragments to be used for cloning or construction of probes. Gels contained 0.9 to 1.4% agarose, depending on the degree of resolution required. Gels were made using 1x TBE, except when DNA fragments were to be eluted, in which case 1x TAE was used. Samples for gel electrophoresis were mixed with 10x standard loading buffer. Gels were run with an applied voltage between 40 and 100 V, depending on the time schedule of the experiment.

Denaturing SDS-polyacrylamide gel electrophoresis was used for examination of proteins and for Southwestern blots. The acrylamide stock contained 30% acrylamide and 1.2% DATD (N,N'-Diallyltartardiamide) and was stored at 4°C in a light-protected bottle. The separating gel contained 13% acrylamide, 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.067% APS (freshly made), and 0.067% TEMED. Prior to the addition of TEMED and pouring of the gel, this solution was de-gassed for 15 minutes. The separating gel was overlaid with 95% ethanol and allowed to polymerize for one hour. After removal of the ethanol overlay, a stacking gel, composed of 5% acrylamide, 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% APS, and 0.1% TEMED, was poured and allowed to polymerize for 20 minutes. Samples were mixed with 2x sample buffer (314 mM Tris base, 50% glycerol, 5% SDS, 5% 2-mercaptoethanol, and 0.0025% bromophenol blue) and boiled for five minutes prior to loading. Electrophoresis was performed using SDS-PAGE running buffer (25 mM Tris base, 1.44% glycine, and 0.1% SDS) and an applied voltage between 150 and 200 V.

Once electrophoresis was complete, proteins were visualized in the following manner. The gel was fixed by soaking under gentle agitation in Fix solution (20% methanol, 10% acetic acid) for 1 hour. It was then stained for 2 hours under gentle agitation in Coomassie blue stain (50% methanol, 0.05% Coomassie Brilliant Blue R, 10% acetic acid). The gel was destained in Fix solution with gentle agitation overnight. The gel was preserved by drying for 1 hour on a Bio-Rad gel drier.

2.7. DNA Sequencing

2.7.1. Chain Termination Method

The dideoxynucleic acid chain termination method of DNA sequencing, based on the method of Sanger et al. (1977), was routinely used to generate DNA sequences. For this purpose, a Pharmacia T7 DNA sequencing kit was employed, following the manufacturer's directions, and utilizing denatured double stranded DNA. This method was successful using plasmid DNA purified using the LiCl method or Qiagen columns, as well as with the less pure plasmid DNA prepared by the method of Birnboim and Doly (1979).

2.7.2. Base Specific Chemical Cleavage Method

The base specific cleavage method of DNA sequencing, based on the method of Maxam and Gilbert (1980), was employed to generate a sequencing ladder for amplification by ligation mediated PCR and for use as a control in DNAse I footprinting assays. 20000 cpm of ^{32}P-labelled DNA or 10 μg of unlabelled DNA were used in reactions specific for a single nucleotide. Twice these amounts were used in the reactions specific for two nucleotides.

For the guanine-specific reaction, 5 μL of DNA was mixed with 200 μL of DMS buffer (50 mM sodium cacodylate, 1 mM EDTA (pH 8.0)) on ice, 1 μL of dimethyl sulphate (DMS; Aldrich) was added and the mixture

incubated at room temperature for 80 to 120 seconds (the optimal time was empirically determined). The reaction was terminated by the addition of 50 μL of DMS stop buffer (1.5 M Na acetate (pH 7.0), 1 M 2-mercaptoethanol), followed by precipitation with 750 μL of precooled (-70°C) 95% ethanol.

For the purine-specific reaction, 10 μL of DNA (at 4°C) was mixed with 25 μL of formic acid (Aldrich) and incubated at room temperature for 10 minutes. The reaction was terminated by the addition of 200 μL of Hz stop buffer (0.3 M Na acetate (pH 7.5), 0.1 mM EDTA (pH 8.0)), followed by precipitation with 750 μL of precooled (-70°C) 95% ethanol.

For the pyrimidine-specific reaction, 10 μL of DNA and 10 μL of distilled water (at 4°C) was mixed with 30 μL of hydrazine (Fisher) and incubated at room temperature for 8 minutes. The reaction was terminated by the addition of 200 μL of Hz stop buffer, followed by precipitation with 750 μL of precooled (-70°C) 95% ethanol.

For the thymidine-specific reaction, 5 μL of DNA and 15 μL of 5 M NaCl (at 4°C) were mixed with 30 μL of hydrazine and incubated at room temperature for 8 minutes. The reaction was terminated by the addition of 200 μL Hz stop buffer, followed by precipitation with 750 μL of precooled (-70°C) 95% ethanol.

All samples were then processed as follows. After ethanol precipitation by incubation on dry ice for 20 minutes, samples were centrifuged at 14000 g for 15 minutes at 4°C. DNA pellets were resuspended in 225 μL of distilled water and 25 μL of 3 M Na acetate (pH 5) and then reprecipitated by addition of 750 μL of precooled (-70°C) 95% ethanol and incubation on dry ice for 20 minutes. After collection by centrifugation, the

DNA was washed with 70% ethanol and dried under vacuum. Pellets were then dissolved in 100 μL of 1 M piperidine (freshly diluted; Aldrich), the microfuge tube caps sealed with Teflon^{TM} tape, and the samples heated at 95°C for 30 minutes. Once samples had cooled to room temperature, they were frozen on dry ice, and dried under vacuum. They were then subjected to four wash cycles consisting of resuspension in decreasing volumes (50 μL, 50 μL, 20 μL, and 10 μL) of distilled water, freezing on dry ice, and drying under vacuum; the final drying step was allowed to proceed overnight. Finally, samples were resuspended in 10 μL distilled water and were ready for either examination by gel electrophoresis or further processing by ligation mediated PCR.

2.8. Preparation of a GST Fusion Protein

2.8.1. Induction of Protein and Preparation of Bacterial Lysate

Purified GST fusion proteins were prepared as follows. *E.coli* transformed with plasmid coding for the fusion protein were grown to an optical density at 600 nm of 0.9 in 1 L of TB at 37°C. Production of fusion protein was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM and incubation of the culture for 2 hours at 37°C. All subsequent steps were performed at 4°C. Cells were harvested by centrifugation at 11000 g for 15 minutes. Pellets were washed by resuspension in 400 μL of 50 mM Tris-HCl (pH 8.0) and recovered by a second centrifugation. Cells were lysed in 25 mL

of lysis buffer (13% sucrose, 100 mM EDTA (pH 8.0), 20 mM EGTA (pH 8.0), 50 mM Tris-HCl (pH 8.0), 250 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mg/mL lysozyme). After a 15 minute incubation on ice, Nonidet P40 (NP40) was added to a final concentration of 0.1% and a second incubation for 15 minutes on ice allowed. Cellular debris was pelleted by centrifugation at 150000 g for 1 hour in a Beckman Ti50.2 rotor. The supernatant was then ready for purification of the fusion protein.

2.8.2. Purification of GST fusion protein

GST fusion proteins were separated from the bacterial lysate on the basis of the ability of the GST moiety of the protein to bind to Glutathione-Sepharose 4B (Pharmacia). Sufficient Glutathione-Sepharose 4B was added to a 10 cm Bio-Rad Econo-Column chromatography column to produce a bed volume of 4 to 4.5 mL. Throughout the following wash steps and application of the sample to the column, pressure was applied with a peristaltic pump in order to increase the flow rate. The column bed was washed with 30 to 50 mL of phosphate buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 880 mM KH_2PO_4, 6.41 mM Na_2PO_4, pH 7.3) and equilibrated with 15 mL of PBS containing 1% Triton-X-100. The bacterial lysate was then applied to the column, followed by washes first with 60 mL of PBS containing 1% Triton-X-100, and then with 60 mL of PBS. Bound material was eluted with 22 mL of 50 mM Tris-HCl (pH 8.0) containing 5 mM glutathione. The first few drops were collected to measure the 'background', and then eleven 2 mL fractions were collected. The column was regenerated by the application of

30 mL PBS containing 3 M NaCl. An approximation of the protein concentration of each fraction collected was made using a Bio-Rad Protein Assay kit (based on the method of Bradford) according to the manufacturer's directions. Samples containing an appreciable quantity of protein were pooled and dialyzed against 2 L of Dialysis buffer (100 mM KCl, 20 mM HEPES (pH 7.9), 10% glycerol, 0.5 mM PMSF, 0.5 mM dithiothreitol (DTT)). The dialysis buffer was changed twice; a minimum of six hours was allowed between each change. The final concentration of the fusion protein was measured using the Bio-Rad Protein Assay kit; BSA was used to establish a standard concentration curve. The protein sample was divided into aliquots and stored at -70°C.

2.9. DNA Affinity Chromatography

The method employed for DNA affinity chromatography was based on that of Kadonaga and Tjian (1986). First, multimers of DNA binding sites were prepared. Complementary oligonucleotides encoding the DNA binding site and having complementary 5' protruding ends were purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. 150 μg of each oligonucleotide were mixed with 2 μL of each ^{32}P-labelled oligonucleotide (1/50 of a kinase reaction, see Preparation of Probes) and 20 μL of 10x kinase buffer (500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 50 mM DTT) in a final volume of 200 μL. The annealing reaction was heated to 90°C, cooled slowly (0.5°C per minute) to 37°C , held at 37°C for 20 minutes, and then incubated at room

temperature for 20 minutes. 0.5 μL of the reaction mixture was counted in a scintillation counter. Complete hybridisation was confirmed by examination of 1 μL of the reaction after electrophoresis through a 15% polyacrylamide gel. The 5' ends of the oligonucleotide duplexes were then phosphorylated by incubation at 37°C for 2.5 hours with 100 U polynucleotide kinase (NEB) and 20 mM ATP (final concentration). Ammonium acetate and magnesium chloride were then added to the reaction mixture to final concentrations of 1.96 M and 10 mM respectively and the kinase inactivated by incubation at 65°C for 15 minutes. The oligonucleotide duplexes were ethanol precipitated and resuspended in 50 μL of distilled water. Excess salt was removed by application of the sample to a G-25 spun column (see Preparation of Probes) and centrifugation for 4 minutes at 1600 g. The spun column was then washed three times with 25 μL of TE (pH 7.6). 0.25 μL of each fraction was counted in a scintillation counter and the fractions containing the majority of the counts were pooled. 30 Weiss units of ligase (BRL) and sufficient 10x ligase buffer (supplied with the enzyme) to result in a final concentration of 1x were added and the reaction incubated overnight at 14°C. The degree of multimerisation of the binding site duplexes was examined by separating 2 μL of the reaction on a 15% polyacrylamide gel and exposing Kodak XAR-5 film to the gel for two hours. After adjustment of the sample volume to 200 μL with TE, the sample was extracted twice from an equal volume of 1:1 phenol:chloroform. 44 μL of 9 M ammonium acetate was added to the sample and the binding site multimers ethanol precipitated. After drying under vacuum, the sample was resuspended in 100 μL of distilled water. The quantity of the

multimerised oligonucleotide duplexes recovered was estimated by counting 0.25 μL in a scintillation counter.

The oligonucleotide duplex multimers were then linked to Sepharose. Three grams of CNBr-activated Sepharose 4B (Pharmacia) were swollen in 45 mL of 1 mM HCl for 15 minutes. The resin was then washed with 600 mL of 1 mM HCl in a sintered glass funnel and rinsed with 50 mL of 10 mM potassium phosphate (pH 8.0). Next, the resin was resuspended in 4 mL of 10 mM potassium phosphate (pH 8.0) and mixed with the ligated oligonucleotides on a rotary shaker at room temperature for 16 hours. The resin was collected on a sintered glass funnel and washed first with 5 mL of distilled water, then with 200 mL of distilled water, and finally with 100 mL of 1 M ethanolamine (pH 8.0). Next, the resin was resuspended in sufficient 1 M ethanolamine to give a total volume of 15 mL and mixed for 4 hours at room temperature on a rotary shaker. The resin was collected again on a sintered glass funnel and washed with 100 mL each of (in order): 10 mM potassium phosphate (pH 8.0), 1 M potassium phosphate (pH 8.0), 1 M KCl, distilled water, and finally, Storage buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.3 M NaCl, 0.02% sodium azide). The resin was transferred into a 20 cm Bio-Rad Econo-Column chromatography column and equilibrated with 50 mL of buffer Z (25 mM HEPES (pH 7.8), 12.5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, 0.1% NP40) containing 100 mM KCl. Pressure was applied with a peristaltic pump in order to increase the flow rate. The protein sample (diluted 1:5 in buffer Z containing 100 mM KCl) was applied to the column and the column then rinsed twice with 15 mL of buffer Z containing 100 mM KCl. DNA-bound protein was eluted from the column with 12 mL of

buffer Z containing 1 M KCl. The first few drops were collected to measure the 'background', and then six 2 mL fractions were collected. An approximation of the protein concentration of each fraction collected was made using a Bio-Rad Protein Assay kit according to the manufacturer's directions. Samples containing an appreciable quantity of protein were pooled and dialyzed against 2 L of Dialysis buffer (see Purification of GST Fusion Proteins). The column was regenerated by washing first with 30 mL of 5 mM Tris-HCl (pH 7.6) containing 2.5 M NaCl and 0.5 mM EDTA, and then with 30 mL of Storage buffer. The column was stored at 4°C in Storage buffer.

2.10. Southwestern Blots

The method employed for Southwestern blots was based on that of Michael et al. (1988). The DNA binding protein sample was subjected to electrophoresis through a denaturing SDS-polyacrylamide gel. After soaking the gel in Transfer buffer (48 mM Tris base, 39 mM glycine, 20% methanol) for 10 to 20 minutes., the protein was transferred from the gel to nitrocellulose membrane (soaked first in distilled water and then in Transfer buffer) using a Bio-Rad Trans-Blot SD semi-dry transfer cell. Transfer was effected by application of a voltage of 15 V for 12 minutes. Protein remaining in the gel was visualized by staining with Coomassie Brilliant Blue, and the gel dried and retained for comparison with the blot.

After transfer, the nitrocellulose was subjected to three 1 hour washes with 200 mL of Buffer A (10 mM Tris-HCl (pH 7.2), 5% skim milk,

10% glycerol, 2.5% NP40, 0.1 mM DTT, 150 mM NaCl). After a quick rinse with 50 mL of Buffer B (10 mM Tris-HCl (pH 7.2), 50 mM NaCl, 0.125% skim milk), hybridisation with 5 to 7 million cpm of a ^{32}P-labelled oligonucleotide probe (bearing the DNA binding site) was performed by gentle agitation in 10 mL of Buffer B overnight at room temperature. The nitrocellulose was then washed three times with 200 mL of Buffer B (minus the skim milk) and air-dried. Proteins binding the DNA probe were visualized by exposure of Kodak XAR-5 film to the nitrocellulose overnight.

2.11. Capture Assays

DNA fragments bearing protein binding sites were isolated from a mixture of DNA fragments on the basis of their ability to be bound by a DNA binding protein-GST fusion protein; these were termed capture assays. Plasmid DNA was digested with a restriction enzyme to generate 5' protruding ends, and then labelled with ^{32}P-dATP or dCTP (see Preparation of Probes). 7 µL of Binding buffer 1 (28.6 mM Tris-HCl (pH 7.6), 2.86 mM EDTA, 0.286% NP40, 429 mM KCl); sheared, denatured herring sperm DNA (final concentrations ranging from 25 µg/mL to 3.2 mg/mL); and 10 µg of DNA binding protein-GST fusion protein were mixed in a total volume of 19 µL and incubated for five minutes at room temperature. Then, 1 µL of ^{32}P-labelled DNA (ca. 50000 cpm) was added and the reaction incubated for 45 minutes at room temperature.

Meanwhile, 20 µL per reaction of undrained Glutathione Sepharose 4B was washed five times with 1 mL of Binding buffer 2 (10 mM Tris-HCl

(pH 7.6), 1 mM EDTA, 0.1% NP40, and 150 mM KCl). The resin was then resuspended in sufficient Binding buffer 2 to result in a final volume of 50 µL per reaction.

Once incubation of the binding reaction between the DNA and the fusion protein was complete, 350 µL of Binding buffer 2 containing sufficient sheared, denatured herring sperm DNA to maintain the concentration was added to the reaction tubes along with 50 µL of the washed, resuspended resin. The reactions were then mixed on a rotary shaker for 2 hours at room temperature to allow binding of the DNA-fusion protein complex to the Glutathione Sepharose 4B. After washing of the resin twice with 1 mL of Binding Buffer 2 containing herring sperm DNA, the isolated DNA and fusion protein were eluted from the resin by mixing with 400 µL of urea-SDS (7 M urea, 350 mM NaCl, 10 mM Tris-HCl (pH 7.8), 10 mM EDTA, 1% SDS) for a minimum of 1 hour on a rotary shaker at room temperature. The supernatant was collected and extracted once against 1:1 phenol:chloroform. The DNA was then precipitated twice with ethanol, quantitated by counting in a scintillation counter, and examined by autoradiography following separation by electrophoresis through a urea-polyacrylamide gel.

2.12. Preparation of Probes

2.12.1. Labeling of Double-Stranded DNA

^{32}P-labelled double-stranded DNA was used in capture assays, Southwestern blots, DNAse I footprinting assays, and base-specific cleavage sequencing reactions. 5' protruding ends were generated on plasmid DNA (or a previously isolated plasmid DNA fragment) by restriction enzyme digestion for 2 or 3 hours at 37°C in a 30 µL reaction volume. Next, 5 µL of either (α-^{32}P)dATP or dCTP (10 mCi/mL, 3000 Ci/mmol; NEN), 1 µL each of 100 mM dGTP, dTTP, and dATP or dCTP (as appropriate), and 1 µL (5 U) of the large fragment of DNA polymerase I (Klenow) (NEB) were added and reaction incubated for 1 to 3 hours at room temperature. In cases where a probe labelled at one end only was required, this was accomplished either by codigestion with a second restriction enzyme generating a blunt end, or by digestion with a second restriction enzyme following ethanol precipitation of the labeling reaction. In cases where a labeling reaction was not immediately preceded by a restriction enzyme digest, the labeling reaction was performed according to the enzyme manufacturer's directions, using their supplied buffer. Unincorporated (α-^{32}P)dATP or dCTP was removed by one of the methods described below.

2.12.2. Labeling of Single-Stranded Oligonucleotides

^{32}P-labelled oligonucleotides were used in colony hybridisations, primer extension assays, DNA affinity chromatography and ligation mediated PCR. 20 to 40 ng of oligonucleotide was mixed on ice with 5 µL of 10x kinase buffer (supplied by enzyme manufacturer) in a total volume of 44 µL. 5 µL of (γ-^{32}P)ATP (10 mCi/mL, 3000 Ci/mmol; NEN) was added, followed by 1 µL (10 U) of polynucleotide kinase (NEB). The reaction was incubated for one hour at 37°C and then terminated by the addition of 200 µL of 2.5 M ammonium acetate (pH 7.5). The sample was applied to a G-50 Sephadex spun column to remove unincorporated (γ-^{32}P)ATP (see below).

2.12.3. Random Primer Labeling of DNA

Random primer labelled DNA probes were used in Southern blots. 50 to 200 ng of plasmid DNA and 2 µL of pd(N)$_6$ primers (Pharmacia) were mixed in a final volume of 38 µL, boiled for five minutes, and then quickly cooled at -20°C in a Stratacooler. The tube was spun briefly in a microfuge to collect the liquid at the bottom of the tube. Next, the following were added (in order): 6 µL 10x Klenow buffer (500 mM Tris-HCl (pH 7.2), 100 mM magnesium sulphate, 60 mM 2-mercaptoethanol, 500 µg/mL BSA), 3 µL each of 1 mM dATP, dGTP, and dTTP, 5 µL of (α-^{32}P)dCTP, and 2 µL (10 U) of the large fragment of DNA polymerase I (Klenow). The mixture was incubated at room temperature for 2 to 48 hours. 150 µL of distilled water was then added to the reaction and the sample applied to a G-50 Sephadex

spun column to remove unincorporated deoxynucleotides (see below). Before use for hybridisation, the probe was denatured at 95°C for several minutes.

2.12.4. RNA Probes

In vitro transcribed RNA probes were used in ligation mediated PCR. An in vitro transcription kit (Promega) was used to make the riboprobe following a modified version of the manufacturer's protocol. The technique utilized a recombinant plasmid in which the desired probe sequences were inserted into the multiple cloning site of pGEM-3Z (Promega). Prior to the transcription reaction, the plasmid was linearized at a restriction site immediately 3' of inserted sequences. The following were mixed in order: 14 µL of distilled water, 12 µL of 5x Transcription buffer (200 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 6 µL of 100 mM DTT, 2 µL (2 µg) of linearized plasmid, 10 µL (α-^{32}P)GTP (10 mCi/mL, 3000 Ci/mmol; NEN), 6 µL of unlabelled GTP, and 3 µL each of 10 mM ATP, CTP, and UTP. Then 2 µL of RNAsin (80 U) and 3 µL (60 U) T7 RNA polymerase were added and the reaction incubated for 1 hour at 37°C. 40 µL of distilled water, 1 µL of 1M $MgCl_2$, and 4 µL of RQ DNase were then added and the reaction incubated for 10 minutes at 37°C. Next, 100 µL of distilled water, 20 µL of 3 M sodium acetate (pH 5), and 15 µL (45 µg) of carrier tRNA were added. The riboprobe was ethanol precipitated and resuspended in 210 µL distilled water. 100 µL probe was used per hybridisation tube.

2.12.5. Elimination of Unincorporated (Deoxy)Nucleotide

DNA Probes were separated from unincorporated (deoxy)nucleotides and/or other DNA fragments by a variety of methods, depending on the intended use of the probe. In some cases, 10 μg of carrier tRNA and sodium acetate or ammonium acetate (to final concentrations of 0.3 M and 2 M, respectively) were added to the reaction, and the DNA ethanol precipitated. After a second ethanol precipitation, most of the unincorporated (deoxy)nucleotides were eliminated. Probes were usually resuspended in 100 μL distilled water.

Alternatively, in some cases unincorporated (deoxy)nucleotides were eliminated by application to a Sephadex G-50 column. The bottom of a disposable 1 mL syringe was plugged with a small quantity of sterile siliconized glass wool. The syringe was then filled with Sephadex G-50 equilibrated in 1x TEN buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), and 100 mM NaCl). Excess buffer was squeezed out with the syringe plunger. More Sephadex G-50 was added and the process repeated until the syringe was full. The syringe was inserted into a 15 mL tube and centrifuged at 1600g for 4 minutes at room temperature. The DNA sample, its volume adjusted to 100-200 μL, was then applied to the column. The column was placed in a fresh 15 mL tube and centrifuged at 1600g for 4 minutes at room temperature. The column was then discarded and the eluted DNA transferred from the 15 mL tube to a microfuge tube.

In some instances, the desired DNA probe was one of several species of fragments present in the reaction. In these cases, the sample was

separated by electrophoresis through a standard polyacrylamide gel (or, for oligonucleotides to be used in primer extension assays, a urea-polyacrylamide gel) and the chosen fragment eluted from the gel (see Elution of DNA fragments from Gels). If the reaction volume was inconveniently large for loading onto a gel, the DNA was ethanol precipitated and resuspended in a smaller volume.

2.12.6. Elution of DNA Fragments from Gels

DNA fragments were eluted from agarose and polyacrylamide gels for use in cloning and in the production of labelled DNA probes. Unlabelled DNA fragments were visualized by staining with ethidium bromide (see gel electrophoresis). Labelled DNA was visualized by autoradiography. Bands in the gel were located by aligning dots of ^{32}P-containing ink which had been placed in the corners of the gel with their autoradiographic images. A thin slice of the gel containing the selected DNA fragment was excised with a razor blade and transferred to a microfuge tube. DNA fragments were extracted from agarose gels by the Qiaex agarose gel extraction procedure (Qiagen), according to the manufacturer's directions.

Gel slices from polyacrylamide gels were crushed with a Teflon plunger (not required for slices from thin urea-polyacrylamide gels). Sufficient Elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS) to cover the acrylamide (typically, 400 µL) was added. 10 to 20 µg of carrier tRNA was also added if it would not interfere with later uses of the DNA. DNA was eluted from the gel

overnight at 37°C. The sample was then centrifuged at 14000 g for 5 minutes to pellet the acrylamide. After the supernatant was transferred to a fresh tube, a volume of elution buffer equal to the volume used for the elution step was added. The sample was vortexed and centrifuged at 14000 g for 5 minutes. The supernatants were pooled and applied to a column of sterile siliconized glass wool in a disposable 1 mL syringe. The column was placed in a 15 mL tube and centrifuged at 1600 g for 2 minutes at room temperature. The sample was ethanol precipitated twice and resuspended in distilled water.

2.13. DNase I Footprinting

The DNase I footprinting procedure was based on that of Galas and Schmitz (1978) with some of the modifications of Brenowitz et al. (1989). In the preparation of DNA fragments from plasmids for use as footprinting probes, restriction enzymes were selected such that one would cut 25 to 100 bp from the predicted DNA binding site (generating 5' protruding ends), and the second would cut 100 to 600 bp from the other side of the DNA binding site (preferably generating blunt ends). After restriction enzyme digest, the DNA fragment was ^{32}P-labelled at the 5' protruding end (see Preparation of Probes). After separation from the other products of the restriction digest by gel electrophoresis, the probe fragment was excised and eluted from the gel.

25 µL of 2x Binding buffer (50 mM Tris-HCl (pH 7.9), 4 mM $MgCl_2$, 20% glycerol, 1 mM EDTA, 1 mM DTT), 0.5 µg of sheared herring sperm

DNA, and 0.15 to 20 μg of DNA binding protein-GST fusion protein were mixed in a total volume of 50 μL and incubated for 5 minutes at room temperature. 2 μL (20000 cpm) of ^{32}P-labelled DNA probe was added and the binding reaction incubated for 20 minutes at 30°C. The protein-bound DNA was then digested for 60 seconds at room temperature with 1-5 μL of DNase I (Pharmacia) freshly diluted to a concentration of 94 U/mL (1:80) in DNase I dilution buffer (50 mM Tris-HCl (pH 7.2), 10 mM $MgSO_4$, 1 mM DTT, 50% glycerol, 25 mM $CaCl_2$). DNase I digestion was terminated by addition of 100 μL of DNAse I Stop buffer (1% SDS, 100 μg/mL tRNA, 200 mM NaCl, 20 mM EDTA, 200 μg/mL proteinase K), followed by incubation for 10 minutes at 50°C. The DNA was extracted once from an equal volume of 1:1 phenol:chloroform and once from an equal volume of chloroform, then ethanol precipitated, separated on a urea-polyacrylamide gel, and visualized by autoradiography.

