

AN ABSTRACT OF THE THESIS OF

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Title: Molecular Genetic Analysis of Vaccinia Virus Genes
Which Confer Resistance to Alpha-amanitin.

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Dr. Dennis E. Hruby

Several laboratories have shown that the VV replication cycle is dependent on the host cell nucleus. The basic biological observation is that if the host cell nucleus is physically removed by cytochalasin B-mediated enucleation, functionally inactivated by UV-irradiation, or selectively blocked with α -amanitin, the results are the same: VV is able to initiate infection and carry out macromolecular syntheses, but no infectious progeny virions are assembled.

To elucidate the molecular mechanism of this virus-host cell interaction, one approach is to identify the viral gene(s) responsible for interaction with the host nucleus and subject them to a detailed molecular analyses.

The genomic location of the gene(s) which provide an α -amanitin resistant phenotype to viral mutants have been mapped to HindIII N/M region of the genome by the use of marker rescue techniques [E.C. Villarreal and D.E. Hruby (1986) Journal of Virology **57**: 65-70]. In an attempt to understand the genetic organization of this part of the genome, the entire HindIII N and part of the neighboring HindIII M DNA fragments have been sequenced. The sequencing data revealed two complete leftward reading open reading frames (ORFs, N2 and M1). In order to determine which of the two ORFs is responsible for conferring resistance to α -amanitin, marker rescue analyses on a VV dual mutant (α^R ts7) that has both temperature sensitive and resistant to α -amanitin was carried out. This showed that N2 ORF is the gene responsible for both phenotypes. To analyze regulatory sequences responsible for expression of the N2 and M1 genes, putative cis-acting regulatory sequences upstream from the N2 and M1 genes were isolated and abutted it to a bacterial reporter gene [chloramphenicol acetyl transferase (CAT)]. To assay for regulatory sequences, these promoter:CAT plasmids were initially used in transient expression protocols and subsequently these plasmids were used to make recombinant viruses.

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Genes Which Confer Resistance
to Alpha-amanitin

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Scott L. Weinrich has contributed in the transcriptional analysis work. Elcira C. Villarreal contributed in the sequencing work and some of the transcriptional analysis as well.

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MOLECULAR GENETIC ANALYSIS OF VACCINIA VIRUS GENES
WHICH CONFER RESISTANCE TO ALPHA-AMANITIN

Chapter 1

Introduction

Poxviridae are a family of DNA viruses with a wide range of susceptible hosts, both among the vertebrates and invertebrates, including mammals, birds and insects (Moss, 1985). Poxviruses within the genus orthopoxvirus mainly infect mammals. Poxviruses are among the largest animal viruses and structurally quite complex. Poxviruses have been known to man for hundreds of years and have had a significant impact on the history of virology and medicine.

Due to its large size, vaccinia virus was the first animal virus seen under the light microscope (von Borries et al., 1938) and accurately titrated. However, the ability of a number of poxviruses to cause diseases in man and animals is the key reason why it is one of the most important virus families known to man. Smallpox, a disease caused by variola major, a member of genus orthopoxvirus, has been responsible for more deaths and disfigurements than any other known human pathogen (Conrat et al., 1988).

In 1798 Edward Jenner pioneered the art of vaccination by inoculating samples from cowpox lesions into man, this resulted in a mild disease but saved them from smallpox. Many years later, it was shown that the samples contained vaccinia virus which was shown to have serological relatedness to variola major but less pathogenicity. VV was then used in the World Health Organization global vaccination programs which culminated in its unprecedented eradication of smallpox in 1977. Since smallpox has been eradicated and the discovery that VV infections can occasionally have serious side-effects, the vaccination program has been stopped. However, there remain many reasons for continued interest in VV research, especially with the advent of genetic engineering techniques.

Vaccinia virus has 185 kb, double-stranded, linear DNA genome with crosslinked termini capable of encoding between 150 to 200 polypeptides. (Geshelin and Berns, 1974; Holowczak, 1974; DeFillipes, 1976). The virion lacks symmetrical nucleocapsids, instead it is slightly ovoid and brick-shaped with an approximate dimension of about 270 x 218 nm. Electron microscopic analysis of virion thin sections reveal the presence of a biconcave core with a lateral body found in each concavity.

The whole structure is enclosed with a bilayer lipoprotein envelope which in turn is wrapped with

surface tubular elements (STE) (Dales and Pogo, 1981; Moss, 1985). Two-dimensional SDS polyacrylamide gel analysis, the virion was found to be composed of more than 100 different proteins (Esani and Dales, 1979; Sarov and Joklik, 1972; Oie and Ichihashi, 1981). The majority of these proteins are believed to be virally encoded but some of them may be cellular origin which are acquired during packaging process.

It has been shown that recombinant VV will package virally encoded foreign proteins in mature virions (Franke and Hruby, 1987; Huang et al., 1988). Besides the structural proteins, regulatory proteins such as DNA binding factors (Wilson et al., 1988; Yuen et al., 1987) and virally encoded enzymes are found packaged within virions. A variety of enzymes involved with transcription are found packaged within the mature virion, including; DNA dependant RNA polymerase (Kates and McAuslan, 1967; Munyon et al., 1967), poly(A) polymerase (Moss et al., 1975), capping and methylating enzymes (Barbosa and Moss, 1978; Martin et al., 1975; Schuman et al., 1980; Monroy et al., 1978; Venkatesan et al., 1980; Martin and Moss, 1975), RNA guanylyl-transferase (Martin et al., 1975; Shuman and Hurwitz, 1981), DNA polymerase (Jones and Moss, 1984), nucleases (Pogo and O'Shea, 1977; Rosemond-Hornbeck et al., 1974; Lakritz et al., 1985), DNA topoisomerase (Bauer et al., 1977; Foglesong and Bauer,

Fig. I.1. The genomic map of vaccinia virus genes with known functions. In the middle of the figure is a schematic presentation of VV linear DNA genome and the letters represent the different HindIII fragments. The different numbers under each fragment correspond to its respective size in kilobase pairs. The top part of the figure is an expanded view of the left hand side of the genome and its known mapped genes. Similarly, the bottom part of the figure represent the expanded view of the right hand part of the genome with its corresponding mapped genes. Each arrows of the mapped genes show the orientation of the genes relative to HindIII fragments and VV genome. The long arrow shows the inverted terminal repeats present at both termini.

The list shows the designated names, function and the source of references for each mapped genes shown in the figure, relative to HindIII fragments. A question mark under 'expression' column indicate that the kinetic of expression has not been determined for the gene involved at the time of this thesis preparation.