2.14. Mammalian Tissue Culture

African Green Monkey Kidney (Vero) cells were used throughout this study. Cells were grown as monolayer culture in Corning 150 cm^2 tissue culture flasks or 175 cm^2 plates at 37°C in a humidified environment in the presence of 5% CO_2. Cells were maintained in α minimal essential medium (αMEM) (Gibco) supplemented with 5% fetal bovine serum (FBS) (Bocknek), 2 mM L-glutamine (Gibco), 100 U/mL penicillin G (Gibco), and 100 μg/mL streptomycin sulphate (Gibco). This is designated as complete medium.

2.15. Growth and Titration of Virus

Stocks of HSV-1 and HSV-1 recombinants were prepared as follows. Confluent monolayers of Vero cells in 175 cm^2 plates (containing approximately 1 x 10^7 cells) were infected at a moi of approximately 0.05 in 5 mL of serum free medium (αMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulphate). Infection was allowed to proceed for 2 hours at 37°C, at which point 20 mL of complete medium was added. Cultures were allowed to grow at 37°C in a humidified environment in the presence of 7.5% CO_2 until confluent cpe was observed (2 or 3 days). Cells were scraped from the plates and collected in 50 mL tubes. After centrifugation at 1100 g for 10 minutes at 4°C, cell pellets were resuspended in PBS and pooled into a single tube. Cells were collected again by centrifugation and the pellet resuspended in 1 mL serum free medium per 175 cm^2 plate harvested. Cells were disrupted by sonification using a Braun-Sonic 2000 sonicator and cellular debris removed by centrifugation at 2000 g for 10 minutes at 4°C. The virus-containing supernatant was aliquoted into cryovials (Nunc) and stored at -70°C.

Virus stocks were titrated by making serial dilutions of 10^{-2} to 10^{-7} in serum free medium and using 0.5 mL of each dilution to infect confluent monolayers of Vero cells in 6-well dishes (Corning). After incubation at 37°C for 2 hours, 3 mL per well of complete medium supplemented with 0.05% human immune serum (Connaught Laboratories) was added. Once plaques had developed (generally after 3 days), medium was carefully

removed, the monolayer stained with crystal violet (61% ethanol, 8.7% Formalin, 4.3% acetic acid, 1% crystal violet), and the plaques counted.

2.16. Construction of Recombinant Viruses

Recombinant viruses were constructed as described by Smiley (1980) and Smiley et al. (1981). Mutations were introduced into HSV-1 strain PAA^{r}5 by coinfection of cells with infectious HSV-1 DNA and linearized plasmid containing the desired sequences flanked by sequences from the HSV-1 thymidine kinase (tk) gene. This was followed by selection for tk deficient viruses.

2.16.1. Preparation of Infectious DNA

Ten 175 cm^2 plates of confluent Vero cells were infected with HSV-1 (strain PAA^{r}5) at an moi of 10 in 5 mL of serum free medium. After 2 hours, 20 mL of complete medium was added and the virus allowed to grow for 1 or 2 days until confluent cpe was evident. Cells were scraped from the dishes, collected in 50 mL tubes, and harvested by centrifugation at 1100 g for 10 minutes at 4°C. Cell pellets were pooled after resuspension in a total volume of 25 mL PBS. Cells were collected again by centrifugation and resuspended in 10 mL of 200 mM EDTA (pH 8.0). Proteinase K and SDS were added to final concentrations of 100 mg/mL and 0.5%, respectively, and the suspension incubated overnight at 37°C. It was then extracted four times with equal volumes of 1:1 phenol:chloroform, inverting gently to mix.

The DNA was then dialysed against 2 L of 0.1x SSC at 4°C for a minimum of 3 days; the dialysis buffer was changed daily. The infectious DNA was transferred to a sterile 50 mL tube in a laminar flow hood and stored at 4°C.

The optimal amount of the infectious virus stock to use in transfection assays was determined by transfecting (see Transfection Assays) monolayers of Vero cells in 100 mm plates with a range of volumes (10 to 80 μL) of the virus stock. After three days, plates were examined to determine which had infected optimally.

2.16.2. Transfection Assays

Transfections of cells were performed as follows. 250 μL of 2x HEPES (1% HEPES, 1.6% NaCl, 0.074% KCl, 0.02% Na_2PO_4, 0.2% dextrose), 10 μL of sonicated salmon sperm DNA, infectious viral DNA (optimal amount determined empirically), and 0.5 μg of linearized plasmid DNA were mixed in a total volume of 475 μL. After the addition of 25 μL of 2.5 M $CaCl_2$, the sample was mixed gently and incubated at room temperature for 20 minutes. It was then added to a 100 mm plate of Vero cells (50 to 60% confluent) and incubated for 3 to 5 hours at 37°C. Next, the medium was removed and 0.5 mL of αMEM supplemented with 10% FBS and 15% glycerol added to the cells. After incubation at room temperature for 1.5 minutes, the cells were washed twice with 10 mL serum free medium, then covered with 10 mL of complete medium. Transfected cells were allowed to grow until confluent cpe was observed (4 to 8 days).

Cells were scraped from the plate and collected in a 15 mL tube. After centrifugation at 1100 g for 10 minutes at 4°C, the cell pellet was resuspended in 1 mL of serum free medium. Cells were disrupted by sonication, cellular debris pelleted by centrifugation at 2000 g for 10 minutes at 4°C, and the supernatant transferred to a cryovial and stored at -70°C.

2.16.3. Selection for tk- Virus

Virus deficient in HSV-1 tk activity were selected as follows. Serial dilutions of 10^{-2} to 10^{-7} in serum free medium were made of virus harvested from transfections. 0.5 mL of each dilution and 1.5 mL of serum free medium were added to a 100 mm plate of confluent Vero cells. Plates were incubated for 2 hours at 37°C, then 8 mL of complete medium supplemented with 0.0625% human immune serum and 1.25 μg/mL bromodeoxycytosine (BrdC, the selection reagent) (final concentrations in plate: 0.05% and 100 μg/mL, respectively) were added. The infection was allowed to proceed until distinct plaques were observed (2 or 3 days). Individual large plaques were selected for further examination. Infected cells from each plaque were transferred by stabbing the plaque with a sterile wooden stick and swirling the stick in a well of a 24-well plate of confluent Vero cells. Each well of the 24-well plate contained 1 mL of complete medium supplemented with 100 μg/mL BrdC. When confluent cpe was evident (1 or 2 days), the infected cells were harvested. From one half of the infected cells, DNA was extracted for examination by Southern blotting to select positive recombinants. The other half of the cells was frozen at

-70°C, reserved for use in future cycles of plaque selections to purify positive virus recombinants.

2.16.4. Rapid Extraction of Viral DNA

DNA from one half of a well of infected cells in a 24-well plate was prepared as follows. Cells were centrifuged for five minutes at 14000g. The cell pellet was resuspended in 400 µL of pronase/SDS buffer (10 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% SDS). 10 µL of 20 mg/mL proteinase K was added and the sample incubated at 37°C for 4 hours or overnight. It was then extracted 2 or 3 times against equal volumes of 1:1 phenol:chloroform. The DNA was ethanol precipitated twice and then resuspended in 20 µL distilled water; 10 µL was used for analysis by Southern blot.

2.16.5. Southern Blots

Positive recombinant viruses were identified by Southern blot (Southern, 1975). Viral DNA isolated from one fourth of a well of a 24-well plate (or 10-20 ng of plasmid DNA) was digested with appropriate restriction enzyme(s). The DNA fragments were then separated by electrophoresis through a 1.4% agarose gel. Next, the gel was soaked twice in 0.25 M HCl for 15 minutes, once in 0.5 M NaOH for 30 minutes, once in 1 M Tris-HCl (pH 7.5) containing 1.5 M NaCl for 30 minutes, and finally, in 20x SSC for 30 minutes.

DNA was transferred from the gel to nitrocellulose or nylon membrane in the following manner. A large piece of plastic wrap was placed on a glass plate. Four pieces of 3 MM paper (cut to the size of the gel) soaked in 20x SSC were placed on the plastic wrap. The gel was placed on the paper, followed by a piece of membrane which had been soaked first in water, and then in 20x SSC. Tight contact between the gel and the membrane was ensured by rolling air bubbles out with a pipette. Two more pieces of 3 MM paper soaked in 20x SSC were placed on top of the membrane, rolling out air bubbles between each layer. The edge of the plastic wrap was rolled up to encase the sandwich on all sides. A thick stack of paper towels was placed on top of the sandwich, ensuring that contact between the paper towels and the 3 MM paper was even. A second glass plate was placed on top of the paper towels and a weight placed on the plate. Transfer was allowed to proceed overnight. Alternatively, a vacuum blotter (Stratagene) was used to transfer the DNA from the gel to a membrane, following the manufacturer's directions and using 20x SSC as the transfer buffer. Once transfer was complete, the DNA was crosslinked to the membrane using a UV crosslinker (Stratagene), following the manufacturer's directions.

Hybridisation of a random primed probe to the DNA affixed to the membrane was performed in a hybridisation oven (Techne). The membrane was first incubated for one hour at 65°C in Church buffer (250 mM sodium phosphate (pH 7.2), 7% SDS, 1% BSA, 1 mM EDTA). The buffer was then replaced with fresh Church buffer, the probe added, and hybridisation allowed to proceed overnight at 65°C. The membrane was

washed twice with 500 mL of 2x SSC containing 0.1% SDS and twice with 500 mL of 0.1x SSC containing 0.1% SDS (Both wash buffers were at 60°C). After the membrane was air-dried, it was wrapped in plastic wrap and the probe-bound DNA visualized by autoradiography.

2.17. Preparation of Total Cellular RNA

Total cellular RNA was extracted from infected cells using a modified version of the RNAzol B method (TEL-TEST, Inc.) which is based on the method of Chomczynski and Sacchi (1987). 100 mm plates of confluent Vero cells (containing approximately 5 x 10^6 cells) were infected at an moi of 10 in 2 mL of serum free medium. Where required (to establish a block in DNA replication), 10 μg/mL aphidicolin was added at the time of infection. After two hours, 7 mL of complete medium was added to the cells, maintaining the correct concentration of aphidicolin. To harvest the RNA, cells were scraped to one edge of the plate and 1 mL of RNAzol B added. The lysate was passed through a pipette several times to solubilize RNA and transferred to a microfuge tube. After the addition of 200 μL of chloroform, the suspension was incubated for 5 minutes on ice. Samples were then centrifuged at 14000 g for 15 minutes (4°C). Following an extraction with an equal volume of chloroform, the RNA was precipitated with an equal volume of isopropanol. After a second precipitation with ethanol, the pellet was washed 3 times with 1:3 diethylpyrocarbonate (DEPC)-treated 0.4 M sodium acetate:ethanol, once with 70% ethanol, and once with 95% ethanol. Pellets were dried under vacuum for 10-15 minutes and redissolved in 600

μL DEPC-treated water. RNA was quantified by measurement of the optical density at 260 nm of a 10^{-1} dilution of the RNA, using a UV spectrophotometer (LKB Biochem Ultrospec II).

2.18. Primer Extension Assays

The protocol for primer extension assays was based on the method of Jones et al. (1985). 15 μg of carrier tRNA and DEPC-treated sodium acetate (to a final concentration of 0.3 M) were added to 10-20 μg of RNA and the RNA precipitated overnight at -70°C with three volumes of ethanol. The RNA was collected by centrifugation at 14 000 g for 30 minutes at 4°C, washed with 70%, then 95% ethanol, and dried under vacuum for 45 to 60 minutes. The RNA pellet was resuspended in 8 μL of TE (pH 7.9) containing 40 000 to 80 000 cpm of labelled oligonucleotide. 2 μL of TKE (10 mM Tris-HCl (pH 7.9), 1.25 M KCl, 1 mM EDTA) were added and hybridisation of the oligonucleotide to the RNA allowed to proceed for 1 hour at 62-63°C. The reaction was cooled to room temperature and centrifuged briefly to collect the fluid at the bottom of the tube. 25 μL of Reverse Transcription buffer (20 mM Tris-HCl (pH 8.7), 10 mM $MgCl_2$, 5 mM DTT, 0.33 mM of each dNTP, 10 μg/mL actinomycin D) containing 10 U of AMV reverse transcriptase was added and the extension reaction incubated for one hour at 37°C. The DNA and RNA were precipitated with 500 μL ethanol at -70°C overnight and then collected by centrifugation at 14000g for 30 minutes at 4°C. The pellet was washed first with 70% ethanol, then with 95% ethanol, dried under vacuum, and redissolved in 8 μL of distilled water and 4 μL of

sequencing loading dye. The DNA was subjected to electrophoresis through a sequencing gel and visualized by autoradiography.

2.19. Densitometry

Densitometry was performed using a Hoefer Scientific GS 300 Transmittance/Reflectance Scanning Densitometer. Autoradiograms to be examined by densitometry were made using intensifier screens and Kodak XAR-5 film which had been preflashed such that its absorbance at 545 nm increased by 0.1-0.2 OD units.

2.20. Ligation Mediated Polymerase Chain Reaction

Ligation mediated polymerase chain reaction (PCR) was performed using a protocol based on the methods of Pfeifer et al. (1989), Mueller and Wold (1989), and Steigerwald et al. (1990). Restriction enzyme cut or base specific cleavage cut plasmid DNA was used.

2.20.1. Primer Extension with Sequenase™

A single blunt end was generated on fragments containing the sequence of interest in the following manner. 0.5-5 μg of cut plasmid DNA was mixed with 0.6-2.0 pmoles of primer 1 (17 or 18-mer oligonucleotide with calculated Tm of 50 to 56°C) and 3 μL of 5x Sequenase™ buffer (200 mM Tris-HCl (pH 7.7), 250 mM NaCl) in a total volume of 15 μL. The

oligonucleotide was annealed to the denatured DNA by incubating the mixture for 3 minutes at 95°C and then 30 minutes at 45°C. The reaction was transferred onto ice and centrifuged briefly to collect the fluid at the bottom of the tube. 7.5 µL of cold (4°C) Mg-dNTP mix (20 mM $MgCl_2$, 20 mM DTT, 0.25 mM of each dNTP; freshly prepared) was added, followed by 1.5 µL of 1:4 diluted Sequenase™ (USB, 13 U/µL, diluted in cold 1x TE (pH 8.0)). The extension reaction was allowed to proceed for 15 minutes at 45-48°C and then cooled on ice. After addition of 6 µL of cold 310 mM Tris-HCl (pH 7.7), the enzyme was inactivated by incubation at 67°C for 15 minutes. It was then transferred onto ice and centrifuged briefly to collect the fluid at the bottom of the tube.

2.20.2. Ligation of Linker to DNA fragments

DNA fragments were provided with a second common end by ligation of a linker to the blunt ends formed by primer extension of the DNA. First, the linker was prepared. An 11-mer (5'-GAATTCAGATC-3') was annealed to a 25-mer (5'-GCGGTGACCCGGGAGATCTGAATTC-3') in 250 mM Tris-HCl (pH 7.7) (final concentration of each oligonucleotide was 20 pmol/µL) by heating to 95°C for 3 minutes and then gradual cooling (-0.5°C/minute) to 4°C. Linkers were stored at -20°C and kept on ice while in use.

45 µL of freshly prepared Ligation mix (13.33 mM $MgCl_2$, 30 mM DTT, 1.66 mM ATP, 83.3 µg/mL BSA, 100 pmol/reaction linker, and 3 U/reaction T4 DNA ligase) was added to the primer extended DNA and the reaction incubated overnight at approximately 17°C. Once the ligase had

been inactivated by incubation at 70°C for ten minutes, the sample was centrifuged briefly and 8.5 µL of 3 M sodium acetate (pH 5) and 10 µg of carrier tRNA were added. The DNA was precipitated with 3 volumes of ethanol and redissolved in distilled water to a final concentration of 1 µg/mL.

2.20.3. Amplification by PCR

The conditions used for PCR amplification were subject to much experimental variation. The conditions which most consistently gave the best results are outlined below. The longer oligonucleotide of the linker and a 25 or 26-mer coding which overlapped the 3' end of the oligonucleotide used for primer extension by Sequenase™ (above) were used as PCR primers. For each reaction, 49 µL of distilled water, 10 µL of 10x PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), 10 µL of 10 mM $MgCl_2$, 5 µL of each primer (1 OD/mL), and 1 µL of Amplitaq polymerase (5 u/µL; Perkin-Cetus) were mixed in a 500 µL microfuge tube and overlaid with 75 µL mineral oil. 1 µL containing 10 pg of linker-ligated restriction enzyme cut DNA or 1 ng of linker-ligated base specifically cleaved DNA was added to each reaction and the mixture heated at 80°C for 10 minutes. After addition of 20 µL of 1 mM dNTPs, 18 or 19 PCR cycles were performed. One cycle consisted of incubation for 1 minute at 96°C, 2 minutes at 66°C, and 3 minutes (plus 5 seconds per cycle) at 76°C. Once all cycles were complete, samples were incubated at 72°C for

10 minutes to allow complete extension of all products. Samples were stored at 4°C until ensuing steps could be performed.

The following variations were tried and resulted in minor but inconsistent improvements in results. In some cases, to aid in amplification of GC-rich DNA, 7'-deaza-dGTP was substituted for dGTP and /or 5% glycerol was added to the PCR reaction. Amplification with the more thermostable Vent polymerase and the Stoffel fragment of Amplitaq (Perkin-Cetus) was performed, using the buffers supplied by their manufacturers. In some cases, samples were held at 96°C for one minute prior to PCR cycling in order to enhance the initial denaturation step.

Once amplification by PCR was complete, 20 μL of Stop solution was added, to result in a final concentration of 300 mM sodium acetate (pH 5), 20 mM EDTA, and 5 μg per sample of tRNA. If detection of amplified products was to be done by primer extension, this was performed prior to addition of Stop solution. Samples were transferred to 1.5 mL microfuge tubes, extracted against equal volumes of 1:1 phenol:chloroform, and precipitated twice with 3 volumes of ethanol. The PCR products were then separated by electrophoresis through a urea-polyacrylamide gel.

2.20.4. Detection of PCR-Amplified DNA by Hybridization

After separation through a urea-polyacrylamide gel, PCR-amplified DNA fragments were transferred from the gel to a nylon membrane (GeneScreen, NEN) using the vacuum blotting technique of Gross et al. (1988). First the gel was transferred onto a dry sheet of Whatman 3 MM

paper. A nylon membrane, prewetted in Transfer buffer (50 mM ammonium acetate, pH 6.7), was placed on the gel, and air bubbles between the gel and the membrane forced out. Excess 3 MM paper and nylon were trimmed and the paper/gel/nylon sandwich inverted onto a stack of five sheets of 3 MM paper which were a little larger (2-6 cm) than the sandwich. The lower four sheets had been soaked previously in 500 mM ammonium acetate (pH 6.7) and dried for 10-16 hours at 50-60°C (They were stored at room temperature in a sealed container). The stack was thoroughly wetted with 200 mL Transfer buffer and then placed on a gel drier, allowing at least 1 cm between the stack and the edges of the mesh surface of the gel drier. Four sheets of 3 MM paper (cut approximately 1 cm smaller in both dimensions than the sheets in the lower stack) soaked in Transfer buffer were placed on the stack in a staggered fashion, and an additional 50 mL of Transfer buffer poured over the stack. Transfer was effected by application of a vacuum overnight. Two vacuum sources were successfully used: a tap aspirator or a strong vacuum pump connected to a lyophiliser or dry ice trap.

Once transfer was complete, DNA fragments were covalently linked to the nylon membrane. The wet nylon membrane was covered with plastic wrap and irradiated for 6 minutes using a 254 nm UV crosslinker (Stratagene).

Hybridisation to a riboprobe was performed in a hybridisation oven (Techne) by a modified form of the technique of Church and Gilbert (1984). The membrane was incubated for one hour at 65°C in Church buffer (250 mM sodium phosphate (pH 7.2), 7% SDS, 1% BSA, 1 mM EDTA). The buffer

was then replaced with fresh Church buffer, the probe added, and hybridisation allowed to proceed overnight. The membrane was washed twice with 500 mL of Wash buffer 1 (20 mM sodium phosphate (pH 7.2), 2.5% SDS, 0.25% BSA, 1 mM EDTA) and twice with 500 mL of Wash buffer 2 (20 mM sodium phosphate (pH 7.2), 1 mM EDTA, 1% SDS) (Both wash buffers were at 60°C). After the membrane was air-dried, it was wrapped in plastic wrap and the probe-bound DNA visualized by autoradiography.

2.20.5. Detection of PCR-Amplified DNA by Primer Extension

Once thermal cycling was complete, amplified PCR products were detected by performing an additional PCR cycle using a ^{32}P-end-labelled primer. After addition of 15 µL of 1x PCR buffer (supplemented with an additional 1 mM $MgCl_2$ and containing 2.5 U Amplitaq, 20 nmol each dNTP, and 1-5 pmol of an end-labelled primer), samples were heated 3 minutes at 96°C, allowed to hybridise for 2 minutes at 66°C, and extended for 10 minutes at 76°C. The reaction was terminated and the samples examined by urea-polyacrylamide gel electrophoresis as described above.

III. RESULTS

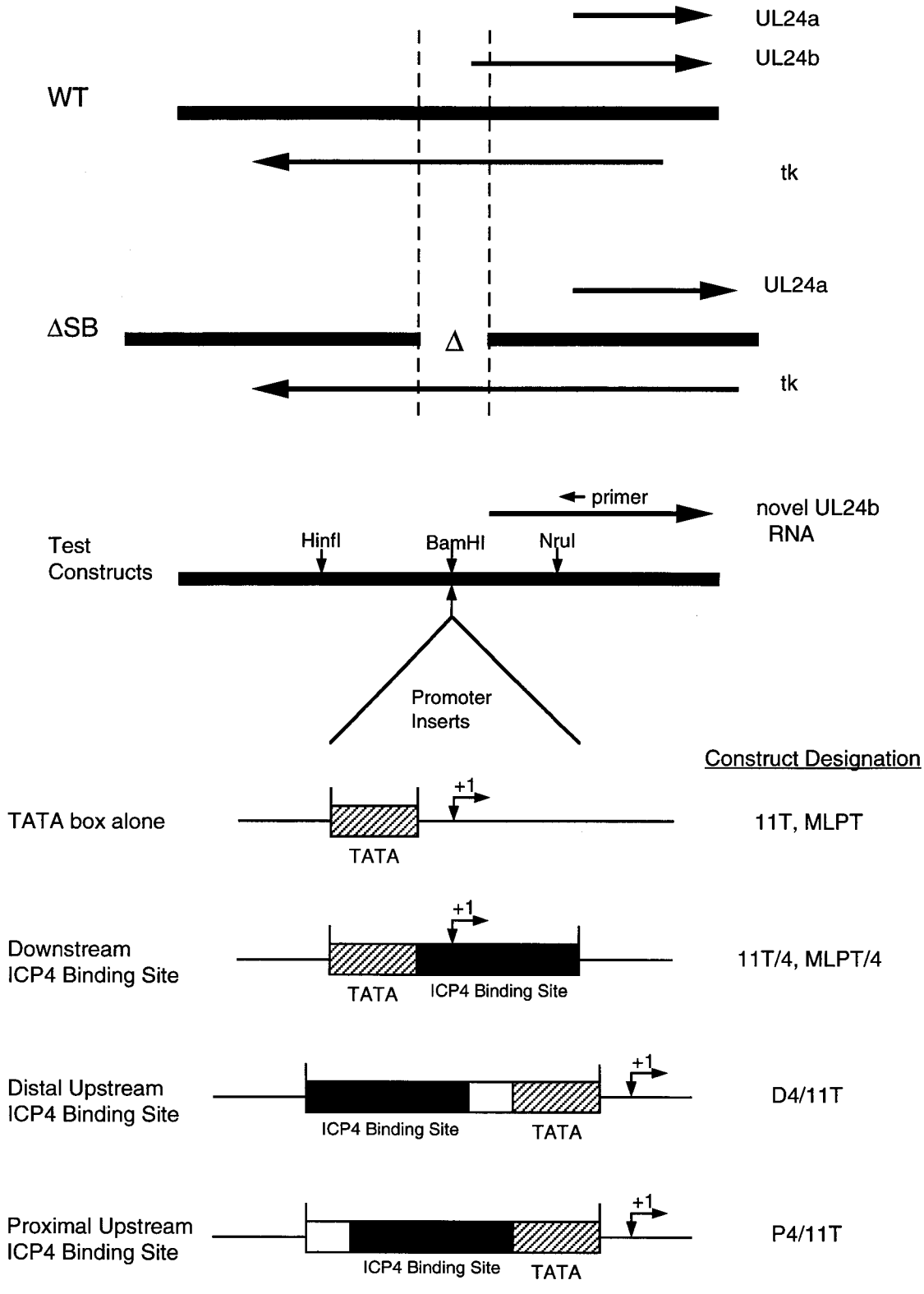
3.1. Experimental Approach

As stated previously, the goal of this study was to gain a better understanding of how ICP4's ability to bind DNA relates to its transregulatory activities. Two separate approaches were used towards achieving this goal. In one approach, a strong ICP4 binding site was introduced into a simple model promoter located within the viral genome; the resultant effect on transcript levels during lytic infection was then assayed by primer extension. A second approach employed a method of in vivo footprinting utilising ligation-mediated polymerase chain reaction; the experimental strategy involved examination of ICP4 interactions with known binding sites in the course of infection.