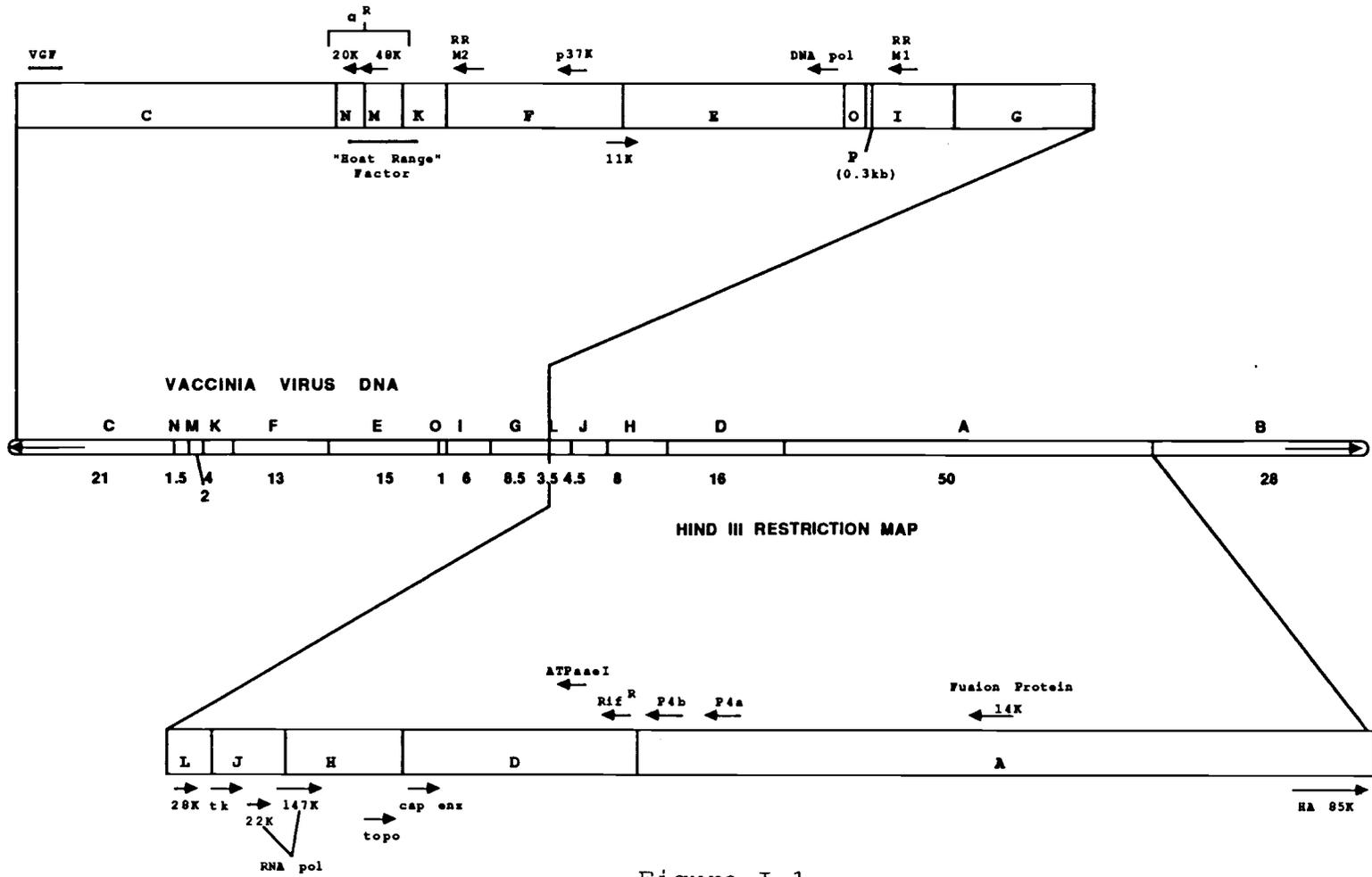


Figure I.1

Figure I.1 continued.

<u>Designation</u>	<u>Location</u>	<u>Reference</u>	<u>Expression</u>
Viral Growth Factor (19K)	C (ITR region)	Wittek, et al. (1980) <i>Cell</i> , 21 , p487-493.	early
Host Range Determinant	N, M, K	Gillard, et al. (1985) <i>J. Virol.</i> 53 (1), p316-318.	?
20K (α -amanitin ^R)	N	Tamin, et al. (1988) <i>Virology</i> , 165 , p141-150.	early
48K (α -amanitin ^R)	M & N	Tamin, <i>ibid.</i>	constitutive
M2 (small subunit of ribonucleotide reductase)	F	Slabaugh, et al. (1988) <i>J. Virol.</i> , 62 (2), p519-527.	early
p37K (envelope antigen)	F	Hirt, et al. (1986) <i>J. Virol.</i> 58 (3), p757-764.	late
11K (structural)	F & E	Wittek, et al. (1984) <i>J. Virol.</i> , 49 (2), p371-378.	late
DNA polymerase (110K)	E	Traktman, et al. (1984) <i>J. Virol.</i> 49 (1), p125-131.	early
M1 (large subunit of ribonucleotide reductase)	I	Tengelsen, et al. (1988) <i>J. Virol.</i> 164 , p121-131.	early
28K (structural)	L	Weir & Moss. (1984) <i>J. Virol.</i> 51 (3), p662-669.	late
Thymidine Kinase (20K)	J	Hruby & Ball. (1982) <i>J. Virol.</i> 43 , p403-409.	early

Figure I.1 continued

22K	J	Broyles & Moss. (1986) <i>PNAS</i> , 83 , p3141-3145.	early
147K (2 subunits of DNA-dep RNA polymerase)	J & H	Jones, et al. (1987) <i>J. Virol.</i> 61 (6), p1765-1771.	early
Topoisomerase (32K)	H	Shuman & Moss. (1987) <i>PNAS</i> , 84 , p7478-7482. Rosel, et al. (1986) <i>J. Virol.</i> 60 (2), p436-449.	late
Guanylmethyl- transferase (large subunit of the capping enzyme)	D	Morgan, et al. (1984) <i>J. Virol.</i> , 52 (1), p206-214.	early
Nucleic acid dependant ATPase I	D	Rodriguez, et al. (1986) <i>PNAS</i> , 83 , p9566-9570.	late
L65 (rifampicin ^R)	D	Weinrich, et al. (1985) <i>J. Virol.</i> 55 (2), p450-457.	late
4b (major core polypeptide)	A	Rosel & Moss. (1985) <i>J. Virol.</i> , 56 (3), p830-838.	late
4a (major core polypeptide)	A	Wittek, et al. (1984) <i>Nuc. Acid.</i> <i>Res.</i> 12 (12), p4835-4848	late
Fusion protein (envelope, 14K)	A	Rodriguez & Estaban (1987) <i>J. Virol.</i> 61 (11), p3550-3554.	late
Hemagglutinin (structural, 85K)	A	Shida. (1986) <i>Virol.</i> 150 , p451-462.	late

1984), and nicking-joining enzyme (Reddy et al., 1989). In fact, extracts from virion cores can be used as an in vitro transcription system; capable of synthesizing mature, functional mRNA. The genes encoding some of these enzymes have been mapped to the VV genome as shown in Figure I.1.

During an analysis of the structure of the genome, it was found that the restriction fragments at each end cross-hybridized (Wittek et al, 1977) and by detailed restriction endonuclease analysis they found a terminal repeat motif about 10 kb in size. In other work, using electron microscopy, Garon and co-workers observed that each single DNA strand of the genome (obtained by denaturing of virion genomic DNA after treatment with single-stranded specific endonuclease to remove terminal cross-links) could self anneal, forming a single-stranded circle with a 'panhandle' structure, thus strongly suggesting the presence of inverted terminal repeats (Garon et al., 1978). A 70 base pair repetition in two blocks of 13 and 17 copies respectively was found within these terminal inverted repeats (Wittek and Moss, 1980).

Unannealed single stranded regions exist within this terminal repeat and are believed to have roles in the DNA replication. Using comparative restriction analysis and hybridization techniques, it was found that members of the orthopoxvirus family, possess a conserved internal

Fig. I.2. Vaccinia virus replication cycle. The explanation of the figure is described in the text.

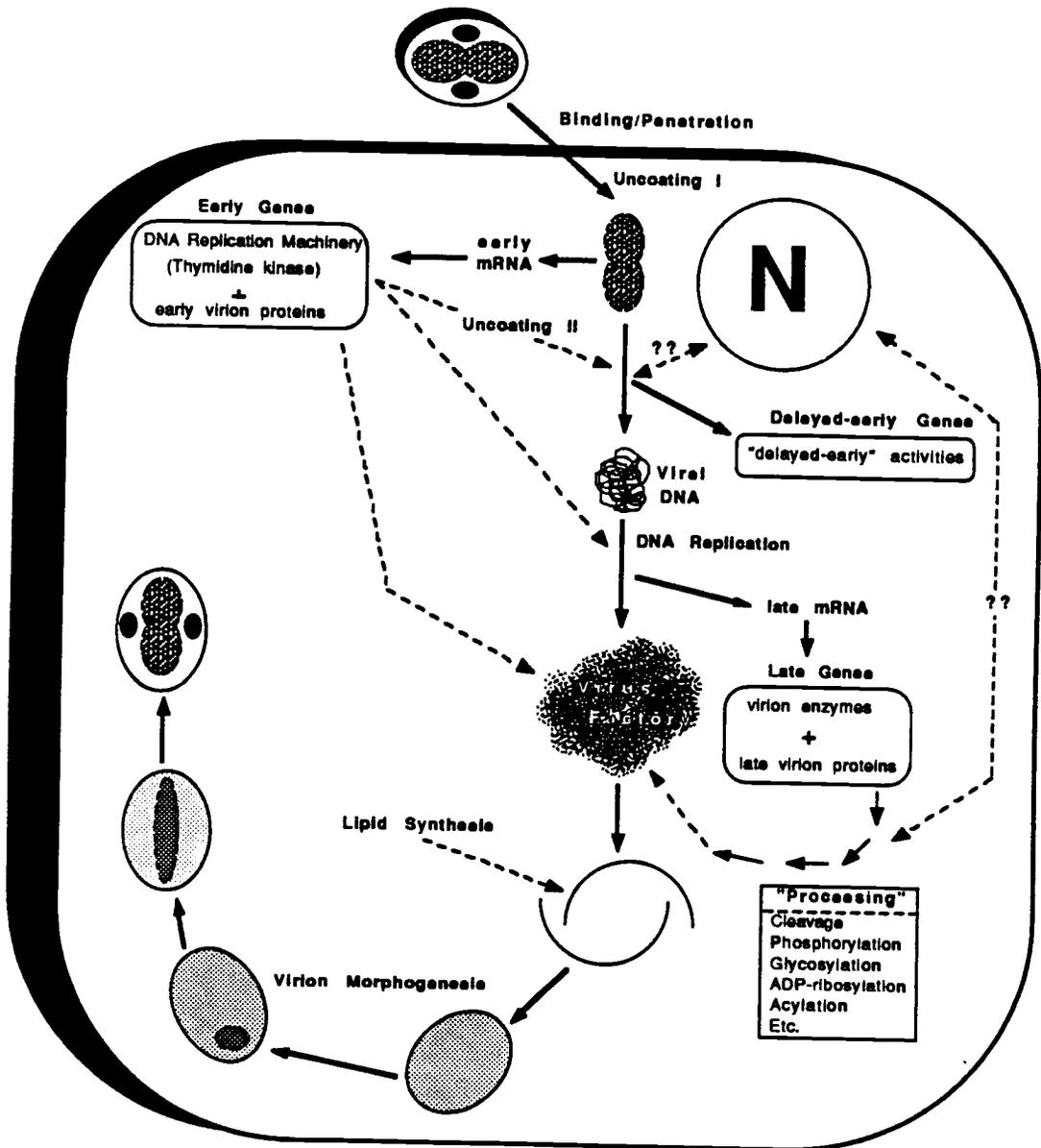


Figure I.2

portion of the genome while the regions at the termini were found to be quite variable (Wittek et al., 1977; Muller et al., 1977; Esposito et al., 1978; Mackett and Archad, 1979, Dumbell and Archad, 1980; Esposito and Knight, 1985). A similar observation was made for avipoxviruses (Schnitzlein et al., 1988). Except for the inverted terminal repeats at both termini, 90% of the VV DNA genome is believed to represent unique sequences (Baroudy and Moss, 1980). Previous workers have suggested that the left terminal portion of VV DNA genome is the most variable and as such was believed to contain "non-essential" genes (Perkus et al., 1986). However the finding that a *ts* mutation can be mapped within this region challenges this notion (Villarreal and Hruby, 1986).

Figure 1.2 schematically illustrates some aspects of the VV replication cycle within infected cells. VV infects susceptible cells by direct penetration or phagocytosis. The exact mechanisms regarding it's attachment and entry into the cell, the involvement of virion attachment proteins and/or cellular receptors for specific binding of virions are still unclear.

Following penetration into the host, two successive stages of uncoating release the viral DNA genome into the host cell cytoplasm. Host cell enzymes and factors remove the viral envelope in the first uncoating process,

leaving nucleoprotein core intact. The immediate early genes are transcribed by virally encoded enzymes in the core. The message produced is capped, methylated and polyadenylated.

At least one or more protein product from this immediate early gene class appears to be required for second uncoating step. This second uncoating results in the release of the viral DNA into the cytoplasm which turns on the expression of the delayed early class of genes. The pattern of gene expression changes dramatically concomitant with the onset of DNA replication at which point late genes are expressed and early genes are switched off. The molecular nature of this switch is unknown.

The late gene protein products include most of the structural proteins required for the mature virion (Salzman and Sebring, 1967). The late stage of viral development involves DNA synthesis, various categories of post-transcriptional and post-translational modifications, and morphogenesis. These processes take place in electron dense cytoplasmic inclusion bodies termed viroplasm (Dahl and Kates, 1970) or viral factories (Cairns, 1960). Some of the known post-translational modifications which occur prior to assembly, include the cleavage of precursor polypeptides (Katz and Moss, 1970; Moss and Rosenblum, 1973;

Pennington, 1974; Van Slyke et al., manuscript submitted; Yang et al., 1988), glycosylation (Moss et al., 1973), phosphorylation (Rosemond and Moss, 1973; Pogo et al., 1975; Kao and Bauer, 1987), ADP-ribosylation (Child et al., 1988) and acylation (Franke et al., manuscript submitted).

During the late stages of replication, lipid crescents can be seen surrounding the viroplasm. Mature VV progeny, unlike many other enveloped viruses, acquire a virally synthesized envelope rather than pirating host cell membranes. These virions are termed intracellular enveloped virus (IEV). When the IEV leave the host cell it acquires an extra envelope from the host cell golgi, and are called extracellular enveloped virus (EEV) which are antigenically different from the IEV. Depending on the type of host cell this complex replication cycle completes itself in about 12 hours.

In order for VV to complete its life cycle in such a short time, it requires extremely efficient control of its gene expression. This suggests that the expression and subsequent modification of all the approximately 200 encoded genes must be very tightly regulated. Based on results of inhibitor studies, expression of VV genes are categorized into two major kinetic classes: early and late, though this does not necessarily reflect the actual

in vivo situation (Pennington, 1974; Hruby and Ball, 1982).

The early genes (about 100) are expressed prior to DNA replication, whereas the late genes are those which are expressed after or concomitant with the onset of DNA synthesis. Early gene transcription is amplified by the presence of the protein synthesis inhibitor cycloheximide.

Late gene expression is sensitive to DNA replication inhibitors like hydroxyurea and Ara C, while the transcription of early genes is not. There are exceptions to this simple classification, because there are VV genes which are expressed continuously throughout infection, before and after DNA replication (Cochran et al., 1985; Weinrich and Hruby, 1986; Roseman and Hruby, 1987; Tamin et al., 1988).

The early gene class, is further divided into two subclasses; first, immediate early genes are expressed from the intact viral cores upon infection (Puckett and Moss, 1983; Foglesong, 1985). A second subclass of early genes, termed delayed early, are those expressed after the stage II uncoating but before the onset DNA replication. Stage II uncoating results in core breakdown which releases the VV genomic DNA into the cytoplasm.

The presence of the drug cycloheximide, which blocks translation prevents expression of the delayed early

genes but does not affect transcription of the immediate early genes (Woodson, 1967). Stage II uncoating process requires one or more proteins from the immediate early gene class.

Transcripts derived from early genes are , polyadenylated and have a distinct transcriptional termination site, producing mRNA products with distinct 3' termini. As such, the transcript size corresponds well with the size of the encoded polypeptide (Mahr and Roberts, 1984a; Hruby et al., 1983; Wittek et al., 1980). Yuen and Moss demonstrated that the consensus TTTT(N)T is a termination signal for early mRNA. This signal is located approximately upstream from the 3' end of the RNA and is never found in the coding region of early genes. A surprising observation was made by Shuman et al., (1987) regarding VV transcriptional termination, namely that VV capping enzyme, which acts at the 5' end of mRNA for capping process, is identical to the VV transcriptional termination factor.