3.2. Effect of Introduction of an ICP4 Binding Site into a Simple Model Promoter within the Viral Genome - Experimental Design

A series of constructs were made in which a strong ICP4 binding site was introduced into a simple model promoter within the viral genome, allowing the effect of the ICP4 binding site on transcript levels to be assayed with a minimum of background due to other cis-acting sequences. In these constructs, an artificial promoter was linked to the *UL24* gene using the following system (previously described by Kibler et al., 1991). The *UL24* gene

overlaps the *tk* gene such that the promoters of two *UL24* transcripts are embedded in *tk* coding sequences but drive transcription from the opposite strand (Holland et al., 1984; McGeoch et al., 1988; Read et al., 1984). Sequences extending from -172 to +24 of the larger transcript (*UL24b*) were deleted in a previously described construct (ΔSB; Varmuza and Smiley, 1985) and spanned by a *Bam*HI linker (Fig. 3.1). This deletion eliminates production of *UL24b* mRNA (Kibler et al., 1991); however, growth of the viral mutant is not noticeably affected, presumably because the smaller UL24 transcript is still produced. When a series of different TATA sequences were inserted into the site of the deletion, transcription of *UL24b* was restored, albeit to varying levels (Kibler et al., 1991). For example, levels of transcripts arising from a construct bearing the Ad2 MLP TATA were relatively high whereas those from a construct bearing the HSV-1 *US11* TATA were relatively low. However, all TATA sequences resulted in E/leaky L kinetics of expression. To allow for subsequent modification of these model promoters, they were constructed with a unique *Bam*HI site immediately downstream of the TATA box and unique *Xho*I and *Sac*I sites immediately upstream. In this study, an ICP4 binding site was introduced either upstream or downstream of the TATA sequence. In one set of constructs, the ICP4 binding site was linked downstream of a TATA box, reproducing the spacing found in the native ICP4 promoter in which an ICP4 binding site straddles the transcriptional start site (Faber and Wilcox, 1988; Muller, 1987). Constructs were made with either a strong (MLP) or weak (*US11*) TATA box against the possible dependency of any effect observed on the strength of the TATA element. In a second set of


UL24a
UL24b
WT
tk
UL24a
ΔSB
Δ
tk
primer
novel UL24b RNA
Test Constructs
HinfI
BamHI
NruI
Promoter Inserts
Construct Designation
TATA box alone
+1
TATA
11T, MLPT
Downstream ICP4 Binding Site
+1
TATA
ICP4 Binding Site
11T/4, MLPT/4
Distal Upstream ICP4 Binding Site
+1
ICP4 Binding Site
TATA
D4/11T
Proximal Upstream ICP4 Binding Site
+1
ICP4 Binding Site
TATA
P4/11T

constructs, the ICP4 binding site was placed in one of two positions upstream of the *US11* TATA box. In one construct, the spacing mimicked that found in the *ICP0* promoter, in which an ICP4 binding site extends from -71 to -46 relative to the transcriptional start (Kristie and Roizman, 1986b). In a second construct, the binding site was placed 5 bp closer to the TATA element. The sites were staggered thus to control for effects of the helical orientation of the binding site with respect to the TATA element. These artificial promoters were linked to the *UL24* gene using the system described above. As the ΔSB deletion occurs within the *tk* gene, these structures could then be transferred into the *UL24* locus of HSV-1 by DNA mediated marker rescue and subsequent selection for tk deficient recombinants. Transcript levels from these promoter constructs during lytic infection were then assayed by primer extension analysis.

3.2.1. Construction of Recombinant Viruses

Prior to construction of the recombinant viruses, four pairs of plasmids containing an ICP4 binding site (or mutant site) linked to a TATA box were constructed. Oligonucleotide duplexes were made bearing the strong consensus ICP4 binding site from the *ICP4* promoter and appropriate *Bam*HI or *Xho*I protruding 5' ends (Fig. 3.2). For each construct, a control was made in which the 'AT' of the ATCGTC motif of the consensus binding site was deleted. This deletion has been shown to be sufficient to eliminate binding by ICP4 (Roberts et al., 1988). In plasmids p11T/4 and pMLPT/4 (or corresponding plasmids p11T/mut4 and

Figure 3.2. Map of plasmids bearing ICP4 binding site inserts.

ICP4 binding sites were inserted into p28 and pUS11-9. p28 is derived from pTKSB (Varmuza and Smiley, 1985). pTKSB contains the HSV-1 (strain 17) *tk* gene bearing a 200 nt deletion extending from +480 to +680; this corresponds to -172 to +24 relative to the *UL24b* transcriptional start. p28 bears the Ad2 MLP TATA box inserted into the site of the deletion oriented so as to drive transcription of *UL24b* (B.D. Keith, Master's Thesis, McMaster, 1988). pUS11-9 is identical to p28 except that it bears the *US11* TATA box, also oriented so as to drive transcription of *UL24b* (Kibler et al., 1991). The following constructs bearing an ICP4 binding site inserted either upstream or downstream of these TATA boxes were made. p11T/4 contains a binding site (or mutant site in p11T/mut4) inserted into the *Bam*HI downstream of the *US11* TATA box in pUS11-9. pMLPT/4 (or pMLPT/mut4) contains a site inserted into the *Bam*HI site downstream of the Ad2 MLP TATA box in p28. pD4/11T (or pDmut4/11T) contains a **d**istal site inserted into the *Xho*I site upstream of the *US11* TATA box in pUS11-9. pP4/11T (or pPmut4/11T) contains a **p**roximal site inserted into the *Xho*I site upstream of the *US11* TATA box in pUS11-9. All sites were inserted in such a way that the consensus ICP4 binding site (or mutant site) was located on the *UL24b* coding strand.

Sequences of the oligonucleotides used to construct ICP4 binding site inserts are shown. Complementary oligonucleotides encoding the ICP4 binding site found at the *ICP4* transcriptional start site were annealed, forming 5' protruding ends compatible with either a *Bam*HI or *Xho*I restriction enzyme cut. The oligonucleotide duplexes were inserted into

pUS11-9, either at the *Bam*HI site located downstream of the TATA box at the *UL24b* locus, or at the *Xho*I site located upstream of the same TATA box. In addition, a binding site was inserted into the analogous *Bam*HI site located in p28. Sequences from the *ICP4* promoter are shown in uppercase letters; the consensus binding site is underlined. A second set of constructs was also made in which two base pairs, indicated by asterisks, were deleted. Oligonucleotides were (listing coding strand/noncoding strand pairs): downstream site - AB1769/AB1770, downstream mutant site - AB1771/AB1772, distal upstream site - AB1738/AB1739, distal upstream mutant site - AB1742/AB1743, proximal upstream site - AB1740/AB1741, proximal upstream mutant site - AB1744/AB1745.

Downstream ICP4 Binding Site (BamHI ends)

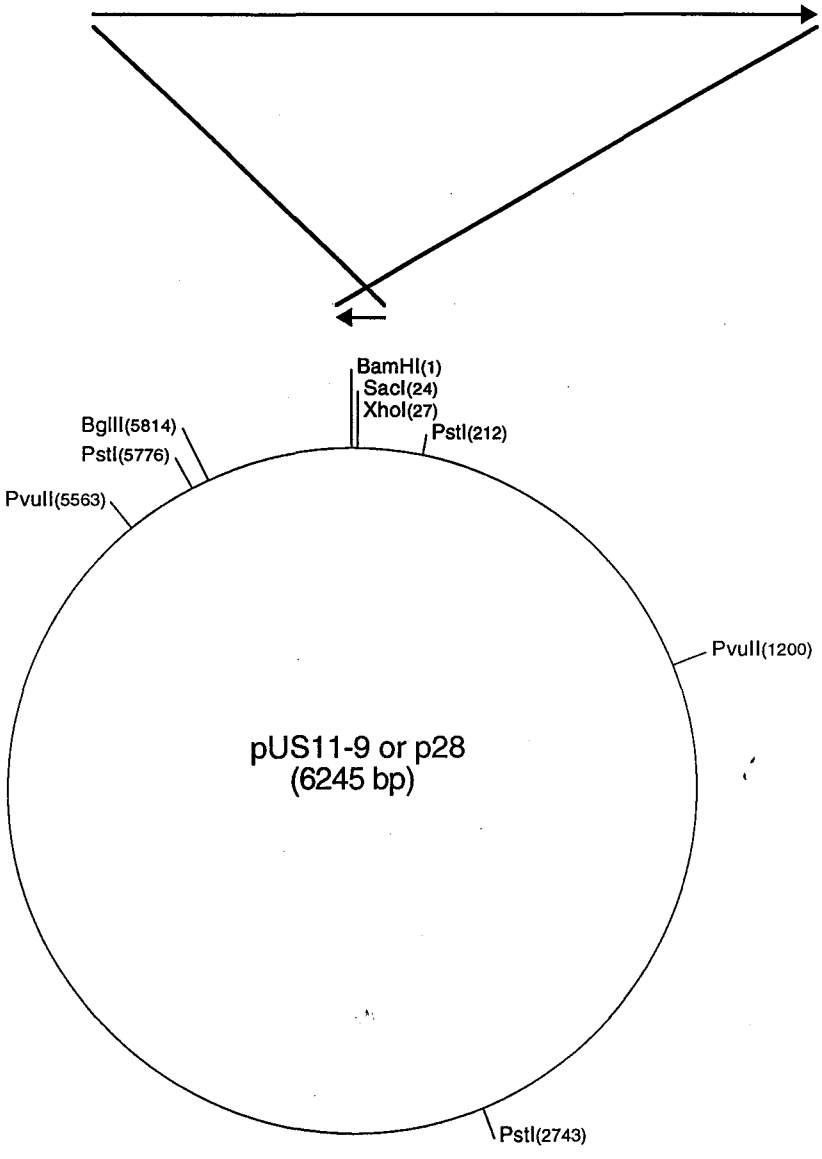
```
                **
gatcaCGCCCCGATCGTCCACACGGAGCGCGGCTG
    tGCGGGGCTAGCAGGTGTGCCTCGCGCCGACctag
                **
```

Distal Upstream ICP4 Binding Site (XhoI ends)

```
                  **
tcgaggaCGCCCCGATCGTCCACACGGAGCGCGGCTGccg
    cctGCGGGGCTAGCAGGTGTGCCTCGCGCCGACggcagct
                  **
```

Proximal Upstream ICP4 Binding Site (XhoI ends)

```
                        **
tcgagccgaggaCGCCCCGATCGTCCACACGGAGCGCGGg
    cggctcctGCGGGGCTAGCAGGTGTGCCTCGCGCCcagct
                        **
```



pMLPT/mut4 bearing mutant ICP4 binding sites), the binding site was inserted into the *Bam*HI site located downstream of the *US11* or Ad2 MLP TATA box in pUS11-9 or p28, respectively (Fig. 3.2), mimicking the spacing found in the native *ICP4* promoter (Faber and Wilcox, 1988; Muller, 1987). In plasmids pD4/11T and pP4/11T (or corresponding plasmids pDmut4/11T and pPmut4/11T), the binding site was inserted into the *Xho*I site upstream of the *US11* TATA box in pUS11-9 (Fig. 3.2). The spacing observed in the *ICP0* promoter was reproduced in pD4/11T (and pDmut4/11T; Kristie and Roizman, 1986b); the binding site was placed 5 bp closer to the TATA box in pP4/11T (and pPmut4/11T). Positive clones were selected after digestion with *Pst*I and *Xho*I (positive clones yielding fragments of 185, 529-536 (varying according to the size of the insert, Fig. 3.2), 2531, and 3033 bp) and their sequences confirmed by the chain termination method (Fig. 3.3, 3.4, 3.5, 3.6; Sanger et al., 1977).

Next, these structures were transferred into the *UL24* locus of HSV-1 by DNA mediated marker rescue (Smiley, 1980; Smiley et al., 1981). Plasmids were linearized by digestion with *Bgl*II and then cotransfected with infectious HSV-1 strain PAA^{r}5 DNA into Vero cells. tk deficient recombinant viruses were selected from the progeny of the cotransfection by growth in medium containing 5-bromodeoxycytidine. After digestion with *Bam*HI and *Pvu*II, recombinants incorporating the artificial promoters were identified by Southern blotting using a probe encoding sequences from the *tk* gene (Fig. 3.7). Positive recombinants yielded two fragments: their predicted sizes were 683-723 bp and 1199-1229 bp, varying according to the size of the insert and its position relative to the TATA box (see Fig. 3.1 and

Figure 3.3. Sequences of p11T/4 and p11T/mut4 inserts.

The sequences of p11T/4 and p11T/mut4 inserts were determined by the dideoxynucleotide chain termination method using AB2051 (5'-ACACAACACCGCCTCGACCAGGGTG-3') as the primer. The sequence shown is on the coding strand of *tk*. Sequences flanking the insert are in lowercase.

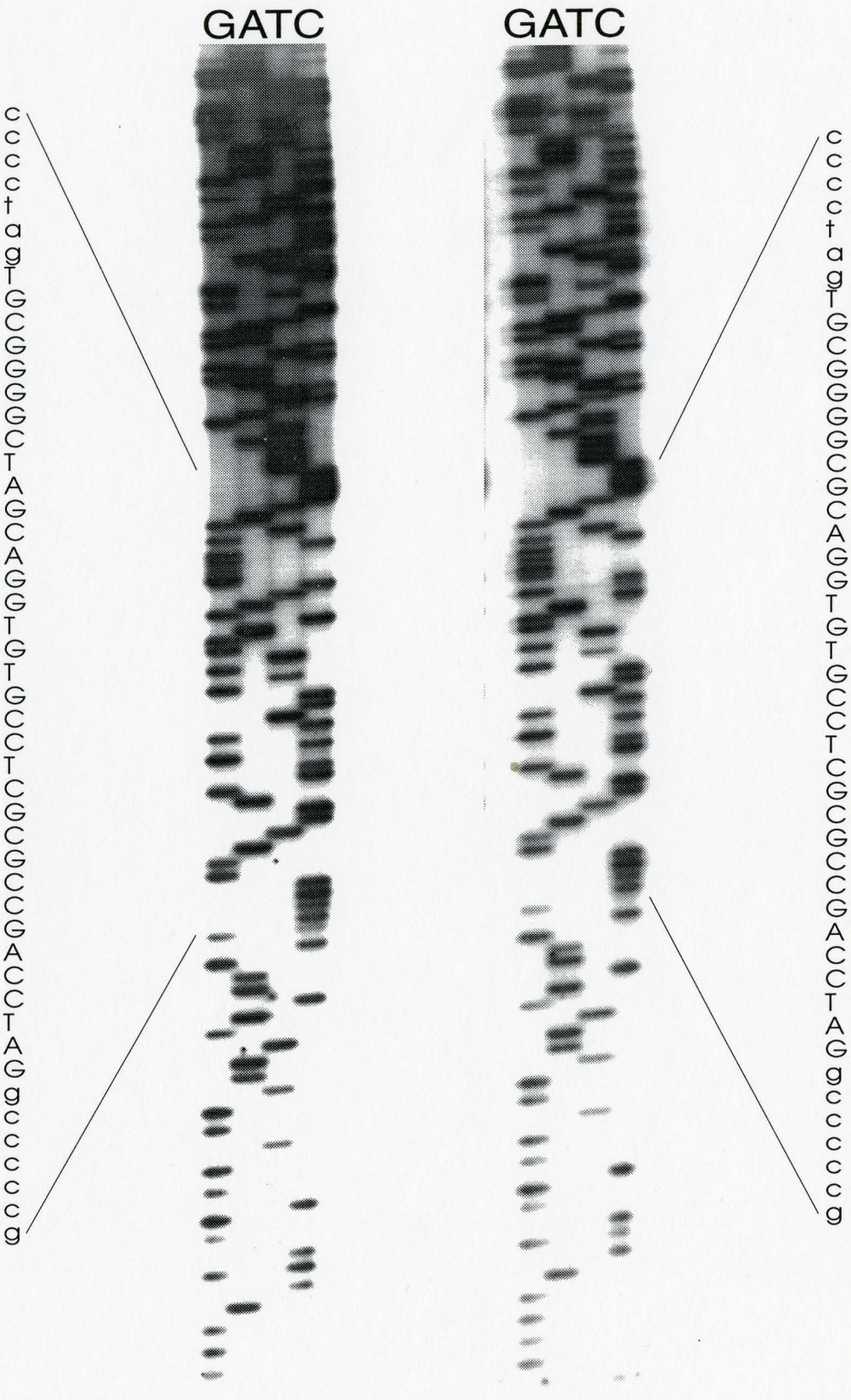

GATC
GATC
ccccctagTGCGGGGGCTAGCAGGTGTGCCTCGCGCCGACCTAGgccccccg
ccccctagTGCGGGGGCGCAGGTGTGCCTCGGCGCCGACCTAGgccccccg

Figure 3.4. Sequences of pMLPT/4 and pMLPT/mut4 inserts.

The sequences of pMLPT/4 and pMLPT/mut4 inserts were determined by the dideoxynucleotide chain termination method using AB2051 (5'-ACACAACACCGCCTCGACCAGGGTG-3') as the primer. The sequence shown is on the coding strand of *tk*. Sequences flanking the insert are in lowercase.

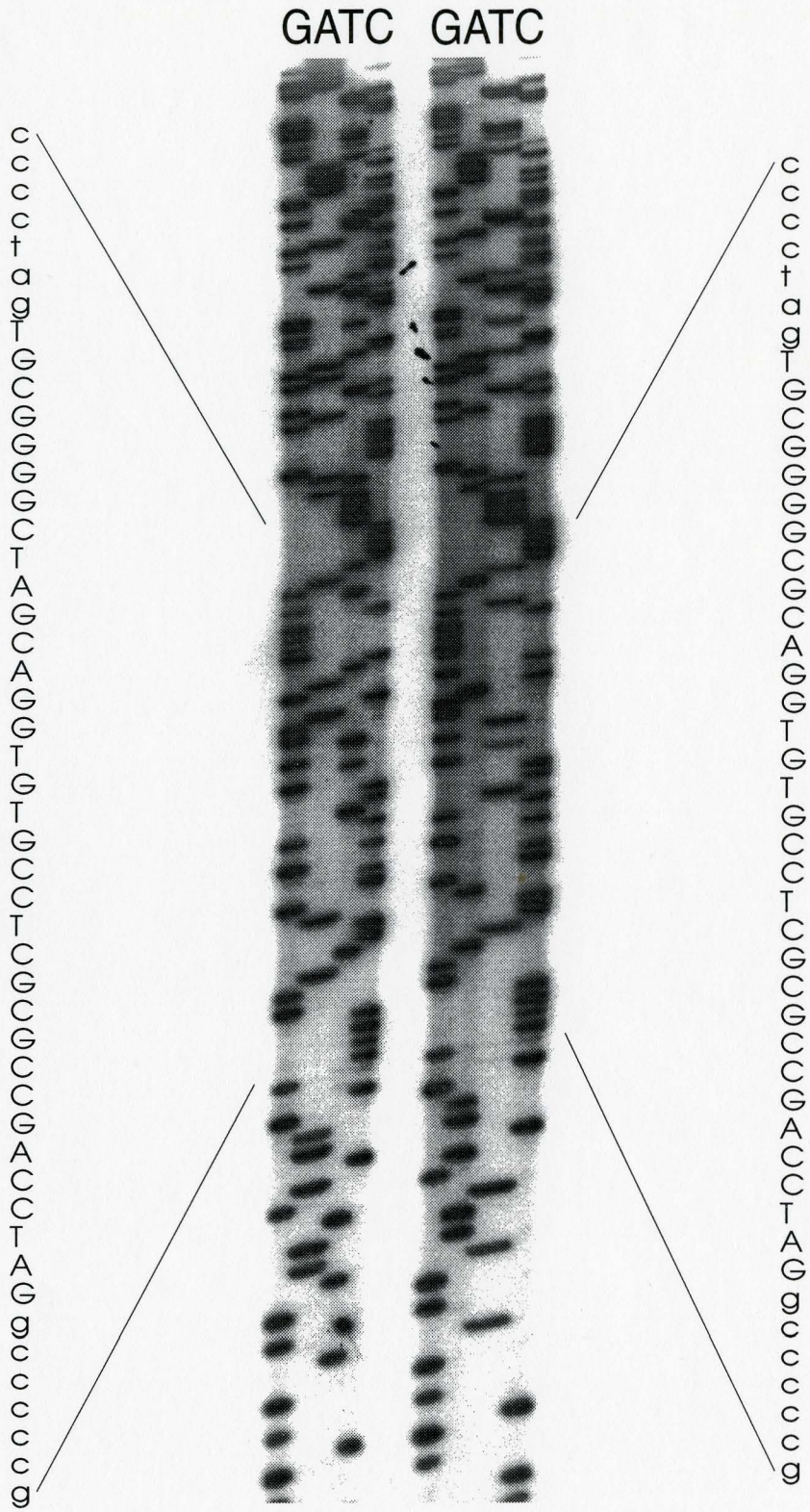

GATC GATC
ccccctagTGCGGGGGCTAGCAGGTGTGCCTCGGCGCCGACCCTAGgccccccg
ccccctagTGCGGGGGCGCAGGTGTGCCTCGGCGCCGACCCTAGgccccccg

Figure 3.5. Sequences of pD4/11T and pDmut4/11T inserts.

The sequences of pD4/11T and pDmut4/11T inserts were determined by the dideoxynucleotide chain termination method using AB2051 (5'-ACACAACACCGCCTCGACCAGGGTG-3') as the primer. The sequence shown is on the coding strand of *tk*. Sequences flanking the insert are in lowercase.

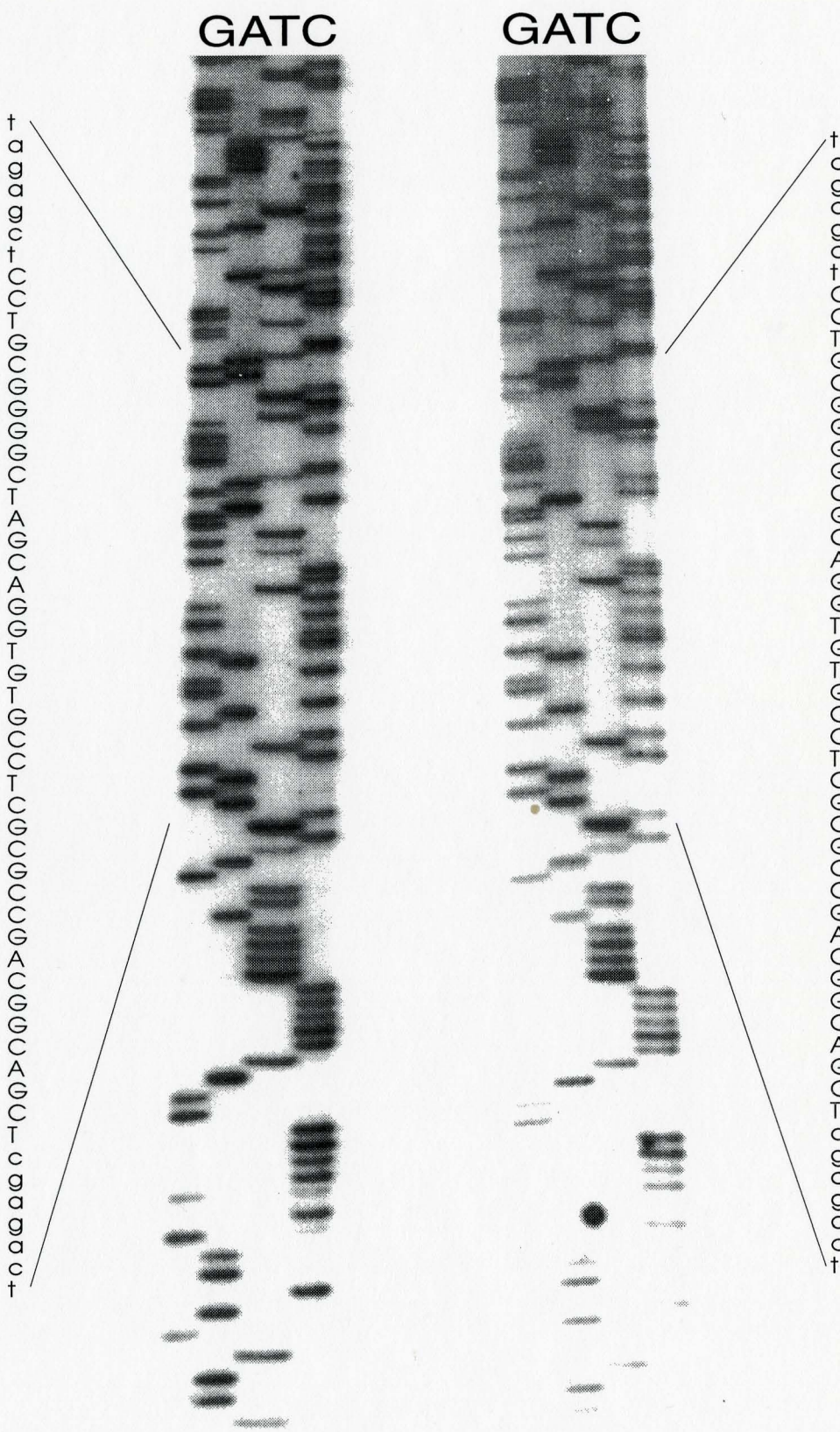

GATC
GATC
taggagctCCTGCGGGGGCTAGCAGGTGTGCCTCGCGCCGACGGCAGCCTcgagact
taggagctCCTGCGGGGCGCAGGTGTGCCTCGCGCCGACGGCAGCCTcgagact

Figure 3.6. Sequences of pP4/11T and pPmut4/11T inserts.

The sequences of pP4/11T and pPmut4/11T inserts were determined by the dideoxynucleotide chain termination method using AB2051 (5'-ACACAACACCGCCTCGACCAGGGTG-3') as the primer. The sequence shown is on the coding strand of *tk*. Sequences flanking the insert are in lowercase.

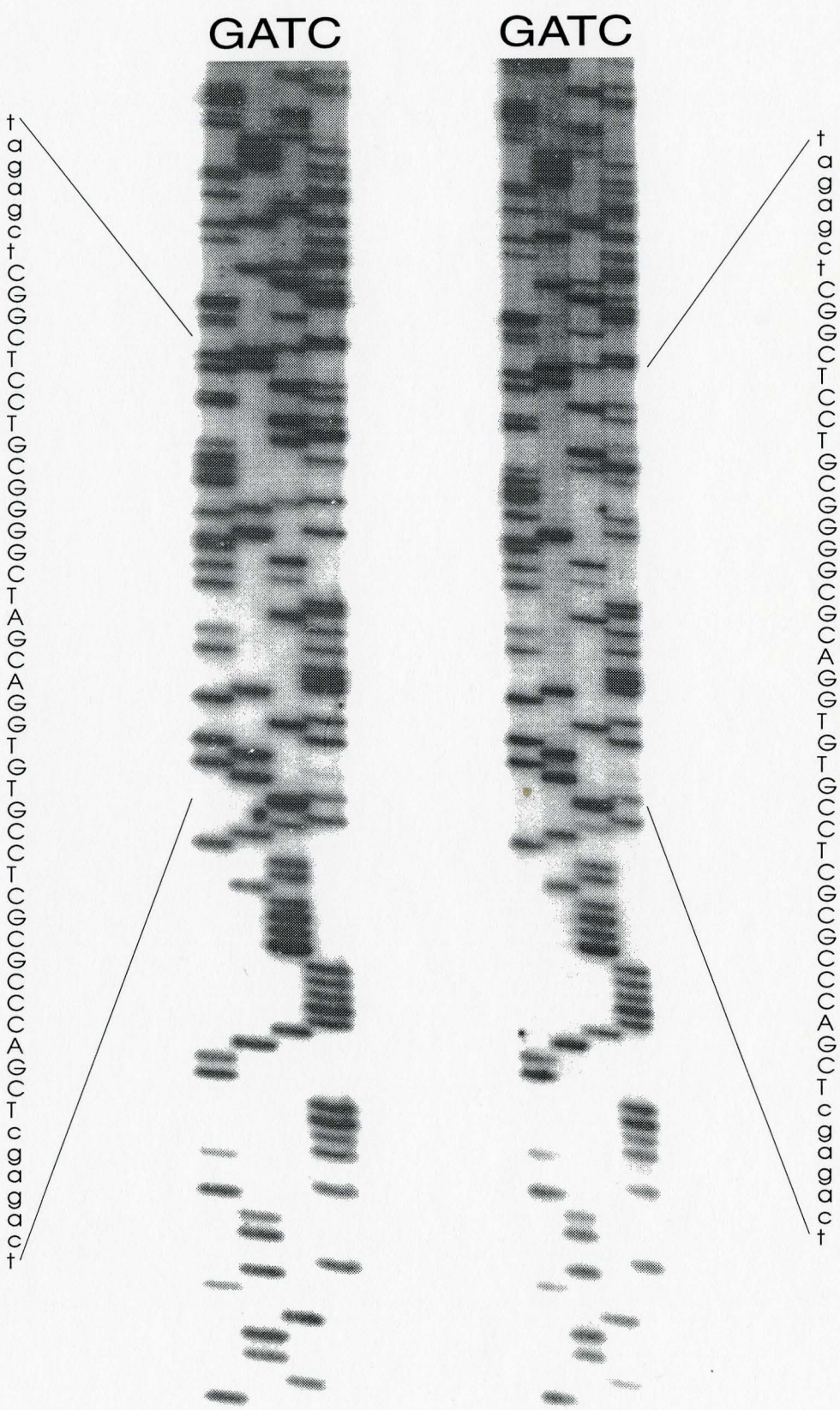
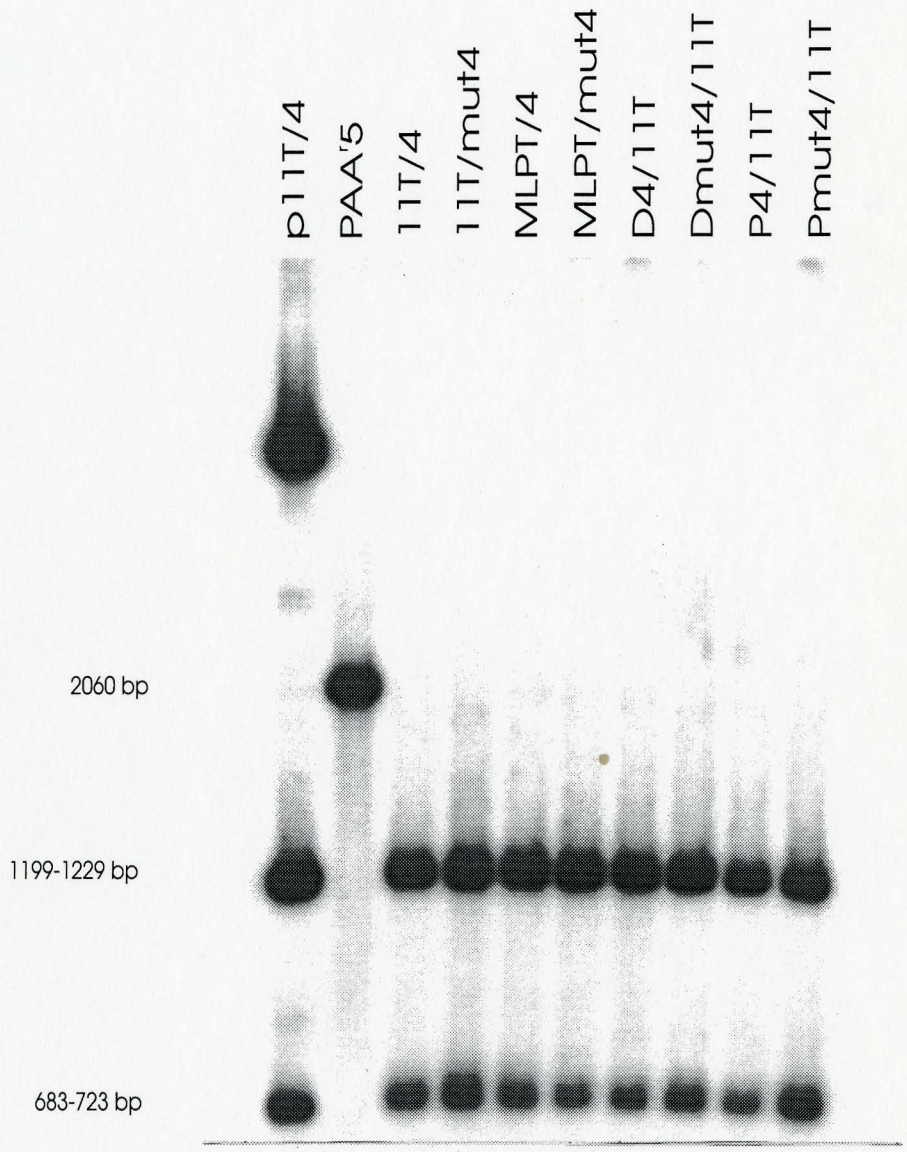

GATC
GATC
taggagctCGGGCTCCTGCGGGGGGCTAGCAGGTGTGCCTCGCGCGCCCAGCTcgagact
taggagctCGGGCTCCTGCGGGGGCGCAGGTGTGCCTCGCGCGCCCAGCTcgagact

Figure 3.7. Southern blot of viral recombinants.

ICP4 binding site constructs and HSV-1 strain PAA^{r}5 infectious DNA were cotransfected into Vero cells. tk deficient viral recombinants were selected by growth in media containing 5-bromo-2-deoxycytidine. Individual plaques were transferred to monolayers in 24 well plates and grown until confluent cpe was apparent. Viral DNA was extracted and cleaved with *Bam*HI and *Pvu*II. PAA^{r}5 DNA and one of the plasmids used to construct the recombinant viruses (p11T/4) were also cut with these restriction enzymes to serve as negative and positive controls, respectively. DNA fragments were separated by electrophoresis through a 1.4% agarose gel and transferred to a nylon membrane. The membrane was then probed with a random-primed ^{32}P-labelled probe made from ptk173 (Smiley et al., 1983), which contains the HSV-1 *tk* gene. Approximate sizes of fragments are shown on the left.


p11T/4
PAA'5
11T/4
11T/mut4
MLPT/4
MLPT/mut4
D4/11T
Dmut4/11T
P4/11T
Pmut4/11T
2060 bp
1199-1229 bp
683-723 bp

3.2). As no *Bam*HI site occurs in this region of the wild type virus, digestion of PAA^{r}5 DNA yielded a single *Pvu*II fragment of ca. 2060 bp (Fig. 3.7). Once positive recombinants were identified, they were plaque-purified twice beyond the point at which no signs of contaminating wild type virus were apparent on an overexposed autoradiogram of a Southern blot. Stocks of the plaque-purified viruses were then made and their titers determined by serial dilution. The recombinant viruses were named for their progenitor plasmids - e.g. - p11T/4 was used to construct recombinant 11T/4. It should be noted that recombinant viruses MLPT/4 and MLPT/mut4 (and their progenitor plasmids) were isolated by Joanne Duncan; Joanne also performed the ligation reaction and transformation of *E.coli* in the construction of p11T/4 and p11T/mut4.

3.2.2. Effect of a Downstream ICP4 Binding Site on Transcript Levels

I first studied the effect of inserting an ICP4 binding site downstream of the *US11* TATA box. Vero cells were infected with recombinant viruses bearing the *US11* TATA element alone (11T), the *US11* TATA box and downstream ICP4 binding site (11T/4), or the *US11* TATA box and downstream mutant ICP4 binding site (11T/mut4). Where indicated, a block in DNA replication was established by the addition of aphidicolin at the time of infection. Total cellular RNA was extracted from infected cells at six and twelve hours postinfection and examined by primer extension analysis using a primer complementary to residues +91 to +67 of the native *UL24b* transcript. In agreement with previous work, RNA isolated from the cells

infected with construct 11T gave rise to primer extension products of ca. 53 nt (Kibler et al., 1991; Fig. 3.8). As expected (based on their inserts downstream of the TATA box), RNA from constructs 11T/4 and 11T/mut4 gave rise to a larger primer extension product of ca. 85 nt. Control experiments in which levels of 7SL and HSV-1 *gD* RNA were assayed confirmed that samples contained similar levels of RNA and that multiplicities of infection were comparable (Fig. 3.8).

Addition of the downstream ICP4 binding site had three effects on *UL24b* expression (Fig. 3.8). First, addition of the downstream ICP4 binding site resulted in reduced levels of *UL24b* transcripts. At six hours postinfection, *UL24b* RNA from 11T/4 was undetectable or barely detectable - an over 100-fold reduction in transcript levels relative to 11T/mut4 (Table 3.1). Second, the presence of the ICP4 binding site resulted in a shift in *UL24b* expression to later times postinfection. Thus, although at six hours postinfection, levels of RNA arising from 11T/4 were over 100-fold lower than those arising from 11T/mut4, expression from 11T/4 increased such that at twelve hours postinfection, transcript levels were only 3-4 fold lower than those from 11T/mut4. Third, the presence of the downstream ICP4 binding site conferred a greatly increased dependence on viral DNA replication. At twelve hours postinfection, infection in the presence of aphidicolin resulted in an over 100-fold reduction in levels of *UL24b* transcripts arising from 11T/4, whereas RNA levels arising from 11T/mut4 were reduced only 4-7 fold (Table 3.2). Thus, the overall effect of the ICP4 binding site was a to shift the kinetics of *UL24b* expression from leaky late to true late as well as a general reduction in transcript levels.

Figure 3.8. Effect of an ICP4 binding site placed downstream of the *US11* TATA element.

Vero cells were infected with 10 PFU per cell of the indicated viral recombinants, and total cellular RNA was harvested at 6 and 12 hours postinfection. 20 μg samples were then assayed for *UL24* transcripts by primer extension using a primer complementary to residues +91 to +67 of the native *UL24b* transcript (5'-ACACAACACCGCCTCGACCAGGGTG-3', AB2051). Samples were also scored for *7SL* and *gD* RNAs by primer extension, using 0.5 and 5 mg of RNA, respectively. The sequence of the *7SL* and *gD* primers were 5'-AACTTAGTGCGGACACCCGATCGGC-3' (AB697) and 5'-CCCCATACCGGAACGCACCACACAA-3' (AB1514), respectively. Where indicated, 10 μg of aphidicolin (aph) per mL was added at the time of infection and maintained continuously. Molecular weight markers (M) were 3'-end-labelled *Hpa*II fragments of pBR322. Marker fragment sizes (in nucleotides) are shown on the left.

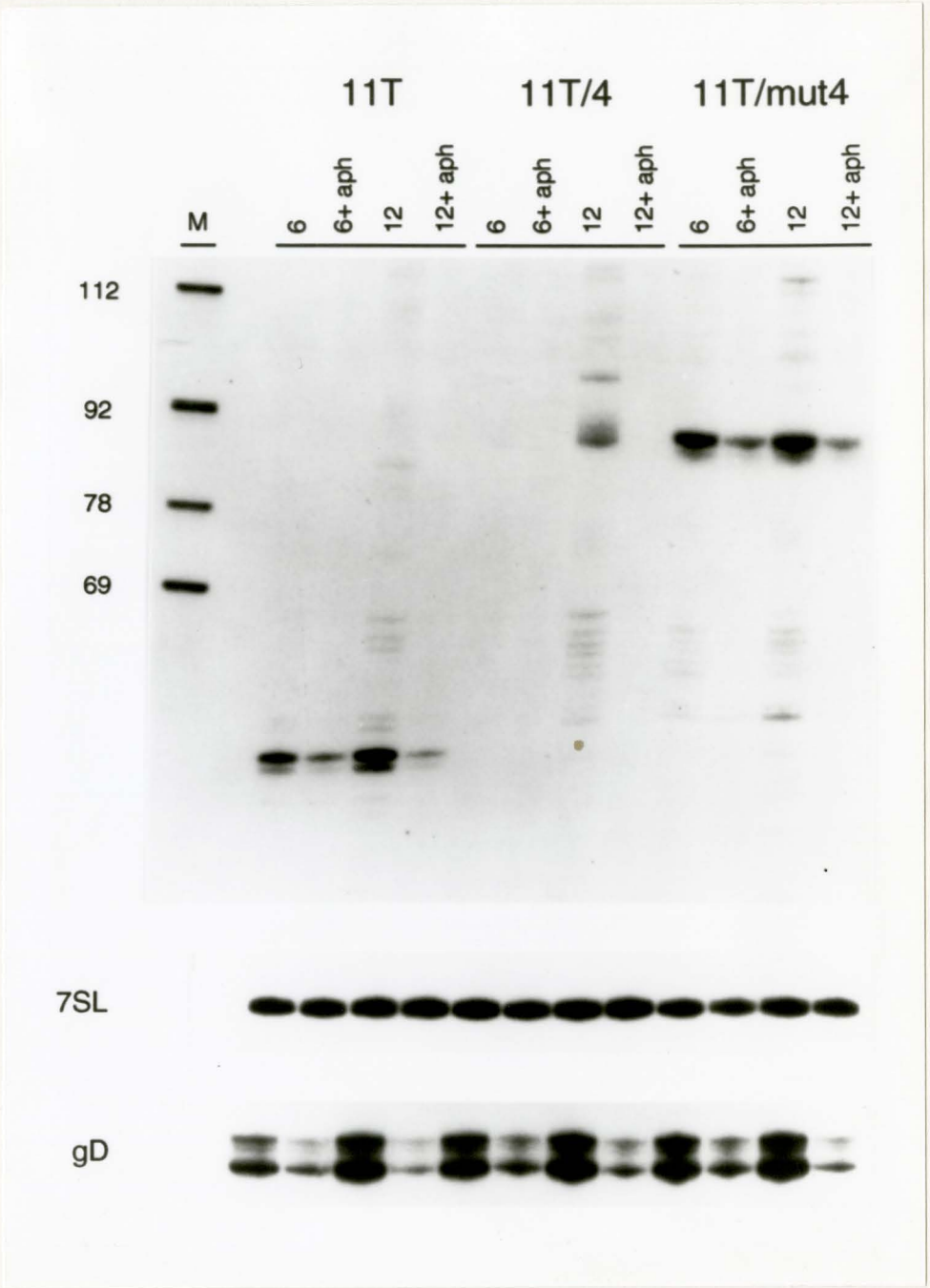

11T
11T/4
11T/mut4
M
6
6+ aph
12
12+ aph
6
6+ aph
12
12+ aph
6
6+ aph
12
12+ aph
112
92
78
69
7SL
gD

Table 1. Inhibition conferred by ICP4 binding site

Virus pair	Fold inhibition: Ratio of activity due to mutant site relative to that due to intact site [a]	
	6 hours	12 hours
11T/mut4:11T/4	>100, >100	3.8, 3.0
MLPT/mut4:MLPT/4	>100, >100, >100, 43	3.7, 1.9, 4.4, 8
Dmut4/11T:D4/11T	22, 6.0, 7.6	2.6, 2.2, 2.3
Pmut4/11T:P4/11T	10, 6.5, 11	2.9, 2.2, 2.8

[a] Gels were exposed to preflashed Kodak XAR-5 film with an intensifer screen, and relative signal intensities quantified by microdensitometry. The degree of inhibition conferred by the ICP4 binding site relative to the mutant site was calculated by dividing the signal obtained for the mutant site by that obtained for the intact site. In some cases, the signal from the construct bearing the the wild-type site could not be detected, representing an inhibition of more than 100-fold relative to the control construct. For each virus pair, the results of several experiments are listed.

Table 2. Inhibition by aphidicolin observed at 12 hours with ICP4 binding site and mutant binding site

Virus pair	Fold inhibition by aphidicolin [a]	
	ICP4 binding site	mutant ICP4 binding site
11T/4; 11T/mut4	>100, >100	6.8, 4.1
MLPT/4; MLPT/mut4	>100, >100, >100, >100	24, 8.5, 16, 69
D4/11T; Dmut4/11T	44, 21, 24	5.8, 5.3, 6.7
P4/11T; Pmut4/11T	34, 57, 20	3.8, 7.7, 6.4

[a] Gels were exposed to preflashed Kodak XAR-5 film with an intensifier screen, and relative signal intensities were quantified by microdensitometry. Fold inhibition by aphidicolin was calculated by dividing the signal obtained at 12 hours without aphidicolin by that obtained at 12 hours in the presence of aphidicolin. In some cases signals could not be detected in the presence of aphidocolin, representing a decrease of more than 100-fold. For each virus pair, the results of several experiments are shown.

Based on findings that gene induction by ICP4 is affected by TATA box sequence (Imbalzano and Deluca, 1992), I next examined whether the downregulation and shift in kinetics of expression conferred by an ICP4 binding site were dependent on the TATA element. For this purpose, I constructed a similar set of recombinant viruses (viral recombinants MLPT, MLPT/4, and MLPT/mut4) in which the weak *US11* TATA box was replaced with the much stronger Ad2 MLP TATA box. Primer extension analysis revealed that addition of an ICP4 binding site downstream of the MLP TATA box had virtually the same effect as it had when inserted downstream of the *US11* TATA box (Fig. 3.9, Tables 3.1 and 3.2). Thus, I conclude that the effects observed are independent of the sequence of the TATA box.

3.2.3. Effect of an Upstream ICP4 Binding Site on Transcript Levels

Having observed that an ICP4 binding site placed downstream of a TATA box could confer a shift in kinetics from leaky late to true late, I next investigated whether an ICP4 binding site placed upstream of the TATA element could confer a similar effect. Using viral recombinants D4/11T and Dmut4/11T, I found that an ICP4 binding site placed upstream of the *US11* TATA box, mimicking the spacing observed in the *ICP0* promoter, resulted in a similar, but less pronounced, effect as had been observed with the downstream binding site (Fig. 3.10). Relative to Dmut4/11T, levels of *UL24b* RNA arising from D4/11T were reduced over 6-fold at six hours postinfection, and 2- or 3-fold at twelve hours postinfection (Table 3.1). A

Figure 3.9. Effect of an ICP4 binding site placed downstream of the Ad2 MLP TATA element.

Vero cells were infected with 10 PFU per cell of the indicated viral recombinants, and RNA samples extracted 6 and 12 hours postinfection were scored for *UL24b*, *7SL*, and *gD* RNAs as described in the legend to Figure 3.8. Where indicated, 10 μg of aphidicolin (aph) per mL was added at the time of infection and maintained continuously. Molecular weight markers (M) were 3'-end-labelled *Hpa*II fragments of pBR322. Marker fragment sizes (in nucleotides) are shown on the left.

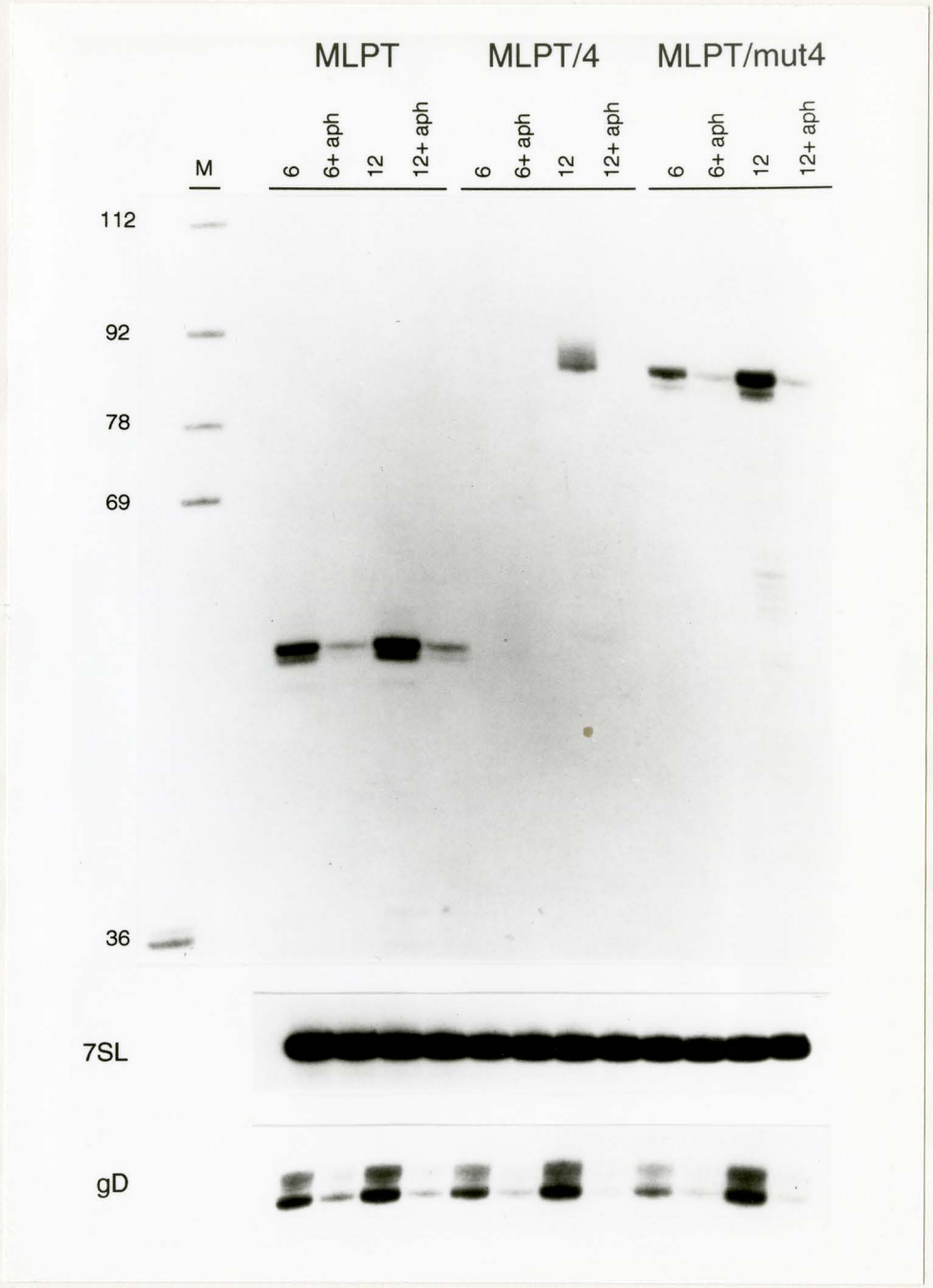
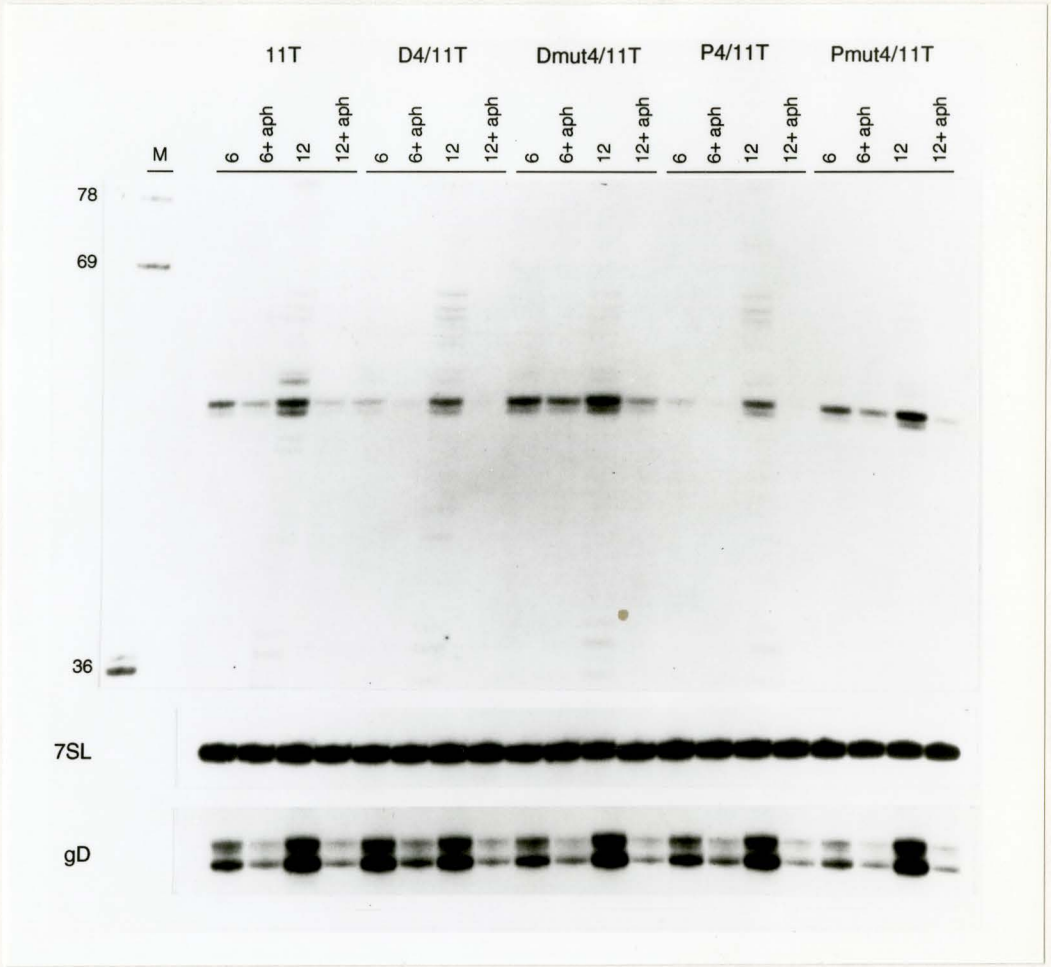

MLPT
MLPT/4
MLPT/mut4
6
6+ aph
12
12+ aph
M
112
92
78
69
36
7SL
gD

Figure 3.10. Effect of ICP4 binding sites placed upstream of the *US11* TATA element.

Vero cells were infected with 10 PFU per cell of the indicated viral recombinants, and RNA samples extracted 6 and 12 hours postinfection were scored for *UL24b*, *7SL*, and *gD* RNAs as described in the legend to Figure 3.8. Where indicated, 10 μg of aphidicolin (aph) per mL was added at the time of infection and maintained continuously. Molecular weight markers (M) were 3'-end-labelled *Hpa*II fragments of pBR322. Marker fragment sizes (in nucleotides) are shown on the left.