With the onset of DNA synthesis, transcription of early genes ceases and late genes are expressed. At late times, some of the early gene transcripts are still present and fully functional if translated in vitro, but they are not translated in vivo (Hruby and Ball, 1981). This suggest that besides transcriptional controls, there are other post-transcriptional controls affecting the

levels of expression from a particular gene (Hruby, 1984).

Transcriptional regulation of late genes differs markedly from that of early genes. The DNA-dependant RNA polymerase complex found packaged in mature virions, and which is responsible for early gene transcription does not recognize late promoters. In addition, there is no distinct mRNA termination for late genes and as such late transcripts also contain sequences from early genes and appear to be several fold larger than the size of polypeptides they encode (Oda and Joklik, 1967; Boone et al., 1979; Colby et al., 1971).

A consensus pentanucleotide TAAAT was found to be located at most of the late transcriptional start sites and is believed to have regulatory role for promoter activity of late genes expression (Weir and Moss, 1987; Rosel et al., 1986). Another unique feature of VV late mRNA was the presence of a 5' poly(A) leader sequence. There are about about 35 A residues at the start of the RNA which are not encoded by the DNA (Bertholet et al., 1987; Schwar et al., 1987). Patel and Pickup (1987) made a similar observation in late mRNA of cowpox virus. By utilizing an in vitro late transcription system, Wright and Moss (1987) found that a DNA template containing a VV late promoter element is active. Kinetic experiments which compared the level of full length transcripts

produced to levels of correctly initiated transcripts appeared to rule out a cis-splicing mechanism for generation of leader sequence, and suggested a RNA polymerase stuttering mechanism.

Observations from several laboratories show that there is lack of temporal organization of early and late genes in the genome (Belle Isle et al., 1981; Chipchase et al., 1980; Rosel and Moss, 1985; Oda and Joklik, 1967; Paoletti and Grady, 1977; Kaverin et al., 1975). Initial transcriptional mapping utilizing RNA:DNA hybridization analysis did not reveal any preferred location for early or late genes groups, rather indicated that they are distributed throughout the VV genome. However, from detailed transcriptional analysis on subgenomic DNA fragments, it appears that genes which have similar kinetics of expression may be clustered.

Within these clusters they are tightly packed and arranged in a tandem orientation (Cooper et al., 1981; Mahr and Roberts, 1984a,b; Weinrich and Hruby, 1986). Since they are so tightly packed, the cis-acting promoter elements required for their transcriptional start sites are often part of the coding sequence of the upstream genes. Similarly for early genes, the 3' end of their mRNA can be mapped within the downstream genes. Recent work by Miner et al., (1988) suggested that although a set of late genes is in a tandemly oriented cluster, each

of them is expressed individually from their respective upstream promoter elements rather than being expressed as a large poly-cistronic transcript and subsequently cleaved.

Several approaches have been used to dissect the upstream promoter elements of VV genes including transient expression (Cochran et al., 1985a), run-off transcription assays using a virion-derived template dependent in vitro transcription assay (Rohrman and Moss, 1985) and the production of recombinant virus (Cochran et al., 1985b). By utilizing these methods, several investigators have demonstrated the presence of promoter elements upstream of early (Mars and Beaud, 1987; Weir and Moss, 1987a; Mackett et al., 1984; Vaseff et al., 1985), late (Weir and Moss, 1984; Bertholet et al., 1985; Miner et al., 1988) and constitutively expressed genes (Cochran et al., 1985b). Each of them seems to be unique since none of these promoter elements has any similarities to consensus sequences found in eucaryotic promoters.

It has been demonstrated that a factor with an apparent molecular weight about 130 kd derived from purified virions binds specifically to a distinct region in the early promoter elements of VV growth factor gene (Yuen et al., 1987). A similar finding was observed by Wilson et al., (1988) where VV thymidine kinase PBF (tk

promoter binding factor) bound to the promoter element of another VV early gene of thymidine kinase.

Both laboratories have shown the factor(s) they studied also bound to other early promoters. They could not show with certainty whether these factor(s) are viral encoded or cellular factors which are packaged in mature virions. Circumstantial evidence, which include the lack of any similarities between VV early promoters to eukaryotic promoter (Cochran et al., 1985), the host protein shut-off upon infection and the presence of VV tk PBF in VV-infected cells but not in non-infected cells (Wilson et al., 1988) indicate they are most probably VV encoded but the final proof will be the mapping of the corresponding genes in VV genome. At present, work is in progress in different laboratories to isolate the late genes promoter trans-acting factor(s) and to understand the nature of the molecular interaction(s) involved (Miner and Hruby, in press).

Although smallpox has been eradicated worldwide, many laboratories are actively studying VV. With the development of powerful genetic engineering and molecular biological techniques, VV has become an important research tool. There are several reasons for this: 1) VV is an ideal model eukaryotic system to investigate various questions regarding development and the regulation of gene expression, 2) VV is used as a cloning

and expression vector for eukaryotic genes, and 3) VV as model system for studying virus-host interactions such as molecular pathogenesis and host function requirement for successful VV replication.

Investigations are currently proceeding in the areas of protein-DNA and protein-protein interactions involved in transcription, translation, post-transcriptional and post-translational processes, development and morphogenesis. Several investigators have found strong homologies between VV RNA Polymerase (Broyles and Moss, 1986), VV DNA polymerase (Earl et al., 1986) and their cellular counterparts, suggesting similar mechanisms of regulation and replication.

The cytoplasmic site of VV replication offers an opportunity to carry out these analyses with little interference from host nuclear reactions. Its haploid genome is an excellent target for classical and molecular genetics. Heterologous foreign genes of interest, derived from other viruses, mammals, and prokaryotes, have been successfully inserted into non-essential regions of the VV genome and found to be correctly expressed and processed.

In a similar manner, VV is widely used as a vector for recombinant vaccine development for immunization purposes against a variety of medical and veterinary diseases. Foreign proteins expressed by VV are very

antigenic and as such induce strong protective immunity to the vaccinee and recipient animals (Franke et al., 1985a; Mackett et al., 1985; Gillespie et al., 1986; Jones et al., 1986; Hruby et al., 1988; Chambers et al., 1988; Tomley et al., 1988).

There are many reasons that VV is an excellent viral vector for vaccine development. It has been used as vaccine against smallpox for hundreds of years and is relatively safe to work with, at least to non-immunocompromised workers and moreover it is non-oncogenic. It has broad host range (Franke and Hruby, 1985), and acts as a suitable shuttle vector between different cell, organs or species types. Its genome is large and it has no rigorous packaging requirements which offer flexibility on the size of foreign insert (Rice et al., 1985). Since it has many non-essential sites in the genome, it is an excellent choice for polyvalent or multi-subunit vaccine development. This will ease the administration of vaccination and lower costs of production. VV can be grown and maintained in tissue culture and thus the process of constructing recombinant VV is relatively convenient and not expensive.

Several laboratories currently are engaged in research pertaining to increasing the efficiency and detection of recombinant viruses (Franke et al., 1985; Fathi et al., 1986). A well orchestrated basic research

program on the biology of VV which includes VV gene regulation, alternative sites of foreign gene insertion and its molecular pathogenesis will provide information in developing VV as a better research tool and also a better candidate for recombinant vaccines. The ultimate goal is to have technology which will allow us to express or modify a particular gene at will.

An important area of research which has not been discussed in detail above is the understanding of the biological interaction between VV and its host cell during infection. This is the central interest of this investigation. The work reported here specifically addresses some aspects of VV interaction with host cell nuclear functions, in particular the potential VV gene(s) involved and a preliminary study on their regulation during VV replication.

As mentioned earlier, vaccinia virus replication occurs entirely within the cytoplasmic compartment of the infected host cell. Neither the VV genomic DNA nor any of its fragments become integrated into the host cell genome. Thus it is not surprising that until early 1970's it was a popular belief that vaccinia virus replication was totally independent from any kind of host cell nuclear involvement.