11T
D4/11T
Dmut4/11T
P4/11T
Pmut4/11T
M
6
6+ aph
12
12+ aph
78
69
36
7SL
gD

block in DNA replication resulted in a 5- to 7-fold reduction in *UL24b* transcript levels in Dmut4/11T at twelve hours postinfection, whereas the reduction was over 20-fold in D4/11T (Table 3.2).

DiDonato and Muller (1989) have observed that ICP4 and TFIID are on opposite helical faces when bound at their respective sites in both the *ICP4* and the *ICP0* promoters, and that in these promoters, binding of ICP4 results in an alteration in the helical geometry of the TATA region. Based on these observations, they proposed that stereospecific orientation and conformational changes may be important to ICP4-induced repression of IE gene expression. To examine the importance of the helical orientation of bound ICP4 with respect to TFIID for the effects that I have observed, I constructed an additional set of viral recombinants (P4/11T and Pmut4/11T) in which the ICP4 binding site was positioned 5 bp (approximately 1/2 helical turn) closer to the TATA box than in the previous set of viral constructs. Primer extension analysis revealed that this shift had no effect on levels of UL24b transcripts relative to those observed with D4/11T and Dmut4/11T (Fig. 3.10, Tables 3.1 and 3.2). Therefore, I conclude that the helical orientation of ICP4 binding to DNA with respect to the TATA binding protein (TBP) is not important for the effects that I have observed.

In summary, the results of these experiments indicated that an ICP4 binding site located ca. 35 bp upstream or ca. 26 bp downstream of the TATA sequence inhibited expression, and that viral DNA replication partially reverses this effect. In the discussion, I speculate about possible mechanisms and the relevance of these findings for temporal regulation of HSV gene expression.

3.3. Construction and Characterization of an ICP4-GST Fusion Protein

I wished to confirm the ability of ICP4 to bind the minimal ICP4 binding site used in the recombinant viruses described in the previous sections. For this purpose, I constructed a fusion protein in which the DNA binding domain of ICP4 (Wu and Wilcox, 1990) was linked to glutathione *S*-transferase (GST) from *Schistosoma japonicum* (Smith et al., 1986). The specificity of DNA binding by the ICP4-GST fusion protein was characterized by capture assays and DNase I footprinting. Binding of ICP4-GST to the binding site used in the above constructs was then examined by DNase I footprinting.

3.3.1. Construction of the ICP4-GST Fusion Protein

First, the sequences encoding the DNA binding domain of ICP4 (Wu and Wilcox, 1990) were linked to the glutathione *S*-transferase (GST) gene from *Schistosoma japonicum* (Smith et al., 1986). The 681 bp *Sac*II fragment (encoding residues 262-490 of ICP4) of pGX58 (Everett, 1984b) were inserted into the *Bam*HI site of pGEX-2T (Pharmacia), using a *Bam*HI - *Sac*II linker to maintain the coding frame (Fig. 3.11). A positive clone was selected after digestion with *Sma*I (positive clones yielding fragments of 39, 184, 285, and 5135 bp) and the sequence of the junction between the ICP4 and GST coding regions confirmed by the chain termination method (Fig. 3.12; Sanger et al., 1977). The resultant plasmid was designated pIGF-17. Since

Figure 3.11. Map of pIGF-17

Plasmid pIGF-17 was made as follows. Oligonucleotide AB1731 (5'-GATCCTCCGCGGAG-3') was self-annealed to form a *Bam*HI-*Sac*II-*Bam*HI linker, which was then inserted into the *Bam*HI site of pGEX-2T (Pharmacia). A 681 bp *Sac*II fragment of pGX58 (Everett, 1984b) encoding residues 262 to 490 of ICP4 was inserted into the *Sac*II site of the linker. The ICP4 coding strand was on the same strand as that of the glutathione *S*-transferase (GST) gene encoded by pGEX-2T; the linker maintained the coding frame between these sequences.

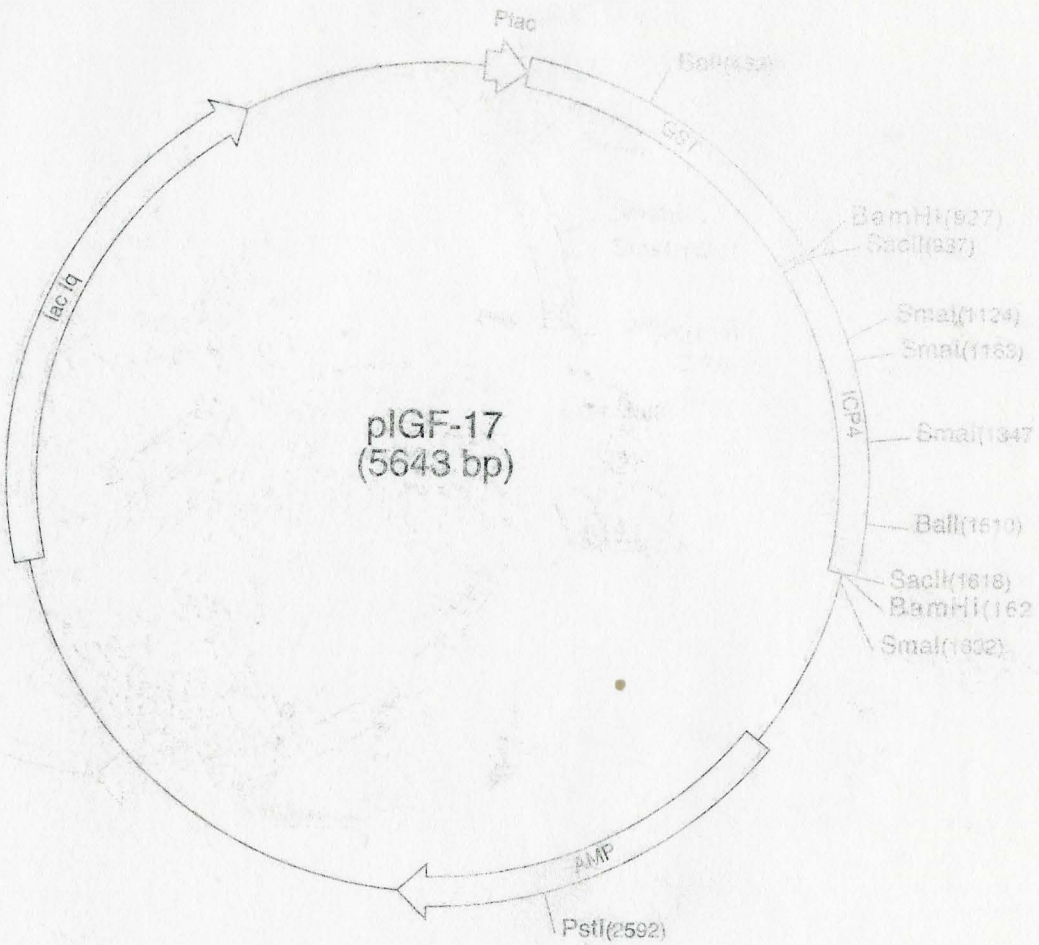
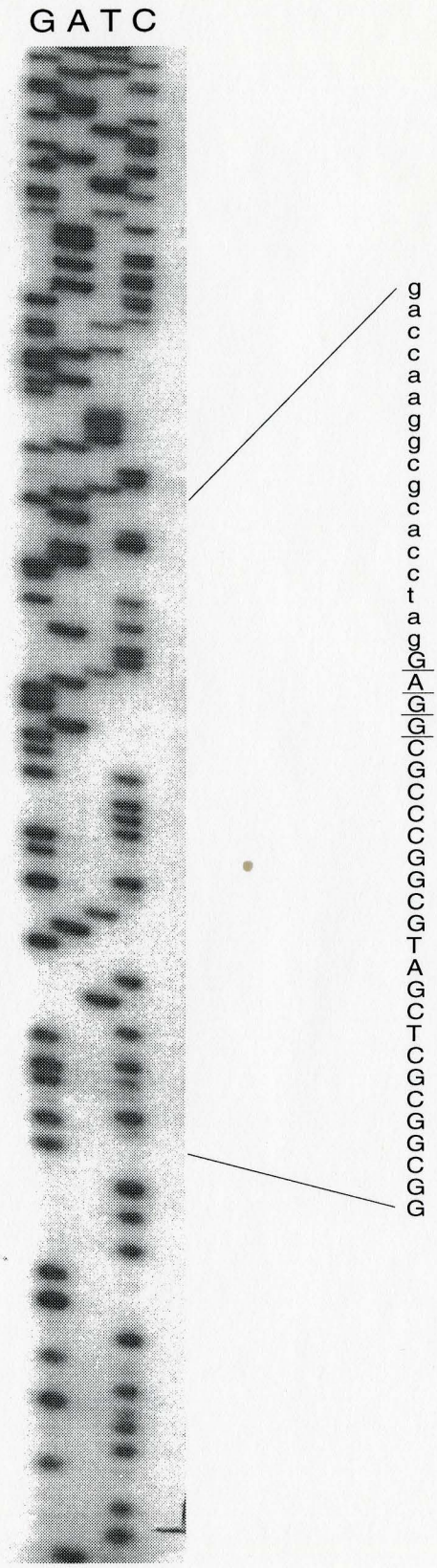

Ptac
GST
BamHI(927)
SacII(937)
SmaI(1124)
SmaI(1163)
ICPA
SmaI(1347
BalI(1610)
SacII(1618)
BamHI(162
SmaI(1632)
pIGF-17
(5643 bp)
AMP
PstI(2592)
lac Iq

Figure 3.12. Sequence across the GST-ICP4 junction in pIGF-17

The sequence spanning the junction between the GST and ICP4 coding regions in pIGF-17 was determined by the dideoxy chain termination method using AB1731 (5'-GTGAAGCGGCCCGTGGCGTC-3') as the primer. This is complementary to residues +1154 to +1135 relative to the *ICP4* transcriptional start. The sequence of the GST-ICP4 noncoding strand is shown (linker sequences are underlined, vector sequences are in lowercase):

5'-GGCGGCGCTCGATGCGGCCCGCGGAGgatccacgcggaaccag-3'


G A T C
gaccaaggcgcacctagGAGGCGCCCGGGCGTAGCTCGCGGCGG

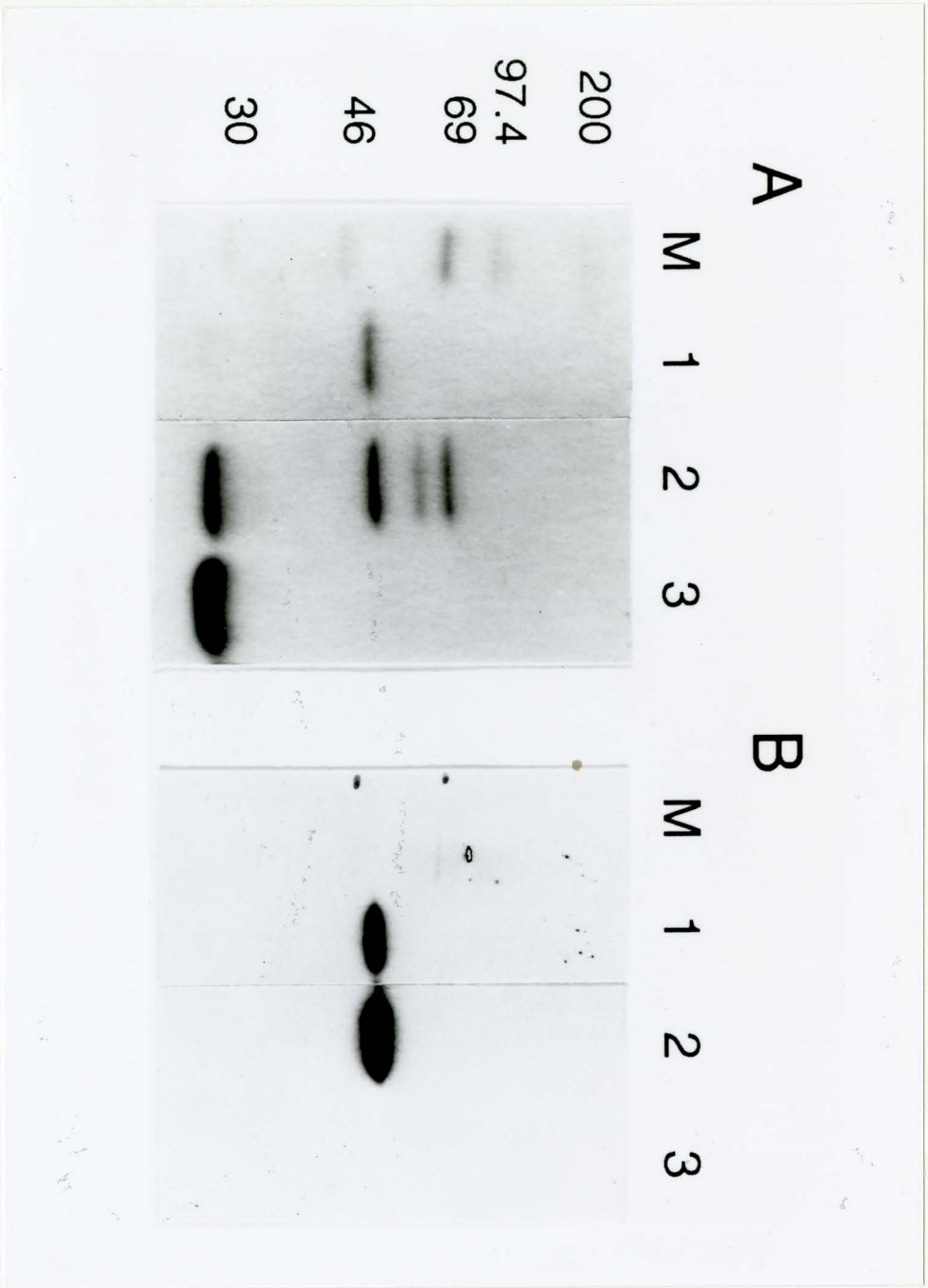
the GST gene in pIGF-17 is under the control of *tac* promoter, expression of the ICP4-GST fusion protein was induced by addition of IPTG to bacterial cultures. ICP4-GST was then isolated from bacterial lysates by affinity chromatography over a glutathione-sepharose column. The major product had an apparent molecular weight of ca. 50 kD, the predicted size of the fusion protein (Fig. 3.13A). In addition, two less abundant larger products were observed, as well as a strong 25 kD band (corresponding to the predicted size of GST). In a Southwestern blot, only the 50 kD product was bound by a probe bearing an ICP4 binding site (Fig. 3.13B). The 50 kD product could be isolated from the other polypeptides by DNA affinity chromatography (Fig. 3.13A), however, the protein isolated by chromatography over glutathione-sepharose was deemed sufficiently pure for the purposes of this study.

3.3.2. Characterization of DNA Binding Specificity of ICP4-GST

Although Wu and Wilcox (1990) found that an ICP4-trpE fusion protein containing ICP4's DNA binding domain had DNA binding activity similar to that of intact ICP4, it was necessary to confirm that the ICP4-GST fusion protein retained this function. The DNA binding specificity of ICP4-GST was characterized by examining its interactions with known ICP4 binding sites present in the *ICP4* and *gD* promoters using capture assays and DNase I footprinting. In capture assays, fragments bound specifically by ICP4-GST are isolated from a mixture of fragments. ICP4-GST is incubated with labelled, restriction enzyme cut plasmid DNA and

Figure 3.13. Analysis of the ICP4-GST fusion protein produced from plasmid pIGF-17.

Protein samples were subjected to electrophoresis through a 13 % SDS-polyacrylamide gel, then transferred to nitrocellulose. (A). Coomassie Brilliant Blue staining pattern of proteins retained in the gel after transfer; (B). Proteins bound to nitrocellulose were reacted with a ^{32}P-labelled oligonucleotide duplex bearing an ICP4 binding site. Lane 1: ICP4-GST purified by DNA affinity chromatography (using an oligomers of duplexes compose of AB1856 (5'-GATCCCGATCGTCCACACGGAGCGCGGCTA-3') and AB1857 (5'-GATCTAGCCGCGCTCCGTGTGGACGATCGG-3')); lane 2: ICP4-GST purified by chromatography on glutathione sepharose; lane 3: GST purified by chromatography on glutathione sepharose. Sizes of protein markers (M; Amersham) are shown on the left.


A
M 1 2 3
200
97.4
69
46
30
B
M 1 2 3

subsequently isolated from unbound DNA fragments by binding of the GST moiety of the fusion protein to glutathione-sepharose beads. DNA fragments retained by the protein-bead complex are then examined by gel electrophoresis.

3.3.2.1. Interaction of ICP4-GST with the *ICP4* Promoter

The ability of ICP4-GST to interact with the strong consensus ICP4 binding site present in the *ICP4* promoter (Fig. 3.14) was first examined by a capture assay. For this purpose, a plasmid, designated p4PRO, containing sequences straddling the *ICP4* promoter was constructed. The 391 bp *Sal*I - *Nco*I fragment (with the *Nco*I terminus filled in to a blunt end) of pGX58 was inserted into the *Sal*I - *Sma*I junction in the multiple cloning site (MCS) of the pGEM-4Z vector (Fig. 3.15). Positive clones were identified by digestion with *Pvu*I (positive clones yielded fragments of 374, 896, and 1852 bp) and the sequence spanning the insert confirmed by the chain termination method (Fig. 3.16). In the capture assay, when ICP4-GST was incubated with *Hpa*II cut p4PRO, retention of the majority of the fragments was competed by addition of 50 μg/mL of sheared herring sperm DNA (Fig. 3.17). However, the 122 bp fragment bearing the ICP4 binding site from the *ICP4* promoter required ca. 32 times this amount of nonspecific competitor to eliminate binding to ICP4-GST, indicating specificity in the binding of this fragment. In addition, a 110 bp fragment of vector origin required ca. 8 times the above amount of competitor to eliminate retention by ICP4-GST; it

Figure 3.14. Arrangement of cis-acting sequences and LM PCR primers in the *ICP4* promoter.

The relative positions of the following regulatory elements located in the *ICP4* promoter between -213 and +203 (relative to the transcriptional start) are shown: Sp1 binding sites (cross-hatched boxes) at -198 to -178, -138 to -120, and -90 to -71, a TAATGARAT motif (grey box) at -119 to -95, the TATA box (open box) at -26 to -22, and an ICP4 binding site (black box) at -10 to +20. The relative positions of sites of several restriction enzymes used in this study are shown. The relative locations of the sense and antisense primers used for ligation mediated PCR are also shown. Primers used for the initial Sequenase extension of DNA fragments are pictured furthest from the line representing the *ICP4* promoter: sense - 5'-GCGGGGTCGTGCATAAT-3' (AB1300), antisense - 5'-CTTGGGTGGGAAAAAGGA-3' (AB1302). PCR primers are shown in the centre: sense - 5'-GGGTCGTGCATAATGGAATTCCGTTC-3' (AB1413), antisense - 5'AAAGGACAGGGACGGCCGATCCC-3' (AB1303). Primers used for the final detection by primer extension are drawn closest to the line representing the *ICP4* promoter: sense - 5'-GGTCGTGCATAATGGAATTCCGTTCG-3' (AB1646), antisense - 5'-ACGGCCGATCCCCCTCCCGCGCTTC-3' (AB1647).

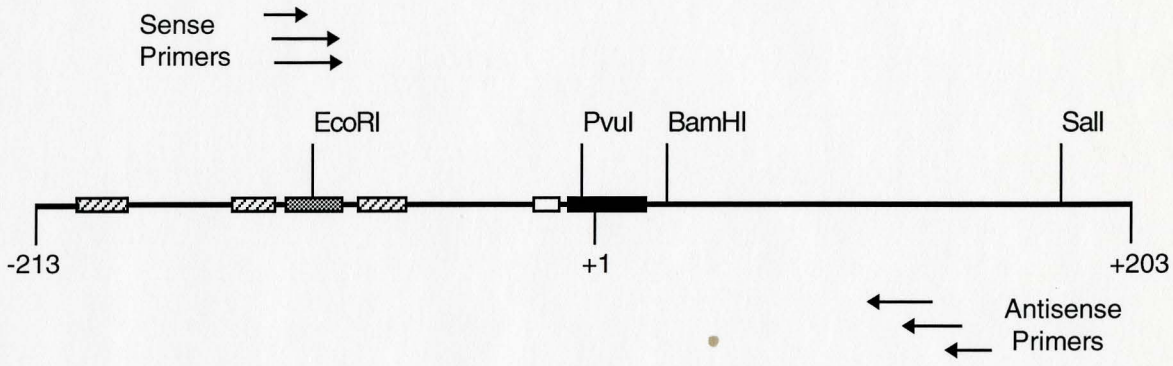

Sense
Primers
EcoRI
PvuI
BamHI
SalI
-213
+1
+203
Antisense
Primers

Figure 3.15. Map of p4PRO.

Plasmid p4PRO was made as follows. The *Nco*I terminus of the 391 bp *Sal*I - *Nco*I fragment of pGX58 (Everett, 1984b) was filled in to form a blunt end. The fragment was then inserted into the *Sal*I-*Sma*I junction in the multiple cloning site of pGEM-4Z (Promega). Sequences from the ICP4 promoter are indicated by the black box within the multiple cloning site (white boxes). The β-lactamase coding region, which confers resistance to ampicillin, is shown by the hatched box.

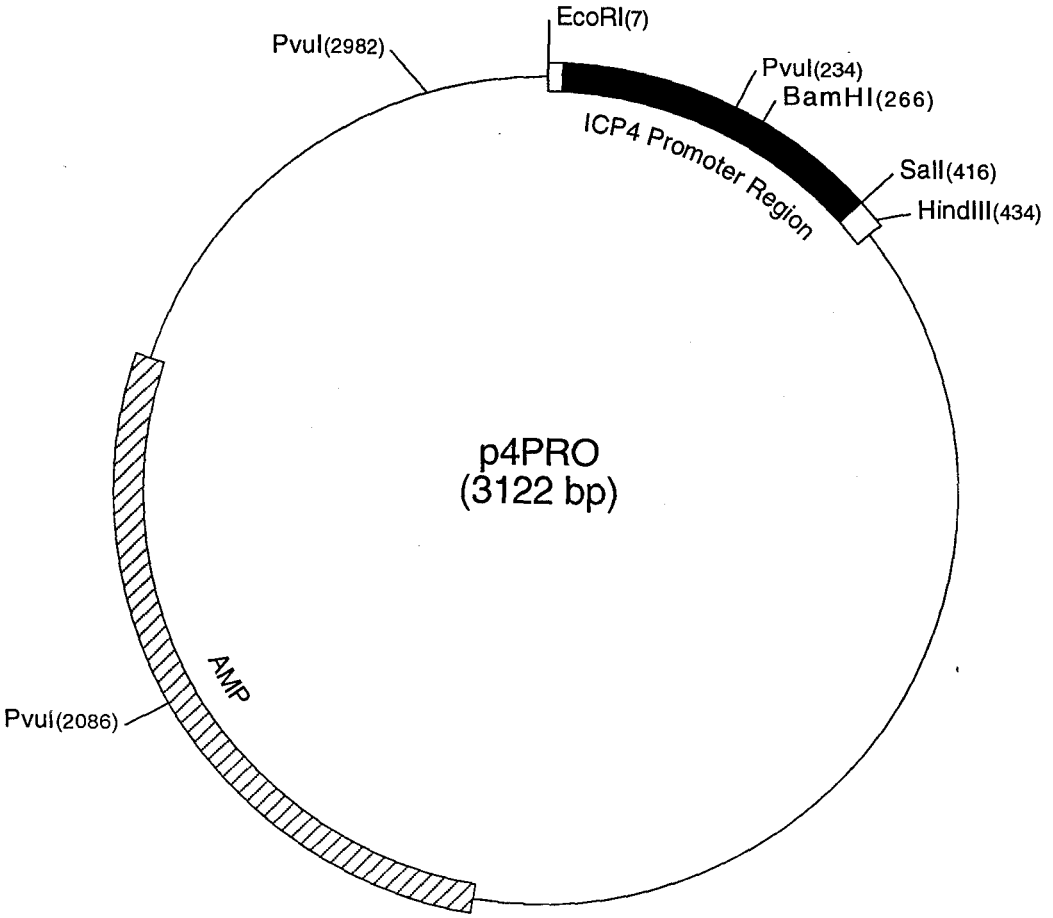

EcoRI(7)
PvuI(2982)
PvuI(234)
BamHI(266)
ICP4 Promoter Region
SalI(416)
HindIII(434)
p4PRO
(3122 bp)
AMP
PvuI(2086)

Figure 3.16. Sequence of p4PRO insert.