However the work of Pennington and Follet in 1974 began the host cell nuclear involvement controversy. They

observed that enucleated host cells, when infected with vaccinia virus did not produce any mature infectious progeny. Subsequently, Hruby et al., (1979a,b) and Silver et al., (1979) demonstrated independently that α -amanitin treated cells, when infected with vaccinia virus, did not allow production of mature progeny. The drug α -amanitin is inhibitor of host RNA polymerase II enzyme but not the viral enzyme. It appeared that active participation of host nucleus is required for successful vaccinia virus replication.

Villarreal et al., (1984) were able to isolate a mutant vaccinia virus that is capable of replicating successfully in the presence of α -amanitin at concentrations inhibitory to wild type vaccinia virus. With this mutant it became possible to map the gene(s) responsible for this host nuclear interaction. This mutant vaccinia virus, α -27, in the absence of α -amanitin, has a wild type phenotype characteristics with respect to its growth kinetics, DNA synthesis, protein synthesis and morphogenesis. However, infections in the presence of the drug demonstrated that WT VV was not able to process proteolytically the major core precursor polypeptides, P94 and P65 to two major capsid proteins, VP62 and VP60 respectively. On the other hand, α -27 showed no difficulties at all with this post-translational modification process. In addition, it has

been shown also that infection of α -27 in enucleated host cells produced infectious progeny.

Subsequently, Villarreal and Hruby (1986) isolated two double mutant vaccinia viruses, $\alpha^{\text{r}}\text{ts7}$ and $\alpha^{\text{r}}\text{ts12}$ which are both resistant to α -amanitin and also temperature sensitive (grow only at 31°C). They have shown that both phenotypes are the result of one or two very closely-linked mutations. The ts mutation of $\alpha^{\text{r}}\text{ts7}$ and $\alpha^{\text{r}}\text{ts12}$ were rescued by 1.5 kb HindIII N and 2.0 kb HindIII M fragments respectively. However, only the rescued progeny of $\alpha^{\text{r}}\text{ts7}$ were sensitive to α -amanitin. Therefore the data suggested that the gene which is responsible for α -amanitin resistance phenotype mapped within HindIII N fragment of the vaccinia virus genome. Armed with this information, in the succeeding chapters are presented work done to further understand the mechanisms of this host-involvement: 1) Sequencing the 1.5 kb HindIII N fragment and part of the neighboring HindIII M of wild type vaccinia virus genome and its molecular genetics analysis, 2) mapping the VV gene responsible for the α -amanitin resistance phenotype, and 3) the isolation and identification of cis-acting promoter elements which regulate the expression of the VV N2 and M1 genes.

CHAPTER II

Nucleotide Sequence and Molecular Genetic
Analysis of the Vaccinia Virus HindIII N/M
Region Encoding the Genes Responsible for
Resistance to Alpha-amanitin.

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Summary

The genomic location of the gene(s) which provide vaccinia virus (VV) α -amanitin resistant mutants with a drug-resistant phenotype have been mapped to the HindIII N/M region of the genome by the use of marker rescue techniques [E.C. Villarreal and D.E. Hruby (1986) Journal of Virology **57**:65-70]. Nucleotide sequencing of a 2,356 base-pair HindIII - Sau3A fragment of the vaccinia virus genome encompassing this region reveals the presence of two complete leftward reading open reading frames (ORFs, N2 and M1) and two incomplete ORFs (N1 and M2). By computer analysis the N2 and M1 ORFs would be predicted to encode soluble VV polypeptides with molecular weights of approximately 20 Kd and 48 Kd, respectively.

The N2 and M1 orfs have extremely A-T rich 5'-proximal sequences, consistent with previous data regarding the location and A-T richness of viral early promoters. Likewise, the consensus signal believed to be involved in terminating VV early gene transcription, TTTTNT, was evident at the 3'- boundary of both the N2 and M1 ORFs suggesting that these genes may be VV early genes. The in vivo transcriptional activity, orientation, and limits of these putative transcriptional units were investigated by northern blot, nuclease S1 and primer extension analysis.

Both N2- and M1-specific transcripts were detected in the cytoplasm of VV-infected cells, suggesting that these loci are bonafide viral genes. Time-course nuclease S1 experiments revealed that the N2 gene was transcribed exclusively prior to VV DNA replication. In contrast, the M1 gene was transcribed throughout infection, although different start sites were used at early versus late times post infection. These results are discussed in relation to the drug-resistant phenotype and future experiments to identify the viral gene product responsible.

Introduction

Vaccinia virus (VV), the prototypic member of the Poxvirus family, is a large complex DNA-containing animal virus which completes all of the detectable stages of its replication cycle within the cytoplasmic compartment of infected host cells (Moss, 1985). Although VV replication was originally believed to be independent of the host cell nucleus (Joklik and Becker, 1964; Prescott et al., 1971), additional experiments have demonstrated an obligatory interaction between the host cell transcriptional apparatus and VV during the course of a productive infection (Pennington and Follett, 1974; Hruby et al., 1979ab; Silver et al., 1979; Silver and Dales, 1982).

The basic biological observation is that if the host cell nucleus is physically removed by cytochalasin-B mediated enucleation, functionally-inactivated by UV-irradiation, or selectively blocked with the transcriptional inhibitor α -amanitin, the results are the same: VV is able to initiate infection and carry out macromolecular syntheses, but no infectious progeny virions are assembled. The nature of the required host factor(s) and the viral constituents with which they interact, have not yet been identified.

As an approach to dissect the molecular nature of this essential virus-host cell interaction, a VV mutant (α -27) was isolated which was able to replicate in the presence of concentrations of α -amanitin which totally inhibited the growth of wild-type VV (Villarreal et al., 1984). The replication of α -27 and wild-type VV were biochemically indistinguishable with a single exception. In the presence of drug, the mutant virus was able to carry out the proteolytic processing of the two major core precursor polypeptides P94 and P65 whereas the wild-type virus was deficient in this reaction. It was not obvious whether this defect was a primary or secondary indicator of the function of the required host factor(s).

In order to map the genomic location of the drug-resistance marker and to facilitate study of the encoded polypeptide, a drug-resistant temperature-sensitive VV double mutant ($\alpha^{\text{r}}\text{ts7}$) was isolated in which both phenotypes were apparently the result of a single or two closely-linked mutations. A series of marker rescue experiments were carried out using this mutant which enabled the genomic location of the $\alpha^{\text{r}}\text{ts7}$ mutation to be mapped to within the 1.5Kb HindIII N fragment (Villarreal and Hruby, 1986).

In this manuscript, we report the nucleotide sequence of a 2,356 bp region of the VV genome which includes the entire HindIII N fragment and the proximal

portion of the adjoining HindIII M fragment. A molecular genetic analysis of this region reveals the presence of two VV genes that are actively expressed during viral infection. Since at least one of these genes is apparently interacting with the required host nuclear factors during replication, this information should provide a basis for unraveling this interesting biological question.

Methods and Materials

Cells and viruses. BSC-40 monkey kidney cells were grown in monolayer and maintained with Eagle's minimal essential media (Flow Laboratories) supplemented with 10% heat-inactivated fetal calf serum, 2mM L-Glutamine and 50mg/ml of gentamycin sulfate. Parental VV (WR strain) was obtained from the American Type Culture Collection in 1977 and has been propagated by low-multiplicity passages and periodic plaque purification since that time. Growth, purification, and plaque assay of infectious VV were carried out as previously described (Hruby et. al., 1979a).

Transcriptional analyses. The procedures employed for the RNA isolation and the subsequent S1 nuclease experiments were as described by Weinrich and Hruby (1986). Primer extension studies on MI orf were carried out as have been described elsewhere (Tengelsen et. al, 1988). For northern analyses, ten micrograms of the indicated VV RNAs were electrophoresed in denaturing agarose-formaldehyde gels. Gels were then blotted onto nitrocellulose, and hybridized with nick-translated probes as previously described (Rice et al., 1985). Autoradiography was done using Kodak XAR-5 X-ray film with an intensifying screen at -70oC.