The sequence of the HSV-1 insert in p4PRO was confirmed by the dideoxynucleotide chain termination method. Primers corresponding to positions 3104 to 3122 (5'-GGATTTAGGTGACACTATA-3') and positions 463 to 447 (5'-AATACGACTCACTATA-3') of p4PRO were used to sequence the upper and lower strands (respectively) of the insert. The sequence spanning the insert was as follows (vector sequences are written in lowercase; overlapping complementary sequences are underlined):

5'-tcggtacccCATGGCAGATGGCGCGGATGGGCGGGGCCGGGGGTTCGACCAACGGGCC

GCGGCCACGGGCCCCCGGCGTGCCGGCGTCGGGGCGGGGTCGTGCATAATGGAATTCCGTT

CGGGGTGGGCCCGCCGGGGGGGCGGGGGGCCGGCGGCCTCCGCTGCTCCTCCTTCCCGCCG

GCCCCTGGGACTATATGAGCCCGAGGACGCCCCGATCGTCCACACGGAGCGCGG-3'
3'-GGACCCTGATATACTCGGGCTCCTGCGGGGCTAGCAGGTGTGCCTCGCGCCGACGGCT

GTGCCTACCTAGGTGCTGGGCTGCGCCCTGGCGGTCTCTGTCTGGCAGTCTGCGAGCGGCG

CGGCCCTGCGGCTATGCGCCTGCTTCGCGCCCTCCCCCTAGCCGGCAGGGACAGGAAAAAG

GGTGGGTTCGTAGCTGGCCAGGCGCGATCAAGGCGCAGCTggacgt-5'

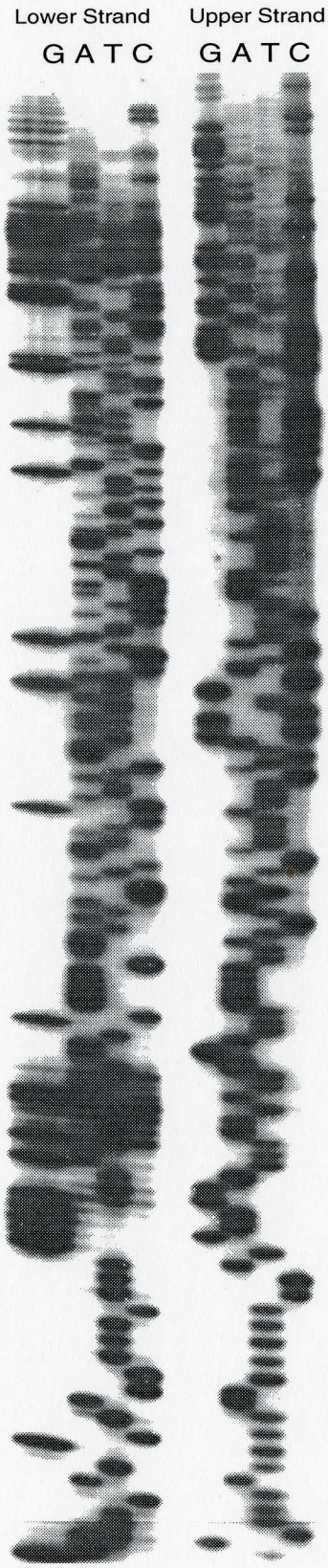
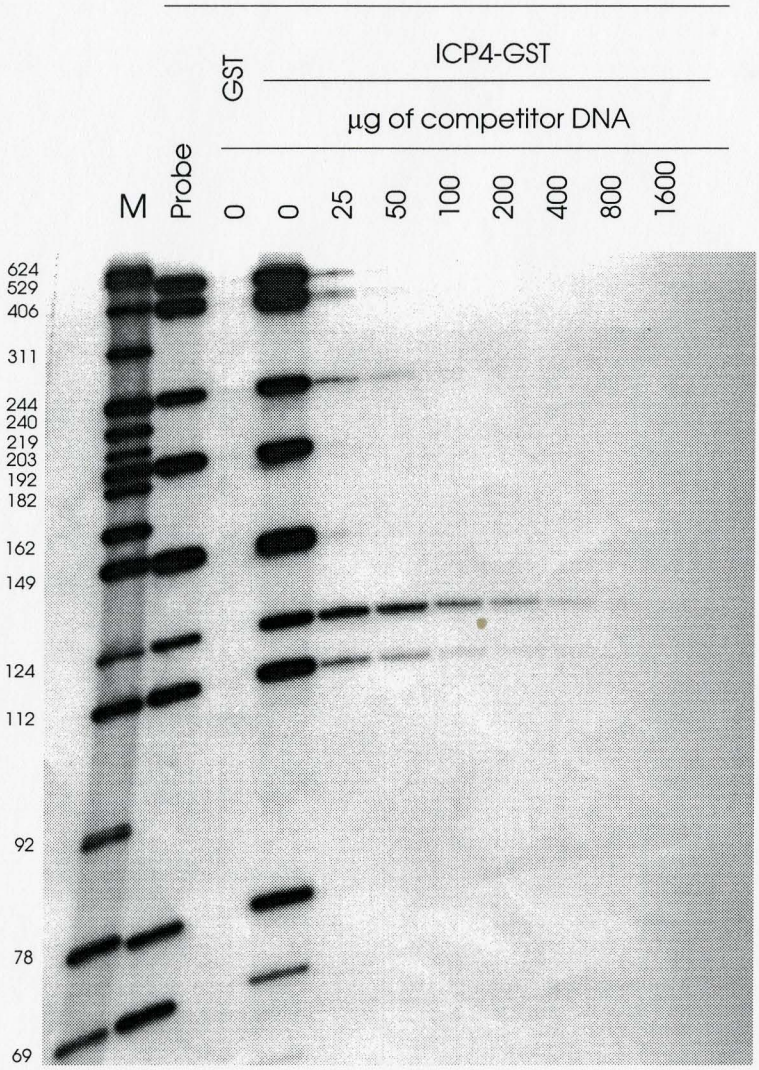

Lower Strand
G A T C
Upper Strand
G A T C

Figure 3.17. Binding of ICP4-GST to a fragment from the *ICP4* promoter.

10 μg of ICP4-GST were incubated with 20 ng of ^{32}P-labelled *Hpa*II cut p4PRO in the presence of 0 to 3200 μg/mL of sheared herring sperm DNA. DNA bound by ICP4-GST was then isolated by reaction with glutathione sepharose. Fragments retained by the ICP4-GST-glutathione sepharose complex were eluted and separated on a 7M urea - 8% polyacrylamide gel. Untreated probe and DNA fragments retained by 10 μg of GST are also shown. Molecular weight markers (M) were 3'-end-labelled *Hpa*II fragments of pBR322. Marker fragment sizes (in nucleotides) are shown on the left.


GST
ICP4-GST
µg of competitor DNA
M
Probe
0
0
25
50
100
200
400
800
1600
624
529
406
311
244
240
219
203
192
182
162
149
124
112
92
78
69

is not known whether this fragment contains a site which is recognised by intact ICP4.

I further examined the interaction between ICP4-GST and the *ICP4* promoter using DNase I footprinting. A 285 bp *Eco*RI - *Sal*I fragment of p4PRO (Fig. 3.15), containing sequences spanning the ICP4 promoter, was 3'-end labelled at the *Eco*RI end. When treated with DNase I, a region of the *ICP4* promoter extending from -9 to +12, relative to the *ICP4* transcriptional start , was protected by ICP4-GST (Fig. 3.18); the location of the footprint was determined by comparison with a base specific chemical cleavage ladder of the probe (Maxam and Gilbert, 1980). In comparison, Faber and Wilcox (1988) have found that intact ICP4 protects a region extending from -10 to +20. Thus, I conclude that ICP4-GST ability to bind a strong consensus ICP4 binding site is similar to that of the intact protein. However, additional footprints not reported in experiments using intact ICP4 were evident, notably one extending from +25 to +41 relative to the transcriptional start (Fig. 3.18). It was not determined whether intact ICP4 could protect these regions under the conditions used in this study.

3.3.2.2. Interaction of ICP4-GST with the *gD* Promoter

Having found that ICP4-GST binds a strong consensus ICP4 binding site, I next examined whether it could interact with weaker consensus or nonconsensus ICP4 binding sites. For this purpose, I used pJB39 (Smiley et al., 1992) which contains sequences spanning the *gD* promoter, including three ICP4 binding sites (Tedder et al., 1989; Fig. 3.19): Site I, a

Figure 3.18. DNase I footprinting of the ICP4 binding site in p4PRO.

The *Eco*RI - *Sal*I fragment spanning the ICP4 binding site in p4PRO (3'-end labelled at the *Eco*RI end) was incubated with 15 μg of ICP4-GST or GST (purified by chromatography on glutathione sepharose), then treated briefly with DNase I. Digestion products were separated by electrophoresis through a 7 M urea - 8% polyacrylamide gel. The regions of the fragment protected by the ICP4-GST fusion product are indicated by the brackets shown on the right. Lanes G, A+G, T+C, and C show base-specific chemical cleavage products of the same end-labelled fragments (Maxam and Gilbert, 1980).

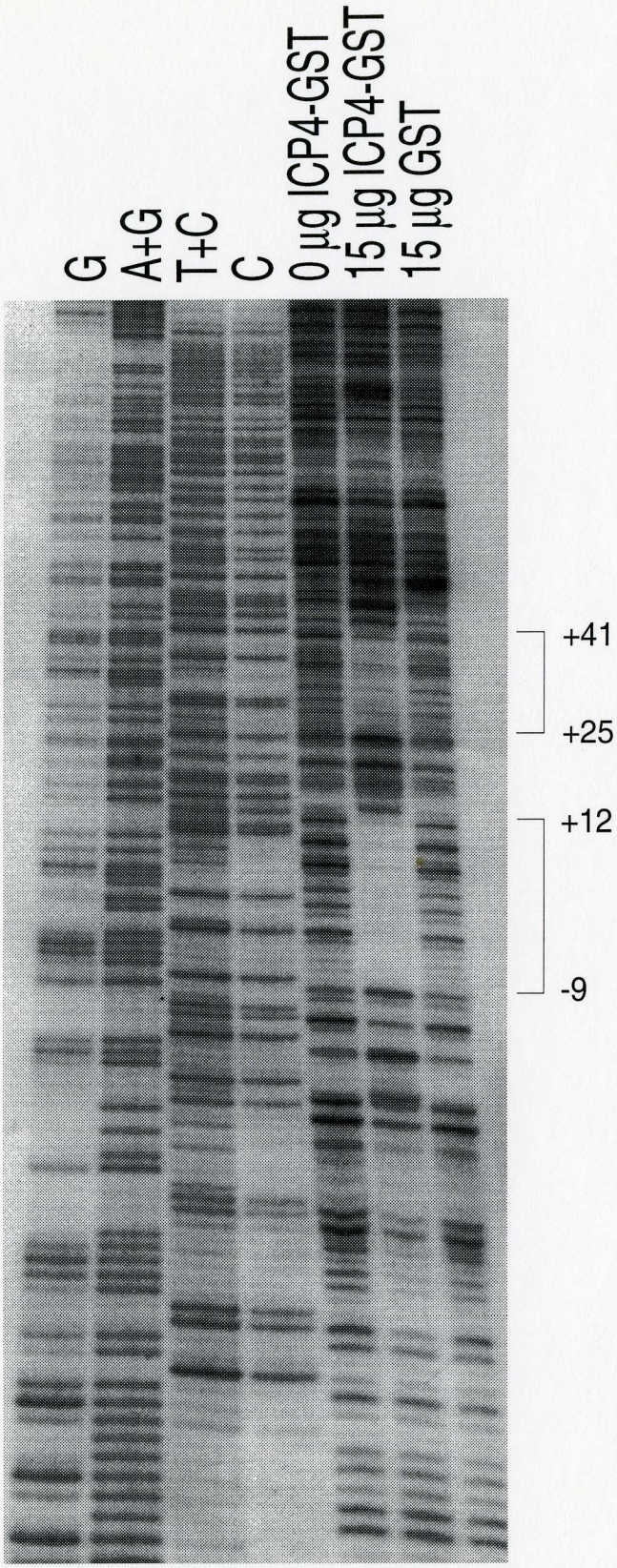
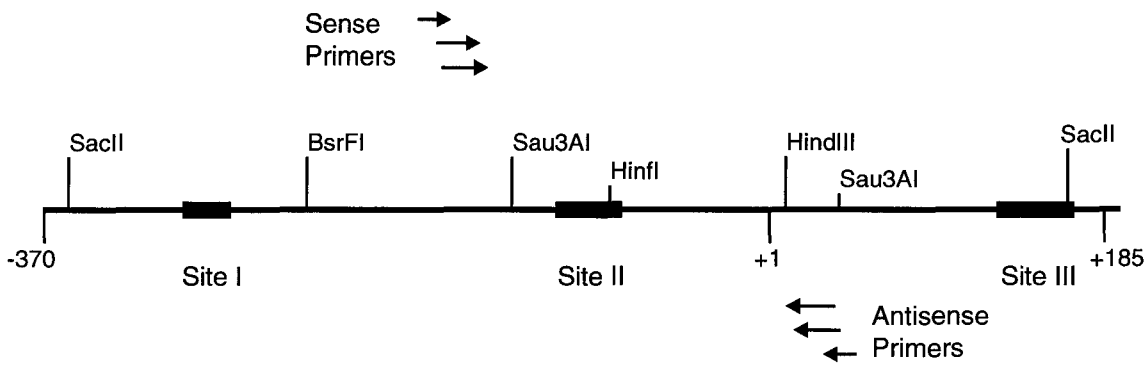

G
A+G
T+C
C
0 µg ICP4-GST
15 µg ICP4-GST
15 µg GST
+41
+25
+12
-9

Figure 3.19. Arrangement of cis-acting sequences and LM PCR primers in the *gD* promoter.

The relative positions of the ICP4 binding sites located in the *gD* promoter are shown as black boxes: Site I, at position -308 to -282 relative to the transcriptional start, Site II, at -111 to -75, and Site III, at +122 to +163. The relative positions of sites of several restriction enzymes used in this study are shown. The relative locations of the sense and antisense primers used for ligation mediated PCR are also shown. Primers used for the initial Sequenase extension of DNA fragments are pictured furthest from the line representing the *gD* promoter: sense - 5'-CCAACAACACCGGGCTAA-3' (AB1702), antisense - 5'-ATGATCGGGGTAGTTGGT-3' (AB1699). PCR primers are shown in the centre: sense - 5'-CGGGCTAACCAGGAAATCCGTGGC-3' (AB1701), antisense - 5'-GGTAGTTGGTCGTTCGCACTGAAGC-3' (AB1698) Primers used for the final detection by primer extension are drawn closest to the line representing the *gD* promoter: sense - 5'-GCTAACCAGGAAATCCGTGGCCCCG-3' (AB1700), antisense - 5'-AGTTGGTCGTTCGCACTGAAGCTTATG-3' (AB1697).


Sense
Primers
SacII
BsrFI
Sau3AI
HinfI
HindIII
Sau3AI
SacII
-370
Site I
Site II
+1
Site III
+185
Antisense
Primers

nonconsensus binding site; Site II, a strong consensus binding site; and Site III, a weaker consensus binding site. Another plasmid, pgD123mut (Smiley et al., 1992), bears *gD* promoter sequences in which Sites I and II are deleted and several point mutations introduced into Site III. Binding by ICP4 to these mutated sites, as examined by gel mobility shift assays, is greatly reduced or eliminated in the cases of Sites I and II, and reduced for Site III (Smiley et al., 1992).

I examined the ability of ICP4-GST to interact with each of the ICP4 binding three sites (or mutant sites) in pJB39 and pgD123mut in a capture assay. The 527 or 494 bp *Sac*II fragment of pJB39 and pgD123mut (respectively) was isolated, cut with *Sau*3AI, and 3'-endlabelled. This *Sac*II fragment spans the *gD* promoter; *Sau*3AI cuts this *Sac*II fragment twice such that ICP4 binding Sites I, II, and II are contained on different fragments. The predicted sizes of these fragments are ca. 234 bp for the fragment bearing Site I (or ca. 222 bp for the deleted Site I), 178 bp for the fragment containing Site II (or 157 bp for the deleted Site II), and ca. 119 bp for the fragment bearing either the intact or mutant Site III. Since these predicted sizes are based on the HSV-1 strain 17 sequence (McGeoch et al., 1985) and plasmids pJB39 and pgD123mut were derived from HSV-1 strain KOS sequences (Smiley et al., 1992), these size estimates are only approximations. When these fragments were separated on an 8% polyacrylamide - 7 M urea gel, four fragments were observed (Fig. 3.20). The approximate sizes of the fragments from pJB39 were 292, 234, 175, and 113 bp; the approximate sizes of the fragments from pdD123mut were 275, 227, 155, 113 bp. By comparison with the predicted fragment sizes and

Figure 3.20. Binding of ICP4-GST to fragments from the *gD* promoter.

pJB39 and pgD123mut (Smiley et al., 1992) were cut with *Sac*II and the 527 or 494 bp fragment (respectively) isolated, cut with *Sau*3AI, and 3'-end labelled. 10 μg of ICP4-GST were incubated with the ^{32}P-labelled fragments in the presence of 0 to 3200 μg/mL of sheared herring sperm DNA. DNA bound by ICP4-GST was isolated by reaction with glutathione sepharose. Fragments retained by the ICP4-GST-glutathione sepharose complex were eluted and separated on a 7M urea - 8% polyacrylamide gel. Untreated probes and DNA fragments retained by 10 μg of GST are also shown. Molecular weight markers (M) were 3'-end-labelled *Hpa*II fragments of pBR322. Marker fragment sizes (in nucleotides) are shown on the left.

Intact ICP4 Binding Sites
Mutated ICP4 Binding Sites
GST
ICP4-GST
µg of competitor DNA
M
Probe
0
0
25
50
100
200
400
800
1600
3200
624
529
406
311
244
240
219
203
192
182
162
149
124
112

restriction enzyme digest with *Xho*I, *Sal*I, and *Ban*II (which are unique sites in the pgD123mut *Sac*II fragment introduced by the ICP4 binding site mutations; Smiley et al., 1992), the fragments were identified as follows. The smallest fragment (ca. 113 bp) contains Site III (or the mutated Site III). The ca. 175 or 155 bp fragment bears Site II or the deleted Site II, respectively. The ca. 234 or 227 bp fragment contains Site I or the deleted Site I, respectively. The largest fragment (ca. 292 or 275 bp) results from incomplete cleavage at the *Sau*3AI site between Sites II and III and thus contains both sites. When these fragments were incubated with ICP4-GST in a capture assay, the following results were obtained. Whereas 100 μg/mL of sheared herring sperm DNA was sufficient to eliminate retention of the ca. 155 bp fragment bearing the deleted Site II, significant amounts of the ca. 175 bp fragment encoding the intact Site II were still bound by ICP4-GST in the presence of 32 times this quantity of competitor (Fig. 3.20). Thus, as expected, ICP4-GST binds a second strong consensus ICP4 binding site. Less competitor DNA (1600 μg/mL) was required to eliminate retention of the ca. 234 bp fragment bearing the nonconsensus Site I. Binding of the ca. 227 bp fragment bearing the mutated Site I to ICP4-GST was eliminated with one quarter of this amount of competitor DNA (400 μg/mL). Thus, the binding of ICP4-GST to a nonconsensus ICP4 binding site occurs with reduced specificity relative to a strong consensus site. Neither the ca. 113 bp fragment bearing the intact Site III nor the fragment containing the mutated Site III were retained by ICP4-GST in a capture assay. This is not unexpected as 5 of the 40 bp protected in DNase I footprinting of this region (Tedder et al., 1989) were removed by the *Sac*II cut used in making this

probe and thus binding may be destabilized. The ca. 292 bp (or 275 bp in the mutant) fragment bearing both Sites II and III was retained in a manner similar to the fragment bearing Site II alone. 3200 μg/mL of competitor DNA was insufficient to eliminate binding of the fragment bearing the wild type sites by ICP4-GST, whereas retention by ICP4-GST of the fragment bearing the mutated sites was eliminated by one 64th of this amount of nonspecific competitor. Thus, I conclude that ICP4-GST retains the ability to bind both consensus and nonconsensus ICP4 binding sites.

I further characterized ICP4-GST's interaction with Sites I and III by DNase I footprinting. Binding of ICP4-GST to Site I was examined using the ca. 362 or 350 bp *Bsr*FI - *Nru*I fragment of pJB39 or pgD123mut (respectively; 3'-endlabelled at the *Bsr*FI end). The location of the footprints was determined by comparison with a base specific chemical cleavage ladder of the appropriate probe (Maxam and Gilbert, 1980). I found that ICP4-GST protected a region extending from -303 to -280 relative to the *gD* transcriptional start (Fig. 3.21), corresponding to Site I. In DNase I footprinting assays using intact ICP4, a region from -307 to -283 is protected (Tedder et al., 1989). As expected, no footprint was observed on the fragment bearing the deleted Site I (Fig. 3.21). Binding of ICP4-GST to Site III was examined using the ca. 261 bp *Hind*III - *Rsa*I fragments of pJB39 and pgD123mut (3'-endlabelled at the *Hind*III end). Protection by ICP4-GST of sequences at Site III was similar to that provided by intact ICP4: +123 to +159 versus +123 to +162 (respectively; Fig. 3.22, Tedder et al., 1989). However, a smaller footprint, extending from +142 to +159, was evident on the fragment bearing the mutated site (Fig. 3.22). This is not entirely

Figure 3.21. DNase I footprinting of *gD* ICP4 binding site I.

*Bsr*FI - *Nru*I fragments spanning the ICP4 binding site in pJB39 and pgD123mut (3'-end labelled at the *Bsr*FI end) were incubated with 15 μg of ICP4-GST or GST (purified by chromatography on glutathione sepharose), then treated briefly with DNase I. Digestion products were separated by electrophoresis through a 7 M urea - 8% polyacrylamide gel. The region of the fragment protected by the ICP4-GST fusion product is indicated by the bracket shown on the left. Wild type: pJB39; mutant : pgD123mut. Lanes G, A+G, T+C, and C show base-specific chemical cleavage products of the same end-labelled fragments (Maxam and Gilbert, 1980).

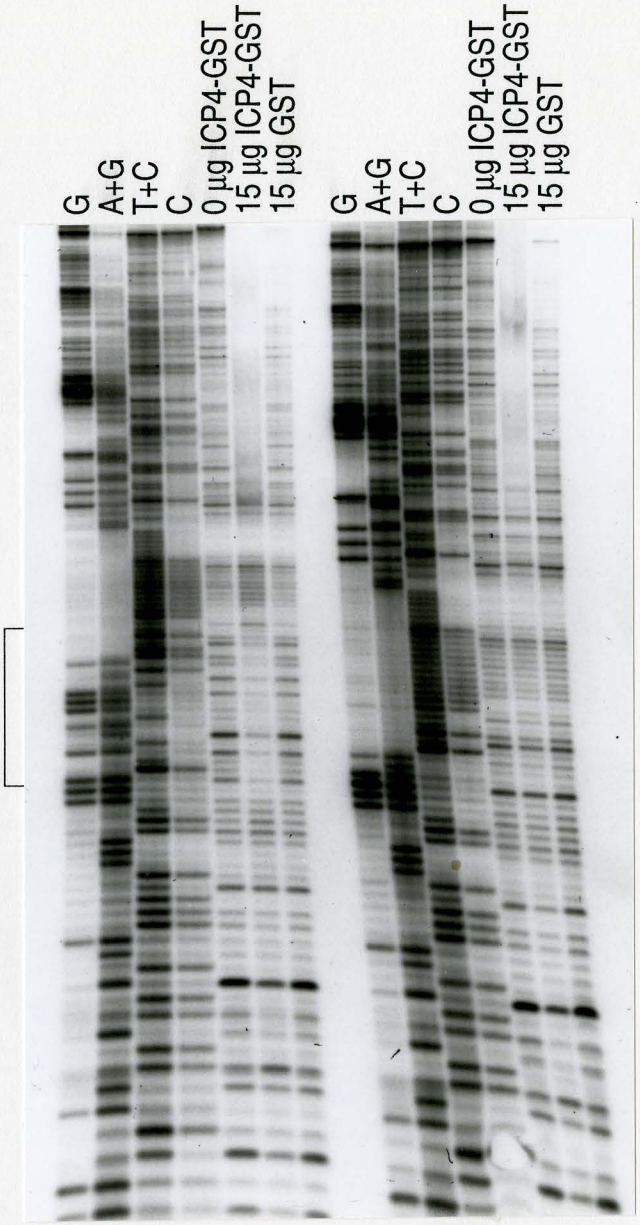
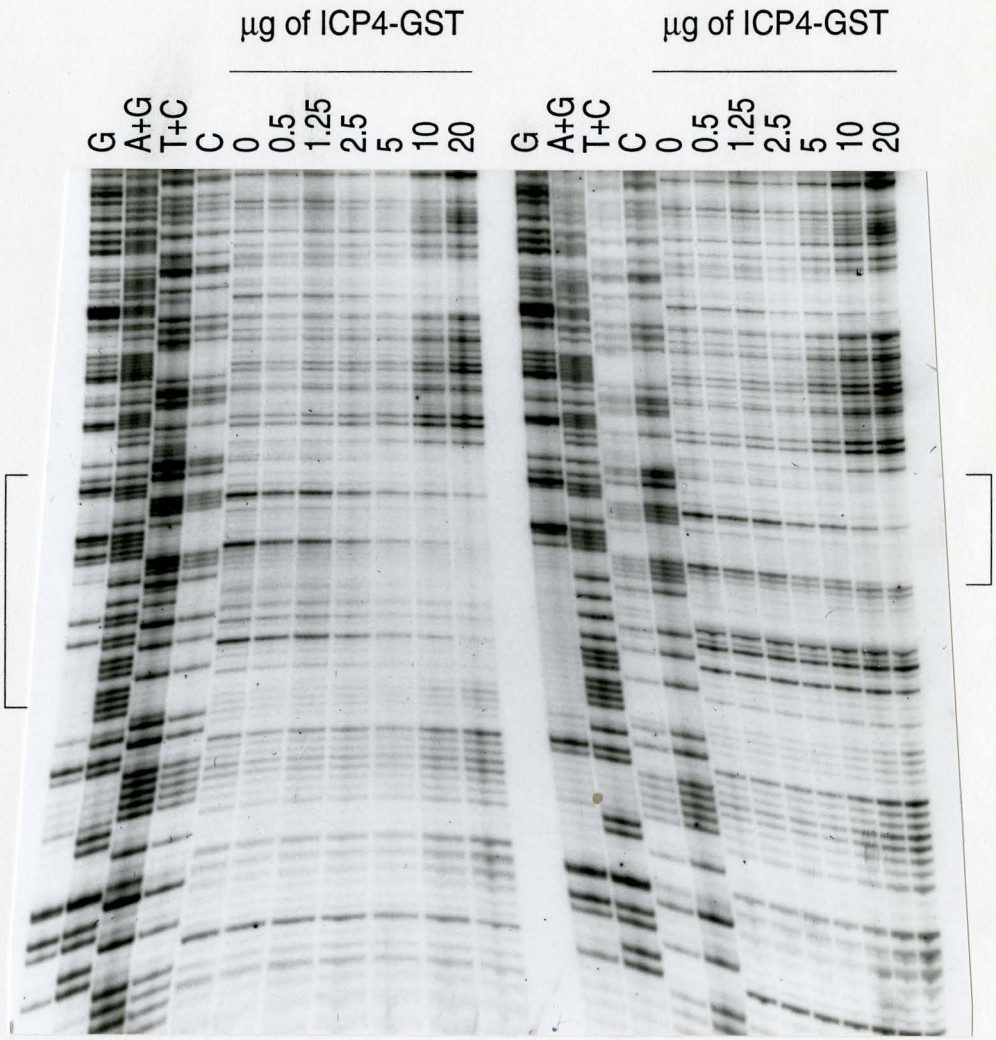

G
A+G
T+C
C
0 µg ICP4-GST
15 µg ICP4-GST
15 µg GST
G
A+G
T+C
C
0 µg ICP4-GST
15 µg ICP4-GST
15 µg GST
Wildtype
Mutant

Figure 3.22. DNase I footprinting of *gD* ICP4 binding site III.

*Hind*III - *Rsa*I fragments spanning the ICP4 binding site in pJB39 and pgD123mut (3'-end labelled at the *Hind*III end) were incubated with 0, 0.5, 1.25, 2.5, 5, 10, or 20 μg ICP4-GST (purified by chromatography on glutathione sepharose), then treated briefly with DNase I. Digestion products were separated by electrophoresis through a 7 M urea - 8% polyacrylamide gel. The region of the fragment protected by the ICP4-GST fusion product is indicated by the brackets. Wild type: pJB39; mutant : pgD123mut. Lanes G, A+G, T+C, and C show base-specific chemical cleavage products of the same end-labelled fragments (Maxam and Gilbert, 1980).