Preparation of nick-translated and 5'-end labeled DNA fragment. Plasmid DNA was isolated and purified as described by Holmes and Quigley (1981), and ethanol precipitated three times. The desired subfragments were released by restriction enzyme cleavage, purified by electrophoresis through neutral agarose gels, and isolated by the freeze-phenol method (Benson, 1985). For use in hybridizations, the fragments were labeled by nick-translation (Rigby et al., 1977) with [α - 32 P]dATP (New England Nuclear). For transcriptional mapping, restriction endonuclease generated DNA fragments were treated with calf intestine alkaline phosphatase (Sigma Chemical) and then labeled at the 5'-end with [γ - 32 P] ATP (New England Nuclear) and polynucleotide kinase (Bethesda Research Laboratory; Maxam and Gilbert, 1980).

DNA sequencing. Selected restriction endonuclease fragments of HindIII N and M were subcloned into M13 mp18 and M13 mp19 phages (Bethesda Research Laboratory) and DNA sequencing reactions were carried out by the dideoxynucleotide chain termination method (Sanger et al., 1977) using either the 17 bp sequencing primer which was purchased from New England Biolabs or synthetic oligonucleotide primers (OSU Center for Gene Research Central Services Laboratory). The Klenow fragment of DNA polymerase I was purchased from Bethesda Research

Laboratory. The completed nucleotide sequence were assembled and analysed using the Microgenie (Beckman) program on a IBM personal computer.

Fig. II.1. VV HindIII map and strategy used to determine the nucleotide sequence of the HindIII N/M region. The top line shows the location of HindIII N/M fragments, relative to their neighboring HindIII C and HindIII K, at the left terminus of the VV genome. The HindIII N/M region of interest is expanded below with salient restriction enzyme cleavage sites indicated. The arrows beneath the HindIII N/M map indicate the extent and orientation of the M13 clones used to determine the nucleotide sequence. The bottom portion of the figure displays the open reading frames and intergenic spaces predicted by computer.

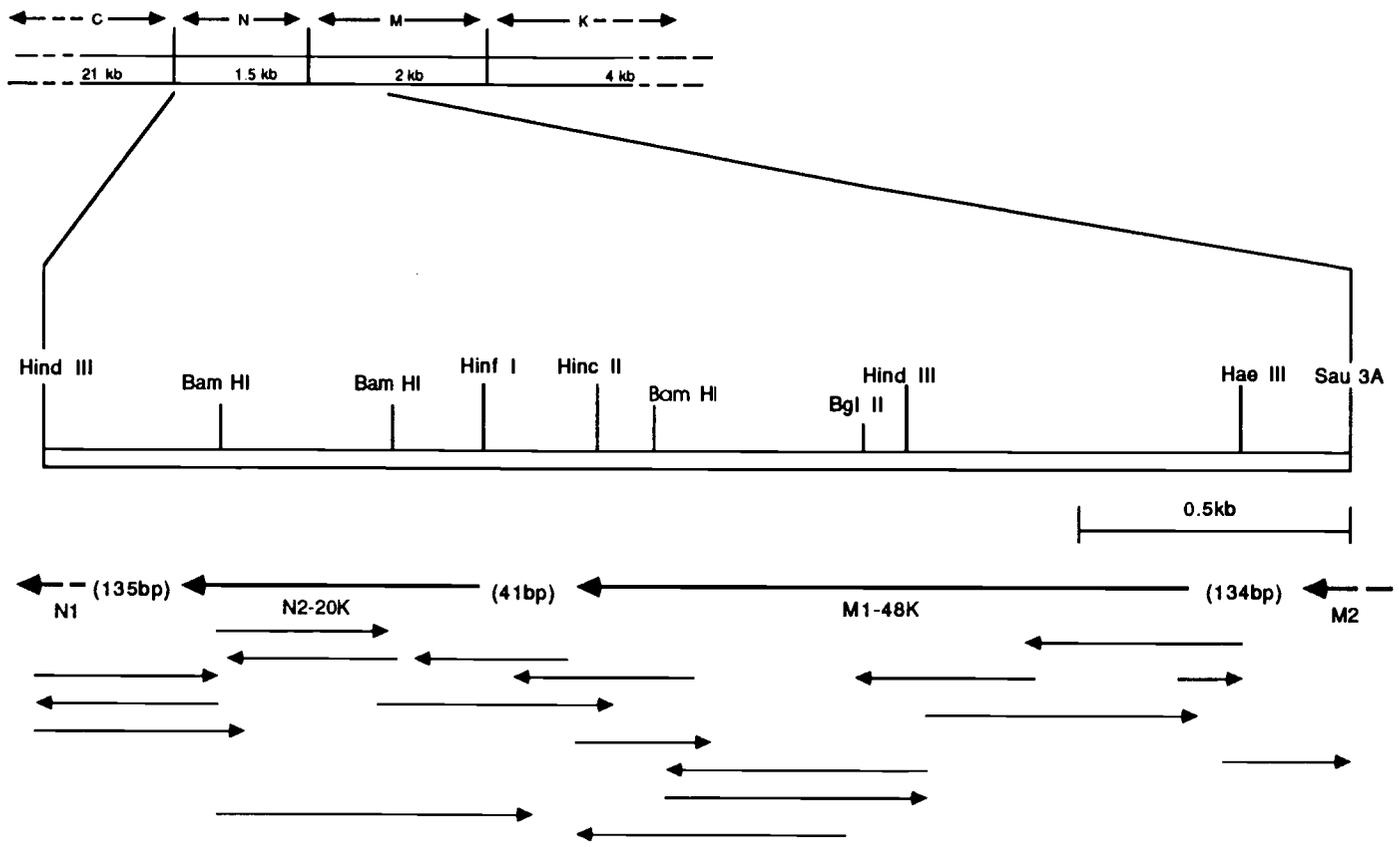


Figure II.1

Results

Mapping and Sequencing of VV HindIII N/M region. Initially, the genomic VV HindIII N and M fragments were cloned into the HindIII site of pBR322. These parental plasmids were digested with a variety of restriction endonucleases and the resulting fragments analyzed by gel electrophoresis in order to produce a restriction map of this region (Figure 1). Once this information was available, individual subfragments of choice were released by enzyme digestion, isolated by preparative gel electrophoresis, and subcloned in both orientations into mp18/19 M13 bacteriophage. Use of these recombinant phage in concert with chain termination nucleotide sequencing procedures allowed a contiguous sequence of 2,356 base pairs (bp) to be determined which included the entire HindIII N fragment and 790 bp of the proximal left side of the HindIII M fragment. The entire sequence was confirmed by sequencing both strands of DNA.

Translation of this region in a rightward direction demonstrated no significant open reading frames (ORFs). In contrast, translation of the complimentary strand of DNA revealed 2 complete and 2 incomplete leftward reading ORFs. These putative genes were tandemly oriented and tightly grouped with only 135 bp, 41 bp, and 134 bp

Fig. II.2. Nucleotide sequence of HindIII N/M region of the genome. The displayed sequence corresponds to 790 bp of the left-hand portion of HindIII M fragment and the whole neighboring HindIII N fragment. HindIII restriction sites are marked. The transcriptional start sites detected at early (e) and late (L*) times are indicated. Arrows show the start and direction of the N2 and M1 open reading frames. The predicted amino acids are shown below the N2 and M1 nucleotide sequence. The underlined sequences are homologs of termination signals for early genes.



Figure II.2

separating the ORFs. In line with the recently suggested VV genetic nomenclature system (Rosel et al., 1986), these ORFs were designated from left to right as N1, N2, M1, and M2 (Figure 1).