µg of ICP4-GST
G
A+G
T+C
C
0
0.5
1.25
2.5
5
10
20
µg of ICP4-GST
G
A+G
T+C
C
0
0.5
1.25
2.5
5
10
20
Wildtype
Mutant

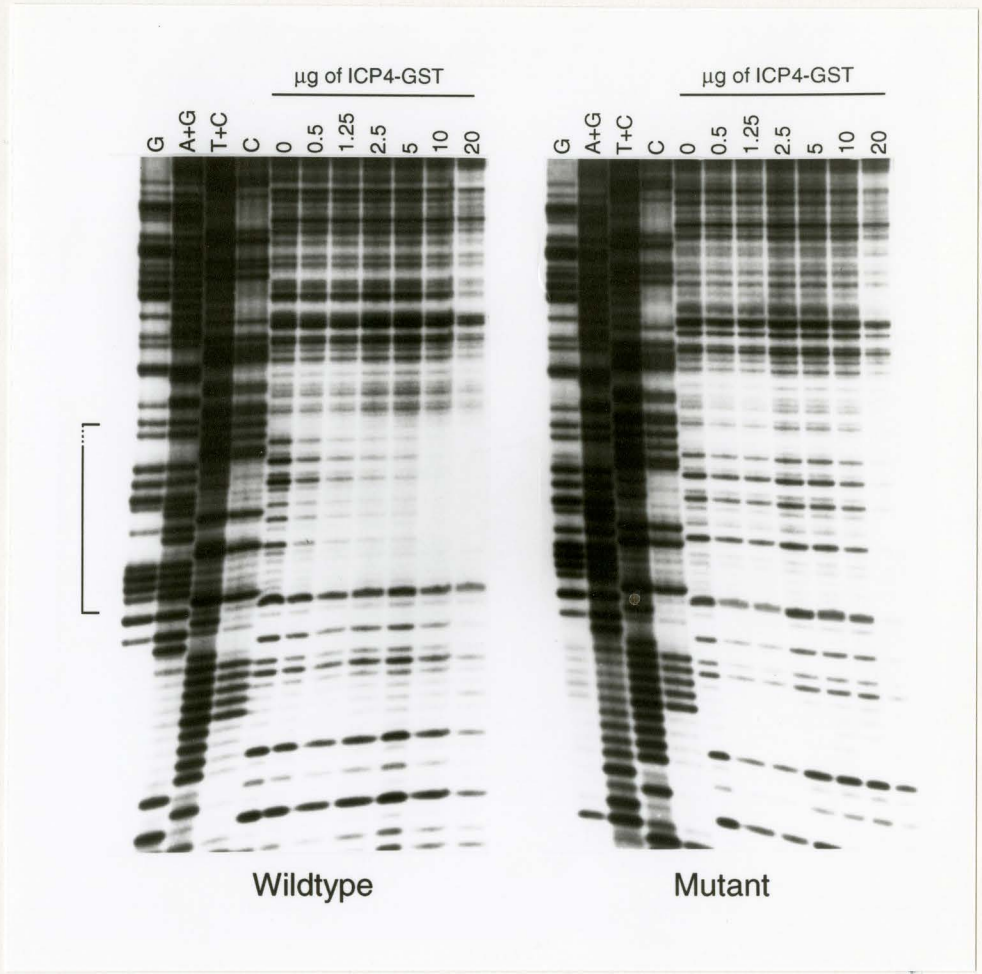
unexpected as a fragment bearing this mutated site is bound by intact ICP4 in gel mobility shift assays (albeit at 15% of the levels found for the intact site; Smiley et al., 1992). The three point mutations made in Site III are located in the 5' half of the site (Smiley et al., 1992); given the large size of the site, it is possible that it actually consists of two tandem sites, only one of which was affected by the mutations. However, on the basis of the results of the capture assays and DNase I footprinting assays, I conclude that ICP4-GST retains the DNA binding specificity of intact ICP4.

3.3.3. DNase I Footprinting of the Promoter Inserts of pMLPT/4 and pMLPT/mut4

Having characterized the DNA binding specificity of the ICP4-GST fusion protein, I next confirmed its ability to bind the ICP4 binding site used to construct the viral recombinants described above. The interaction of ICP4-GST with the ICP4 binding site (or mutant site) was examined using the 744 or 742 bp *Xho*I - *Pvu*II fragment of pMLPT/4 or pMLPT/mut4 (respectively; 3'-endlabelled at the *Xho*I end). In a DNase I footprinting assay, a region corresponding to -9 to +12 relative to the *ICP4* transcriptional start (the source of the site used in the recombinants) in pMLPT/4 (Fig. 3.2) was protected (Fig. 3.23). This is the same region as was protected by ICP4-GST in the intact *ICP4* promoter (Fig. 3.18). As expected, the 'AT' deletion from the 'ATCGTC' motif of the binding site in pMLPT/mut4 (Fig. 3.2) was sufficient to eliminate binding by ICP4-GST

Figure 3.23. DNase I footprinting of the ICP4 binding site in construct MLPT/4.

*Xho*I - *Pvu*II fragments spanning the ICP4 binding site in constructs MLPT/4 and MLPT/mut4 (3'-end labelled at the *Xho*I end) were incubated with 0, 0.5, 1.25, 2.5, 5, 10, or 20 μg ICP4-GST (purified by chromatography on glutathione sepharose), then treated briefly with DNase I. Digestion products were separated by electrophoresis through a 7 M urea - 8% polyacrylamide gel. The region of the fragment protected by the ICP4-GST fusion product is indicated by the bracket shown on the left. Wild type: MLPT/4; mutant : MLPT/mut4. Lanes G, A+G, T+C, and C show base-specific chemical cleavage products of the same end-labelled fragments (Maxam and Gilbert, 1980).


µg of ICP4-GST
G
A+G
T+C
C
0
0.5
1.25
2.5
5
10
20
Wildtype
µg of ICP4-GST
G
A+G
T+C
C
0
0.5
1.25
2.5
5
10
20
Mutant

(Fig. 3.23). Thus, the sequences used to construct the viral recombinants were sufficient or insufficient for binding by ICP4 as per design.

3.4. In Vivo Footprinting - Experimental Design

A second approach towards gaining an improved understanding of the role of ICP4's ability to bind DNA employed a method of in vivo footprinting involving ligation-mediated PCR (Mueller and Wold, 1989; Pfeifer et al., 1989). The intended strategy was to assay for ICP4 binding to previously characterized binding sites during the course of lytic infection by wild type virus or mutants such as pri12, an ICP4 revertant deficient in DNA binding yet having wild type transregulatory activity. Correlations would then be made between the presence or absence of a footprint in the promoters examined and ICP4's transregulatory activities. Unfortunately, these experiments could not be performed as technical difficulties were encountered in the optimization of the ligation-mediated PCR. Although unsuccessful, some of the approaches towards resolving these difficulties are outlined below.

3.4.1. In Vivo Footprinting by Ligation Mediated PCR

In vivo footprinting offers an advantage over the more common in vitro footprinting in that it allows observation of interactions as they occur in their natural setting, with all factors present, and at specific times in the course of the cell cycle (or infectious cycle). This allows a more meaningful

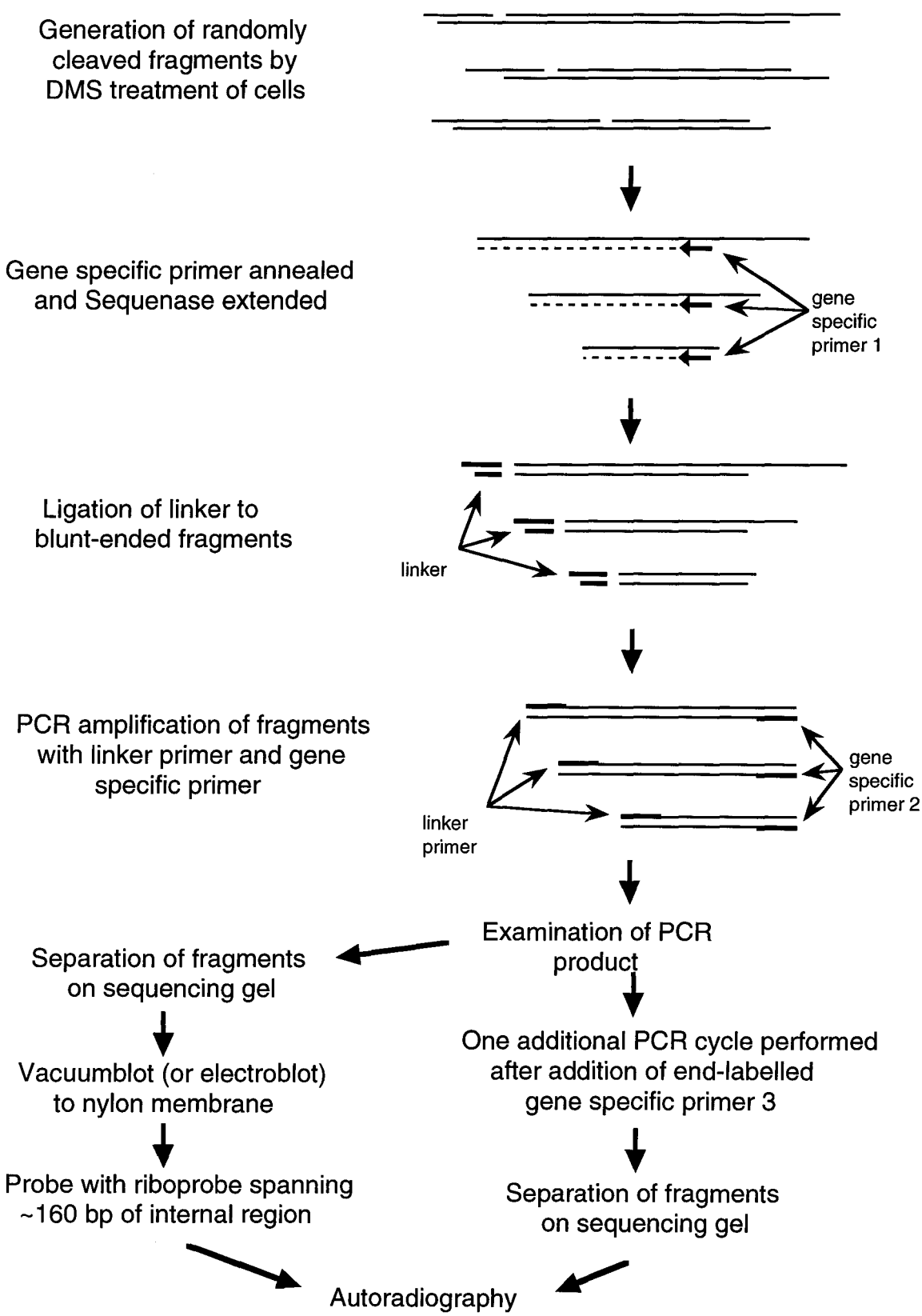
correlation to be made between function and the presence or absence of binding. The major problem associated with in vivo footprinting is that of detecting a signal against the high background of genomic DNA. In vivo footprinting using ligation mediated PCR (LM PCR) addresses this problem by first selectively amplifying the desired DNA fragments and then enhancing specificity through the method of detection of the amplified DNA (Mueller and Wold, 1989; Pfeifer et al., 1989). Cells are first treated with dimethyl sulphate, which selectively modifies guanine residues; these residues are protected from modification in regions of DNA bound by protein. Crude nuclear extracts of the treated cells are made, the genomic DNA denatured, and a gene specific primer annealed and extended by Sequenase polymerase. This yields a set of partially double stranded fragments having one end of the double stranded region defined by the primer and the opposite end a blunt terminal (Fig. 3.24). An oligonucleotide duplex is then ligated to the blunt end; this linker is composed of an 11-mer annealed to a 25-mer and thus has only one blunt end. The DNA fragments now have two defined ends, and are therefore suitable templates for amplification by PCR. PCR is performed using the linker 25-mer and a second gene specific primer overlapping, but extending 3' of, the first gene specific primer; this adds a second level of specificity. Additional specificity is introduced in the detection of the amplified product; this is done by one of two alternate methods. In one method, the PCR products are separated on a sequencing gel, transferred to a nylon membrane, and hybridised to an RNA probe (riboprobe) spanning the region of interest but interior to the PCR primer. In the second method, one additional PCR cycle is performed

Figure 3.24. Sequence of steps in ligation mediated PCR.

The sequence of the linker ligated to the blunt ends generated by primer extension with Sequenase was:

5'-GCGGTGACCCGGGAGATCTGAATTC-3' (AB1298)
3'-CTAGACTTAAG-5' (AB1299)

The longer oligonucleotide was used as the linker primer for amplification by PCR. See text for further details.


Generation of randomly cleaved fragments by DMS treatment of cells
Gene specific primer annealed and Sequenase extended
gene specific primer 1
Ligation of linker to blunt-ended fragments
linker
PCR amplification of fragments with linker primer and gene specific primer
gene specific primer 2
linker primer
Examination of PCR product
Separation of fragments on sequencing gel
Vacuumblot (or electroblot) to nylon membrane
Probe with riboprobe spanning ~160 bp of internal region
One additional PCR cycle performed after addition of end-labelled gene specific primer 3
Separation of fragments on sequencing gel
Autoradiography

after the addition of a third, end-labelled, gene specific primer (overlapping, but extending 3' of, the second gene specific primer). The labelled DNA fragments are then separated by electrophoresis through a sequencing gel.

3.4.2. Ligation Mediated PCR Across the *ICP4* Promoter

The region of HSV-1 initially selected for study by in vivo footprinting was the *ICP4* promoter. This well-characterized promoter has several binding sites for cellular factors, several Vmw65/Oct-1 binding sites, and a strong ICP4 binding site spanning the transcriptional start (Fig. 3.14; Cordingly et al., 1983; Faber and Wilcox, 1988; Jones and Tjian, 1985; Mackem and Roizman, 1982a&b; Muller, 1987). Binding by ICP4 to this latter site is widely believed to be necessary for ICP4 autoregulation (Deluca and Schaffer, 1985; Gelman and Silverstein, 1987a&b; Michael and Roizman, 1993; O'Hare and Hayward, 1987b; Roberts et al., 1988). Thus, the *ICP4* promoter was suitable for study as it has several binding sites for other factors (to serve as positive controls) and it contains an ICP4 binding site considered to be required for regulation by ICP4. As the LM PCR technique is relatively complex, I decided to optimize conditions using plasmid DNA, rather than genomic viral DNA. For this purpose, I used p4PRO, described above (Fig. 3.15). A second plasmid containing sequences spanning the ICP4 promoter, designated pPB10, was constructed for use as a template to make the riboprobe used in the hybridisation method of detection of PCR products. The ends of the 168 bp *Nci*I fragment of pGX58 (Everett, 1984b) were filled in to form blunt ends and the fragment inserted

into the *Hinc*II site in the MCS of the pGEM-3Z vector (Fig. 3.25). Positive clones were selected by colony hybridisation using a ^{32}P-labelled oligonucleotide (AB195, 5'-CGAGCGTCTGACGGTCTGTCTCTGG-3') followed by restriction enzyme analysis with *Pvu*I, which yielded fragments of 267, 896, and 1748 bp.

Specific conditions for LM PCR were determined in stages. First, PCR conditions were optimized using gene specific sense and antisense primers corresponding to positions -123 to -98 and +139 to +117 (respectively) relative to the *ICP4* transcriptional start (Fig. 3.14). Next, a sense (positions -126 to -110) or antisense (positions +151 to +134) primer was annealed to p4PRO cut with either *Bam*HI or *Eco*RI, extended with Sequenase, and the linker ligated to the resultant blunt end. This product was amplified by PCR, using the appropriate gene specific PCR primer (above) and the linker primer, and examined by standard polyacrylamide gel electrophoresis. The predicted product sizes of p4PRO cut with *Bam*HI and amplified using either the sense or antisense primer were 180 or 136 bp, respectively; the predicted product size of p4PRO cut with *Eco*RI and amplified using the antisense primer was 271 bp. Once conditions had been adjusted such that a single band corresponding to the predicted size of the product was observed, these products were used to optimize conditions for the hybridisation method of detection. The major products of LM PCR visualized by this method corresponded to the predicted product sizes (Fig. 3.26), although several other bands (ca. 78 and 90 bp) were observed. Next, LM PCR was performed using p4PRO cut by base specific chemical cleavage (Maxam-Gilbert sequencing). A number of problems were evident;

Figure 3.25. Map of pPB10.

Plasmid pPB10 was made as follows. The ends of the 168 bp *Nci*I fragment of pGX58 (Everett, 1984b) were filled in to form blunt ends. The fragment was then inserted into the *Hinc*II site of the multiple cloning site of pGEM-3Z (Promega). Sequences from the ICP4 promoter are indicated by the black box within the multiple cloning site (MCS; white boxes). The T7 and SP6 RNA polymerase promoters which flank the MCS are shown as grey boxes. The β-lactamase coding region, which confers resistance to ampicillin, is shown by the hatched box.

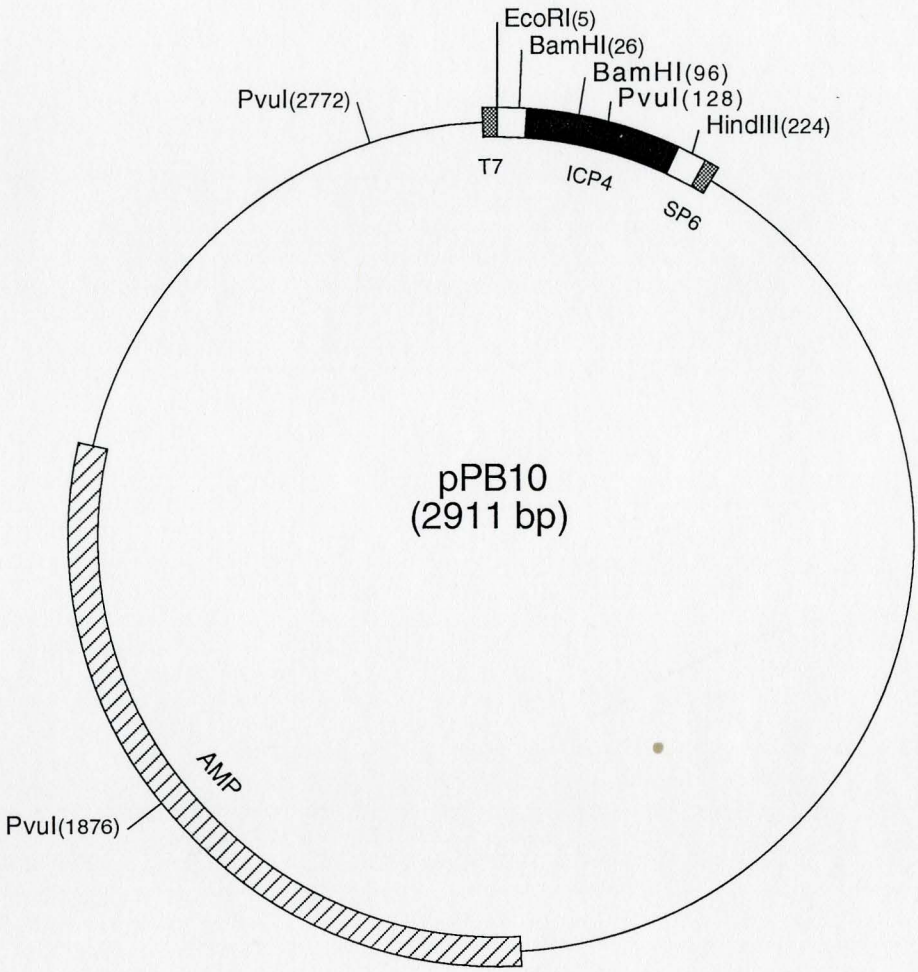

EcoRI(5)
BamHI(26)
BamHI(96)
PvuI(128)
HindIII(224)
PvuI(2772)
T7
ICP4
SP6
pPB10
(2911 bp)
AMP
PvuI(1876)

Figure 3.26. LM PCR of the ICP4 promoter - Detection by hybridisation.

p4PRO cut with a restriction enzyme or cleaved by a base-specific chemical cleavage reaction (Maxam and Gilbert, 1980) was amplified by 30 cycles of LM PCR. Fragments were amplified using the linker primer and either a sense or antisense gene-specific primer. Amplified fragments were separated on a 7M urea - 8% polyacrylamide gel and transferred to a nylon membrane by vacuum blotting. After UV crosslinking of the DNA to the membrane, the fragments were reacted with a ^{32}P-labelled RNA probe made by in vitro transcription from pPB10. Lane 1: *Bam*HI cut p4PRO amplified using the antisense primer. Lane 2: *Bam*HI cut p4PRO amplified using the sense primer. Lane 3: *Eco*RI cut p4PRO amplified using the antisense primer. Lanes 4 and 5: Cytosine-specifically cleaved p4PRO (two separate preparations) amplified using the antisense primer. Molecular weight markers (M) were 3'-end labelled *Hpa*II fragments of pBR322. Marker fragment sizes (in nucleotides) are shown on the left.

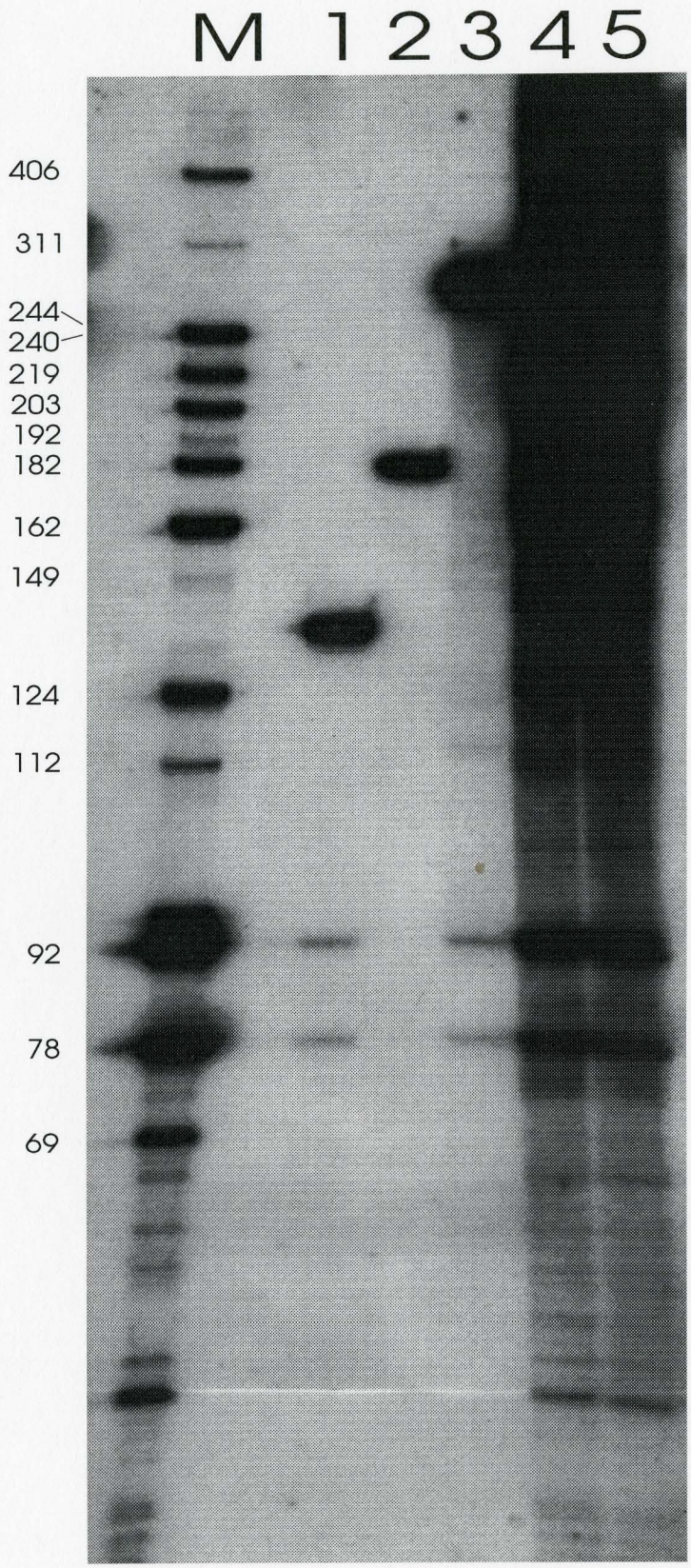
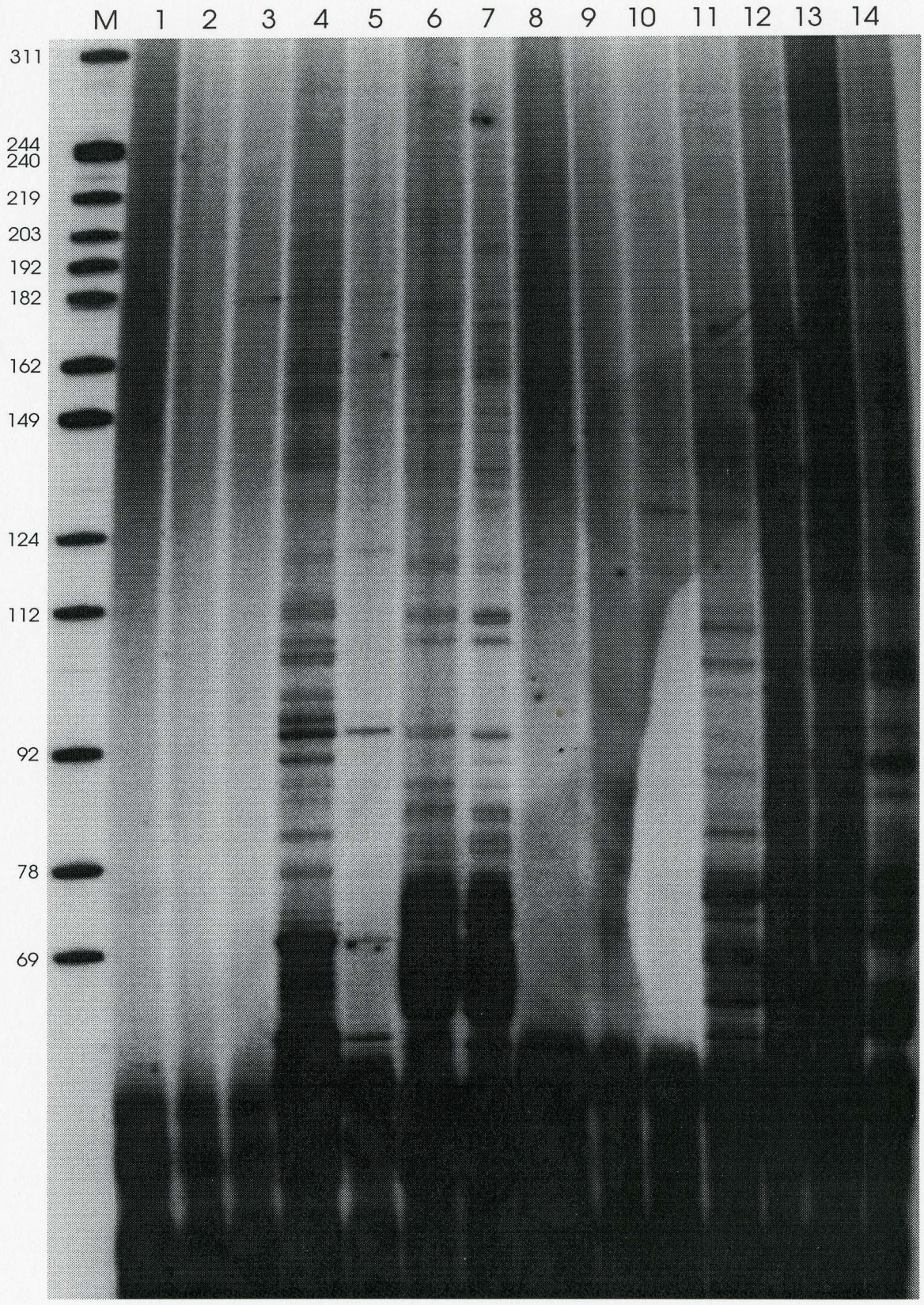

M 1 2 3 4 5
406
311
244
240
219
203
192
182
162
149
124
112
92
78
69

Fig. 3.26 shows one of the most successful experiments; two tracks show the LM PCR products of cytosine specific cleavage of p4PRO. Although bands of various sizes were discernible, they were obscured by high levels of background. The intensity of both the bands and background was greater in areas corresponding to higher molecular weight DNA fragments. In many experiments, no bands were visible above the background; in other experiments, there were high levels of non-specific bands (i.e. the same size bands found in all four base specific cleavage tracks). Despite much manipulation of conditions, these difficulties could not be eliminated entirely.