VV HindIII N/M nucleotide sequence. The nucleotide sequence that was determined is shown in Figure 2. The two complete ORFs M1 and N2 have the potential coding capacity for proteins of 47,901 dalton and 20,854 dalton, respectively. These predicted molecular weights are in close agreement with previous translational mapping studies of Morgan and Roberts (1984) which mapped genes encoding 20Kd and 55 Kd to this region. Furthermore based on this previous work, one would predict that the upstream M2 ORF encodes a 30 Kd protein. The nucleotide sequence of the 50 bp immediately upstream of the initial ATG of the M1 and N2 ORFs were extremely A-T rich, 81% and 87%, respectively. This observation coupled with absence of the characteristic TAAAT sequence found at the 5'-end of VV late genes, would suggest that the M1 and N2 ORFs are likely to represent VV early genes. This suggestion is strengthened by noting that 3'-proximal regions of both ORFs contain homologs of the sequence TTTTNT which has been implicated in the termination of VV early gene transcription (Rohrman et al., 1986). However, the sequence TAAAT characteristic of many VV late genes was

Codon-utilization of M2 orf

Codons	% A+T	% C+G	Codons	% A+T	% C+G
Phe	2.3	2.8	Leu	2.9	2.8
Ile	7.9	2.3	Met	-	5.7
Val	3.9	2.8	Ser	4.0	1.2
Pro	1.7	1.7	Thr	2.8	2.3
Ala	1.7	1.2	Tyr	5.1	2.8
His	1.7	1.1	Gln	2.3	0.6
Asn	4.0	3.4	Lys	6.3	1.1
Asp	5.1	1.1	Glu	4.5	1.1
Cys	2.3	0.0	Arg	3.4	1.2
Gly	2.3	0.0	End	0.6	0.0

Codon-utilization of M1 orf

Codons	% A+T	% C+G	Codons	% A+T	% C+G
Phe	3.1	1.0	Leu	6.7	3.9
Ile	6.9	4.5	Met	-	3.3
Val	4.8	2.1	Ser	4.5	2.2
Pro	3.3	0.9	Thr	3.8	1.5
Ala	1.9	1.9	Tyr	3.5	1.7
His	3.6	0.5	Gln	1.0	0.2
Asn	5.2	1.7	Lys	5.2	1.4
Asp	7.1	1.4	Glu	4.3	0.7
Cys	2.1	0.5	Arg	3.1	0.2
Gly	4.1	0.5	End	0.2	0.0
Trp	-	0.2			

Comparison of amino acids content and secondary features between M1 and M2 orfs

Orf	MW (Daltons)	Composition				2° structures		
		Percent				alpha helix	beta sheet	Asn in context for glycosylation
		acidic	basic	hydro- phobic	aromatic			
N2	20,854	12.0	12.0	41.7	13.1	48.0	17.0	2
M1	47,901	13.5	10.0	37.3	9.3	49.0	22.1	3

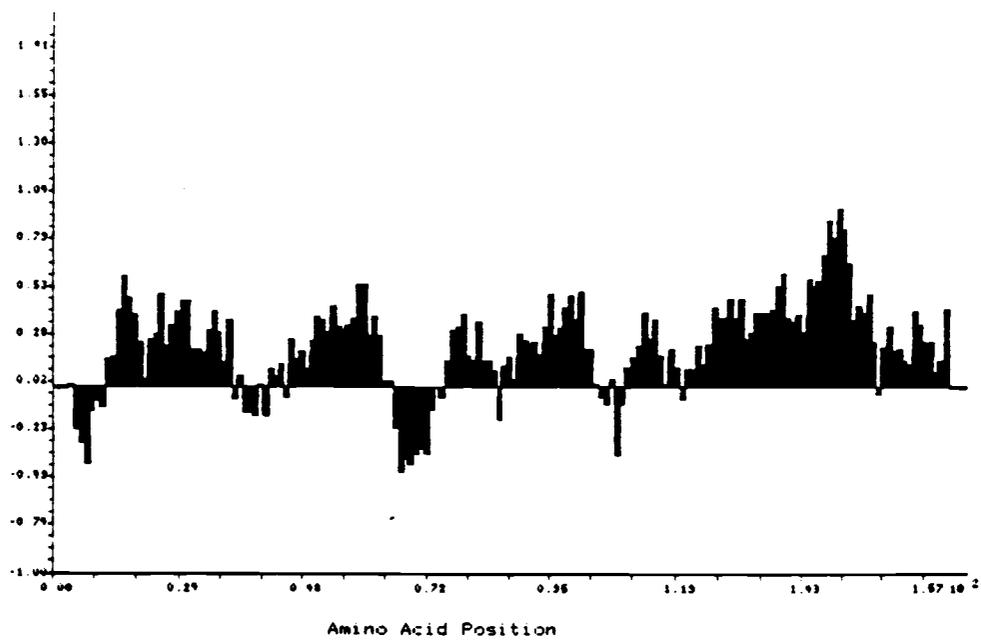
Table II.1. Comparison of the features and codon utilization between M1 and N2 ORFs.

found at the distal start site for M1 orf. The M1 and N2 ORFs do not overlap but they are closely packed, being separated by only 41 bp.

Protein Analysis. A variety of predicted structural features of the M1 and N2 encoded proteins were analyzed by computer (Table 1). As has previously been noted for other VV early genes (Hruby and Guarino, 1984), the M1 and N2 ORFs exhibit distinct codon utilization patterns, preferring codons which end in either A or T. Although this observation can be partially accounted for by the low G+C content (35-40%) of VV DNA, preferences for A or T in the third position of the codon can range upwards of 3- to 5-fold, indicating that additional factors may be involved. Both the 20 Kd and 48 Kd proteins have similar distributions of acidic, basic, aromatic, and hydrophobic amino acids, as well as predicted structural features. Both the 20 Kd (2 sites) and 48 Kd (3 sites) proteins contain asparagine residues in the proper context to serve as sites for N-linked glycosylation modification reactions, although it is not yet known if these sites are modified in vivo. A hydrophobicity plot of both proteins is presented in Figure 3. Neither contains an obvious hydrophobic transmembrane anchor region. When taken together with the other characteristics, this would seem to suggest that both of the proteins are soluble and not membrane-

Fig. II.3 Hydrophobicity plot of the proteins encoded by N2 (A) and M1 (B) open reading frames. Positive values indicate hydrophobic regions, negative values indicate hydrophilic regions.

A



B

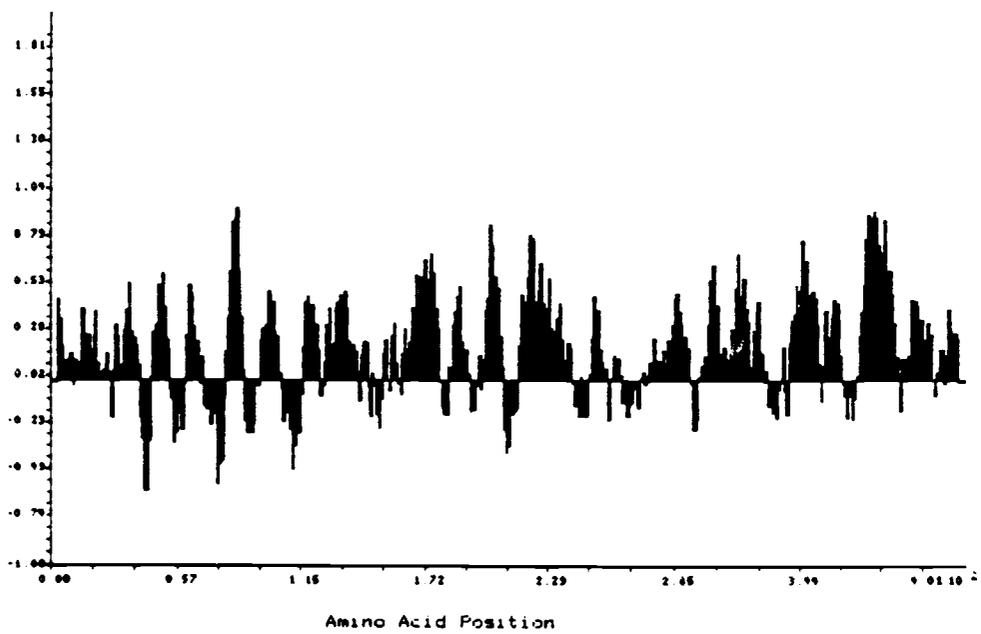


Figure II.3

associated. When the predicted amino acid sequences were compared against those currently present in the NBRF Protein Data Bank, no significant homologies were found.