I decided to attempt detection of the LM PCR amplified sequencing ladder using the primer extension method. An additional PCR cycle was performed using a ^{32}P-end-labelled sense (positions -122 to -97 relative to the *ICP4* transcriptional start) or antisense (positions +128 to +104) primer (Fig. 3.14). An improvement was found over results obtained using the hybridisation method of detection; the amplified base-specific cleavage products had some resemblance to a sequencing ladder. The problems which were typically encountered using this technique are illustrated in Fig. 3.27. Smaller DNA fragments appeared to be preferentially amplified. Furthermore, high background obscured higher molecular weight DNA bands. Results were not always reproducible. For example, the *Bam*HI cut template shown in Fig. 3.27 yielded a stronger signal in other experiments under identical conditions. The adenine and guanine (A+G) specifically cleaved DNA amplified using the sense primer was not amplified as efficiently as the other base specific cleavage reactions, yet in other

Figure 3.27. LM PCR of the ICP4 promoter - Detection by primer extension

p4PRO cut with *Bam*HI or cleaved by base-specific chemical cleavage reactions (Maxam and Gilbert, 1980) was amplified by 30 cycles of LM PCR. Fragments were amplified using the linker primer and either a sense (lanes: 2-7) or antisense (lanes: 9-14) gene-specific primer. A second, ^{32}P-labelled, gene-specific primer (sense or antisense, respectively) was added to the reaction along with additional Amplitaq DNA polymerase and dNTP's and one final PCR cycle performed. Amplified fragments were then separated on a 7M urea - 8% polyacrylamide gel. Labelled primers: Lanes 1 (sense) and 8 (antisense). Lanes 2 and 9: no template DNA. Lanes 3 and 10: *Bam*HI cut p4PRO. Lanes 4 and 11: Guanine specifically cleaved p4PRO. Lanes 5 and 12: Adenine and guanine specifically cleaved p4PRO. Lanes 6 and 13: Thymine and cytosine specifically cleaved p4PRO. Lanes 7 and 14: Cytosine specifically cleaved p4PRO. Molecular weight markers (M) were 3'-end labelled *Hpa*II fragments of pBR322. Marker fragment sizes (in nucleotides) are shown on the left.


M
1
2
3
4
5
6
7
8
9
10
11
12
13
14
311
244
240
219
203
192
182
162
149
124
112
92
78
69

experiments, the A+G cleavage reaction was successfully amplified whereas other reactions were not. The intensity of the background was also found to be variable.

The high GC content of this region (ca. 75% GC) suggested that one source of the observed difficulties might be incomplete denaturation of the template during thermal cycling. Many modifications were made to address this problem: PCR was performed using elevated denaturation, annealing and extension temperatures using the more thermal stable Vent polymerase or Stoffel fragment of Amplitaq, and denaturation times were extended. PCR was performed with 5% glycerol added to the reaction or with 7'-deaza-dGTP substituted for dGTP; both of these have been demonstrated to enhance PCR amplification of GC-rich DNAs (Innis, 1990; Smith et al., 1990). None of these modifications resulted in a substantial improvement in the detected signal.

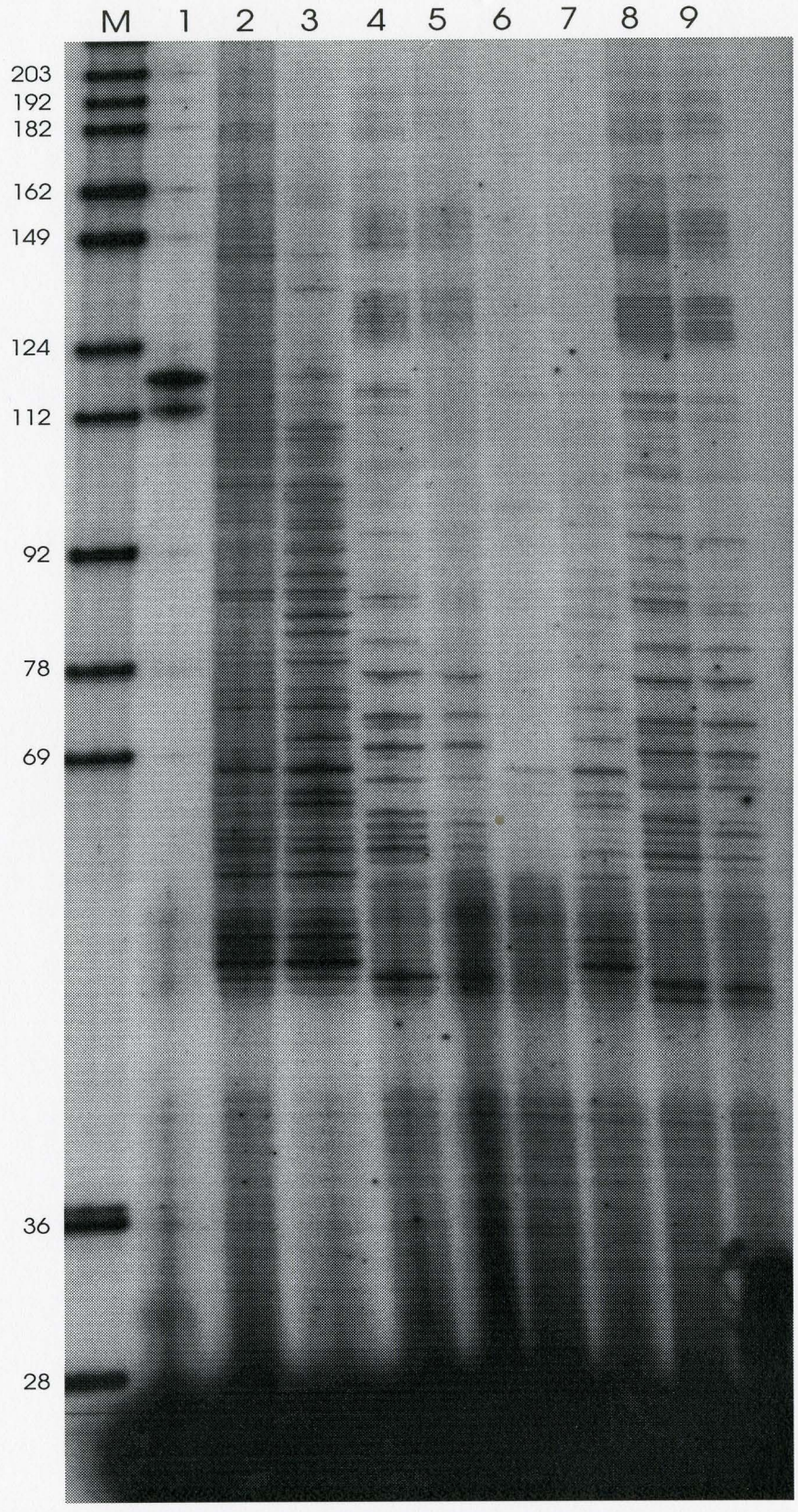
3.4.3. Ligation Mediated PCR Across the *gD* Promoter

In order to circumvent difficulties associated with high GC content, I decided to attempt LM PCR of the region surrounding ICP4 binding site II (-111 to -75 relative to the transcriptional start, Fig. 3.19) in the *gD* promoter (ca. 60% GC). Site II is a strong consensus binding site which has been shown to contribute to stimulation of initiation of RNA synthesis in in vitro transcription assays (Beard et al., 1986; Tedder et al., 1989). The following sense and antisense primers were selected for primer extension, PCR amplification, and detection of the product (respectively): positions -184 to

-167 (sense) and +46 to +29 (antisense), positions -174 to -151 (sense) and +38 to +14 (antisense), and positions -171 to -147 (sense) and +35 to +9 (antisense) (Fig. 3.19). Conditions for LM PCR of the *gD* promoter were optimized as described above, using first restriction enzyme cut plasmid DNA, and then base specifically cleaved plasmid DNA. Plasmid pJB39 (Smiley et al., 1992), which contains the entire *gD* region of HSV-1 strain KOS, was used for this. The premise that high GC content was responsible for the difficulties observed in LM PCR of the *ICP4* promoter was partially supported; it was possible to read further into the amplified *gD* promoter sequence than had been possible in similar experiments with the *ICP4* promoter (Fig. 3.28). However, although the background was reduced and the preferential amplification of smaller fragments partially decreased, the improvement was not sufficient to allow use of the technique for in vivo footprinting of viral DNA. Because of time constraints, this approach in examining the function of ICP4's DNA binding ability was discontinued.

Figure 3.28. LM PCR of the gD promoter.

pJB39 (which contains sequences from HSV-1 strain KOS) cut with *Hin*FI or cleaved by base-specific chemical cleavage reactions (Maxam and Gilbert, 1980) was amplified by 18 cycles of LM PCR. Fragments were amplified using the linker primer and a sense gene-specific primer. A second, ^{32}P-labelled, gene-specific primer was added to the reaction along with additional Amplitaq polymerase and dNTPs and one final PCR cycle performed. Amplified fragments were then separated on a 7M urea - 8% polyacrylamide gel. Lane 1: *Hin*FI cut pJB39. Lanes 2 and 6: Guanine specifically cleaved pJB39. Lanes 3 and 7: Adenine and guanine specifically cleaved pJB39. Lanes 4 and 8: Thymine and cytosine specifically cleaved pJB39. Lanes 5 and 9: Cytosine specifically cleaved pJB39. Either 10 ng or 1 µg of plasmid (lanes 2-5 or 6-9, respectively) were used for the extension reaction with the first gene specific primer and subsequent ligation to the common linker. Molecular weight markers (M) were 3'-end labelled *Hpa*II fragments of pBR322. Marker fragment sizes (in nucleotides) are shown on the left.


M
1
2
3
4
5
6
7
8
9
203
192
182
162
149
124
112
92
78
69
36
28

IV. DISCUSSION

4.1. Effect of an ICP4 Binding Site Introduced into a Model Promoter

In this study, I examined the effect on gene expression of an ICP4 binding site placed in close proximity to a TATA box, independent of any other known regulatory sequences. An ICP4 binding site was placed either upstream or downstream of the TATA box in a simple model promoter within the viral genome, reproducing the spacing which is found in the IE gene *ICP0* or *ICP4* promoters, respectively. In both cases, the binding site greatly reduced accumulation of transcripts early in infection (6 hours postinfection). This repression was partially relieved later in infection (12 hours postinfection). In addition, accumulation of transcripts from promoters bearing an ICP4 binding site was greatly inhibited in the presence of a DNA replication block by aphidicolin. Thus, the net effect of the ICP4 binding site was to shift the kinetics of expression from E to L.

The reduction in transcript levels observed upon addition of an ICP4 binding site to the model promoter is consistent with results of transient expression assays in which expression of a reporter gene driven from either the *ICP4* or *ICP0* promoters is reduced in the presence of ICP4; mutation of the ICP4 binding sites within these promoters restores expression to levels similar to those found in the absence of ICP4 (DeLuca and Schaffer, 1985; Gelman and Silverstein, 1987a,b; O'Hare and Hayward, 1985b; Resnick et al., 1989; Roberts et al., 1988). Furthermore, Michael and

Roizman (1993) have recently shown that the ICP4 binding site(s) located within the *ICP4* promoter mediates repression of that promoter during lytic infection. In contrast, mutation of the high affinity binding site located within the *ICP0* promoter does not affect expression of ICP0 during infection, although the same mutation results in increased gene induction in a transient expression assay (Everett and Orr, 1991). Since the results presented in this study show that an ICP4 binding site located immediately upstream of the TATA box can downregulate expression from that promoter, why was no effect apparent upon mutation of the site within the native *ICP0* promoter? Several factors may account for this apparent contradiction. Firstly, the model promoters examined in this study were inserted into the site of a 200 nt deletion spanning the *UL24b* promoter. Thus, downregulation conferred by the ICP4 binding sites inserted into the model promoters occurred in the apparent absence of other upstream elements. In contrast, the regulated expression of ICP0 during lytic infection results from the cumulative effect of multiple cis-acting sequences. For example, strong activation by Vmw65 may override negative effects due to the ICP4 binding site. Alternatively, if other factors are already associated with promoter sequences, ICP4 binding sites may be inaccessible once ICP4 is produced. Another consideration is that the upstream ICP4 binding site did not inhibit expression from the model promoter to as great a degree as the downstream site did. Furthermore, expression of *ICP4* decreases as the lytic cycle progresses, whereas transcription of *ICP0* increases (Weinheimer and McKnight, 1987). Thus, inhibition of expression by ICP4 during the lytic cycle may be a more

important mechanism for regulation of *ICP4* than of *ICP0*. In contrast to my results that an ICP4 binding site downregulates expression of a promoter lacking additional upstream elements, Gu et al. (1993) have recently reported that ICP4 represses SP1-activated expression from the *ICP4* promoter, but not basal transcription, both in in vitro transcription assays and within the viral genome. The simplest explanation for the difference between our results is that a cis-acting element is present in the sequences flanking the model promoter; thus activated, not basal, levels of expression would have been measured. This possibility could be tested by footprinting assays of the flanking sequences using infected cell extracts, followed by mutation any protected regions and subsequent assay by primer extension of the effect on transcript levels. Alternatively, segments of 'nonspecific' DNA could be inserted upstream of the model promoters to separate them from putative upstream elements.

How does binding of ICP4 in close proximity to a TATA box inhibit expression from that promoter? One possibility is that ICP4 represses transcription simply by steric hindrance or through conformational changes in the DNA induced by its binding. However, Paterson et al. (1990) have isolated an ICP4 mutant which binds DNA but fails to autoregulate, indicating that some activity in addition to DNA binding is required for transrepression by ICP4. Based on observations that ICP4 and TFIID bind opposite helical faces in both the *ICP4* and *ICP0* promoters, DiDonato and Muller (1989) proposed that the orientation of these two factors relative to each other may be important for repression (e.g. repression may involve a direct interaction between ICP4 and TFIID, thus necessitating the correct

positioning of these two factors with respect to each other on the DNA helix). Consistent with this, Smith et al. (1993) have recently demonstrated the formation of a tripartite complex between ICP4, TFIIB, and TFIID; this complex binds DNA fragments which bear both an ICP4 binding site and a TATA box. To address the question of whether the helical orientation of ICP4 with respect to TFIID is important for repression by ICP4, I constructed an additional recombinant virus in which the upstream ICP4 binding site was placed 5 bp (ca. 1/2 helical turn) closer to the TATA box. No apparent difference in transcript levels or kinetics of expression was found between constructs bearing the distal and proximal upstream sites, indicating that the helical orientation of ICP4 with respect to TFIID does not affect transrepression. However, this finding does not argue against repression involving an interaction between ICP4 and TFIID, nor does it exclude the possibility that the positioning of ICP4 with respect to TFIID may affect repression. Association of ICP4 with TFIID and TFIIB may physically prevent formation of the initiation complex and/or block activation by other transcription factors. Perhaps the interaction between these factors is weakened when ICP4 is bound upstream, rather than downstream, of the TATA box; binding of ICP4 to TFIID/TFIIB might then be more readily disrupted, resulting in only partial inhibition of expression. In accordance with this suggestion, inhibition of expression imposed by the downstream ICP4 binding site was virtually complete at six hours whereas inhibition resulting from an upstream site was only partial. Since the ICP4 consensus binding site is asymmetrical (and thus, presumably binding of ICP4 is also), one way of exploring this possibility could be to invert the

ICP4 binding site downstream of the TATA box in a model promoter; this might destabilize the interaction between ICP4 and TFIID/TFIIB and thus reduce repression.

My finding that an ICP4 binding site increases the dependency on DNA replication of a model promoter suggests that this may be one way by which HSV-1 regulates late gene expression. Supporting this possibility, Rivera-Gonzalez and DeLuca (1993) have recently reported that an ICP4 binding site located near the transcriptional start in the *LAT* promoter confers L kinetics of expression. Moreover, ICP4 binding sites have been identified in the vicinity of the transcriptional start sites of true L genes *UL38*, *UL49.5*, *US11*, and *Vmw65* (Flanagan et al., 1991; Michael et al., 1988; P.K. Kibler, J. Duncan, and J.R. Smiley, unpublished results). The ICP4 binding site in the *UL38* promoter is contained within the sequences required for true L regulation (Flanagan et al., 1991; Guzowski and Wagner, 1993); whether ICP4 binding to this site contributes the kinetics of expression of this gene has not been determined. A region of the *UL49.5* promoter bearing two ICP4 binding sites appears to contribute to elevated expression late in infection but does not confer a dependence on DNA replication for expression (Mavromara-Nazos and Roizman, 1989); mutation of one of these sites results in decreased mRNA accumulation but does not alter sensitivity to phosphonoacetate (Romanelli et al., 1992). The role of the ICP4 binding sites in the *US11* and *Vmw65* promoters is not known. Thus, whether the kinetics of expression of any HSV-1 true L genes are determined by ICP4 binding sites remains unclear. One attractive aspect of this possibility relates to my finding that expression from the

promoters bearing the downstream binding site was more sensitive to a DNA replication block than expression driven by the promoters bearing the upstream site. This indicates that the position of the ICP4 binding site affects kinetics of expression. Possibly the different 'degrees of lateness' found among HSV-1 leaky and true L genes is determined by the proximity of the ICP4 binding site to the TATA box. It might then be predicted that the degree of dependency on DNA replication for expression of the promoter bearing the proximal upstream site (ca. 32 bp from the TATA box, measuring from the centre of each site) would be intermediate between that found for the distal upstream site (ca. 37 bp from the TATA box) and the downstream site (ca. 27 bp from the TATA box). Although this was not found to be the case, it is possible that proximity of an ICP4 binding site alters kinetics of expression in a noncontinuous manner. An alternative is that the extent to which expression is dependent on DNA replication is affected by the orientation of ICP4 with respect to the factors interacting with the TATA element - i.e. repression of the general transcription apparatus by ICP4 may be facilitated if ICP4 is bound downstream (as discussed above).

How does DNA replication relieve repression imposed by ICP4? One possibility is that ICP4 is titrated by DNA replication; since each viral genome contains a predicted 500 ICP4 binding sites (DiDonato and Muller, 1989), after replication there are fewer ICP4 molecules than binding sites. Alternatively, perhaps ICP4 is dislodged by the passage of the replicative machinery. A third possibility is that ICP4 remains bound to sites on the viral genome but that conformational changes are induced upon replication

which eliminate ICP4's repressor activity. Relative to the latter suggestion, ICP27 is involved in the switch from E to L gene expression and is thought to post-translationally modify ICP4 (McCarthy et al., 1989; McMahan and Schaffer, 1990; Rice et al., 1989; Sacks et al., 1985; Su and Knipe, 1989). Perhaps L gene expression is initiated through a modification of ICP4 by ICP27 which results in relief of repression. Further investigation of the interaction between ICP4 and ICP27 may yield more information in this regard. For example, although it has been noted that ICP4's electrophoretic mobility is altered in ICP27 mutants, suggesting that ICP27 is involved in post-translational modification of ICP4 (e.g. phosphorylation; McMahan and Schaffer, 1990; Rice et al., 1989; Su and Knipe, 1989), the effects of this on gene regulation by ICP4 have not been examined. Furthermore, it would be interesting to study whether ICP27 is involved in post-translational modification of ICP4 in ICP4 mutants which are defective in L gene expression but not E gene expression (DeLuca et al., 1984; Paterson et al., 1990).

A basic question regarding HSV-1 gene expression is how ICP4 represses transcription of some genes and induces expression of others. If, as suggested by my findings, ICP4 represses some L genes prior to DNA replication, a further question is raised of how it then activates them once DNA replication occurs. One possibility is that activation is mediated through binding to the same site but that replication induces a conformational change in ICP4 which switches it from a repressor to an activator. However, arguing against this suggestion is my finding that expression late in infection from a model promoter bearing an ICP4

binding site remained below that from a similar promoter bearing a mutant binding site. An alternative is that ICP4 induces gene expression from distal binding sites. This would be in accordance with findings that elimination of binding to individual sites does not affect induction by ICP4 and that cellular genes introduced into the viral genome are activated by ICP4, regardless of flanking sequences (Imbalzano et al., 1990; Panning and Smiley, 1989; Smiley et al., 1987, 1992; Smiley and Duncan, 1992). From this it could follow that ICP4 functions as a general transactivator such that all genes within the viral genome are induced unless they are being actively repressed. ICP4 could accomplish this by concentrating transcription factors in the vicinity of the viral genome and/or by facilitating formation of the initiation complex by enhancing binding of TFIIB/TFIID to the TATA box (Smith et al., 1993). One question that arises is how E genes are downregulated late in infection. There is good evidence that at least some IE genes are repressed by ICP4 late in infection and my findings suggest binding of ICP4 may inhibit L gene expression until relieved by DNA replication. There is little information to indicate to how the other half of the switch from E to L gene expression - downregulation of E genes - occurs. ICP27 and ICP22 have both been implicated in the transition to L gene expression; perhaps one or both of them mediates E gene repression.

In the course of this study, a fusion protein, designated ICP4-GST, was constructed in which glutathione S-transferase was linked to the DNA binding domain (amino acids 260-490) of ICP4. After overexpression in E.coli and purification by affinity chromatography, the major product was

found to be a 50 kD polypeptide which bound the ICP4 consensus site. The DNA binding ability of ICP4-GST was further characterized by capture assays and DNase I footprinting analysis. ICP4-GST was found to bind specifically to both consensus and nonconsensus binding sites. It was thus a suitable tool to examine binding to the binding sites used in the construction of recombinant viruses.

4.2. In Vivo Footprinting by Ligation Mediated PCR

Interactions between ICP4 and its binding sites within the viral genome could not be examined by in vivo footprinting due to technical difficulties in the optimization of the ligation mediated PCR technique. A clear ladder could not be obtained that extended sufficiently far into the sequences under examination. One possible reason for this was the high GC content of the sequences being amplified (the ICP4 promoter - 75% GC). However, addition of glycerol or substitution of 7-deaza-dGTP for dGTP in the PCR reaction, both of which have been found to enhance PCR amplification of GC-rich sequences (Innis, 1990; Smith et al., 1990), resulted in only a moderate increase in PCR product yield. Similarly, use of more thermostable DNA polymerases, allowing for elevation of denaturation temperatures and extension of the denaturation period, only partially improved results. The small improvements in PCR product yields resulting from these tactics and the somewhat greater success achieved in LM PCR of the gD promoter (60% GC) indicate that although one factor contributing to the poor results obtained was indeed high GC content of the

template, additional problems also existed. One factor may be that secondary structures within HSV-1 DNA inhibits PCR. A second possibility, related to the high GC content of HSV-1 DNA, is the occurrence of frequent small (6-8 bp) homologies throughout the genome. This made selection of PCR primers exceedingly difficult; since selection of primers was relatively restricted - three overlapping primers had to be found that were located an appropriate distance from the ICP4 binding site under study - self homologies and partial homology to other regions of HSV-1 DNA were inevitable. Although a relatively high annealing temperature was used (66°C), some nonspecific annealing doubtless occurred. Finally, although most stages of LM PCR were subject to optimization, small (not easily detectable) inefficiencies at each step could cumulate in the greater observed failure. How much of my lack of success with this technique was due to the nature of HSV-1 DNA and how much was purely technical is unclear since I did not attempt LM PCR of any template which had previously been amplified successfully by this technique. One approach which could be used to address this question would be to amplify either the α–globin or β-globin gene promoters by LM PCR, from both their endogenous positions within the cellular genome and in viral recombinants containing these genes (Panning and Smiley, 1989; Smiley et al., 1987). In vivo footprinting by LM PCR of the human α–globin and β-globin gene promoters has recently been reported (Ikuta and Kan, 1991; Strauss and Orkin, 1992; Strauss et al., 1992), thus conditions for LM PCR of the endogenous genes are already determined. If identical conditions did not allow successful amplification of genes introduced into the viral genome,

this would suggest that flanking HSV-1 sequences were interfering in some manner. A chief difficulty in attempting this experiment would probably be the isolation of viral and cellular DNAs that were not cross-contaminated; perhaps this could be achieved by cesium chloride gradients or Hirt extractions of DNA. Although the α–globin and β-globin genes are induced by ICP4 when they are contained within the viral genome, the β-globin is repressed during HSV-1 infection at their endogenous locations (Smibert and Smiley, 1990; Smiley and Duncan, 1992). Thus, if successful, this experiment could give insights regarding the role of DNA binding in the induction of viral gene expression by ICP4.

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