Transcription of the VV HindIII N/M region.

To confirm that the VV HindIII N/M ORFs corresponded to bonafide viral transcriptional units, northern blot analyses were carried out on cytoplasmic RNA isolated from VV-infected cells at early (2h) and late times (7h) post infection, (Figure 4). In order to amplify the transcription of VV early sequences, RNA was also extracted from cycloheximide-treated VV-infected cells at 5 hours post infection. Using a HindIII N specific probe revealed the presence of two major 1.6 and 0.8 Kb transcripts, and a minor 2.0 Kb transcript. The 1.6 Kb transcript is sufficient in size to encode the 48Kd protein, and the 0.8 Kb RNA is large enough for the 20 Kd protein. The 2.0 Kb transcript is most likely the N1 transcript which reads into the neighboring HindIII C fragment. The HindIII M probe also hybridized to the 1.6 Kb transcript as well as two smaller highly expressed 1.1 and 1.0 Kb transcripts. The latter two RNAs would be predicted to be derived from the upstream M2 and K1 genes (Morgan and Roberts, 1984). Considering this information in conjunction with the nucleotide sequence allows a rudimentary transcript map to be assembled (Figure 4). This is in agreement with the previous

Fig. II.4. Northern blot hybridization analysis of VV HindIII N/M transcripts. VV early (E), Late (L), or cyclo (C) RNAs were electrophoresed under denaturing conditions, transferred to nitrocellulose, and hybridized with either ^{32}P -labeled HindIII N or HindIII M DNA fragments. The indicated transcript sizes were calculated relative to the migration of BMV RNAs, shown between the panels, which were coelectrophoresed as size markers.

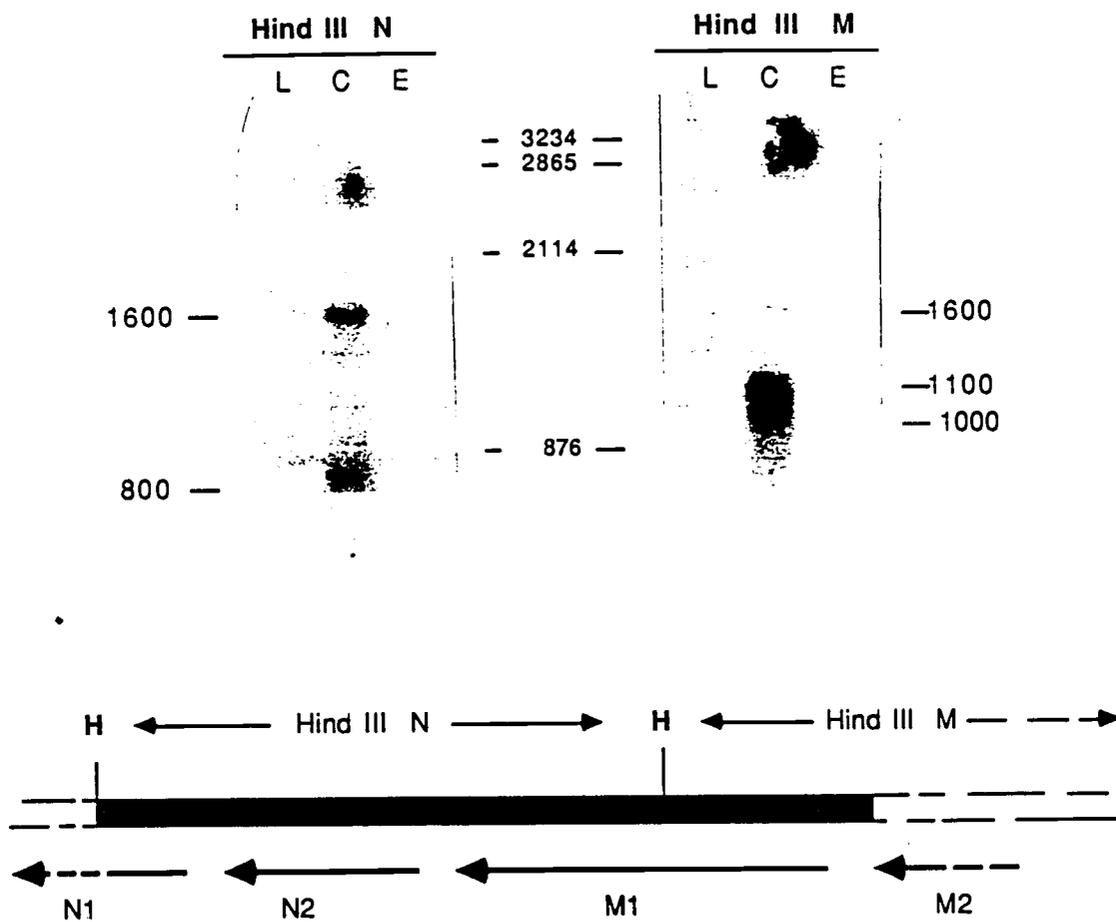


Figure II.4

translational map of this region derived by Morgan and Roberts (1984).

Note in Figure 4 that in all cases, the transcripts were barely detectable in the early VV RNA (although longer film exposures showed them to be present, data not shown), readily observed in the presence of the drug cycloheximide, and absent in VV late RNA (except for some heterodisperse higher molecular weight species). This would suggest that all of these genes are expressed as VV early genes. In order to confirm this hypothesis, the kinetics of ORFs M1 and N2 expression throughout the VV replicative cycle was examined. Cytoplasmic RNA was extracted from VV-infected cells at various times after infection and used in conjunction with nuclease S1 analyses to determine when the individual transcripts were present (Weinrich and Hruby, 1986). To detect the 5'-end of the N2 transcript, a 560 bp BamHI subfragment of VV HindIII N was used as the probe. The data presented in Figure 5 indicates that RNA from cycloheximide- and hydroxyurea-treated VV infected cells protects 100 nucleotides of the probe fragment, thereby mapping the 5'-end of the N2 transcriptional unit. Furthermore, it is apparent that the N2 gene is expressed very early during infection between 0.5 - 1 hpi, and is switched off thereafter. When the proximal end of the HindIII M fragment was end labeled to detect the 5'-end of the M1

Fig. II.5. Nuclease S1 analysis of N2 gene transcription. RNA extracted from VV-infected cells at the indicated times was used in nuclease S1 protection assays with 560 bp HindIII N probe indicated at the bottom of the figure. Control lanes: C (cyclo RNA) and H (VV RNA transcribed in the presence of hydroxyurea) used in S1 assays; markers are a mixture of ³²P-labeled pBR322/MboI and pBR322/TaqI digests.

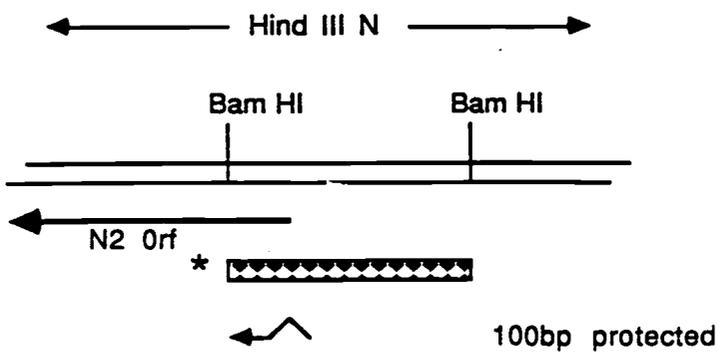
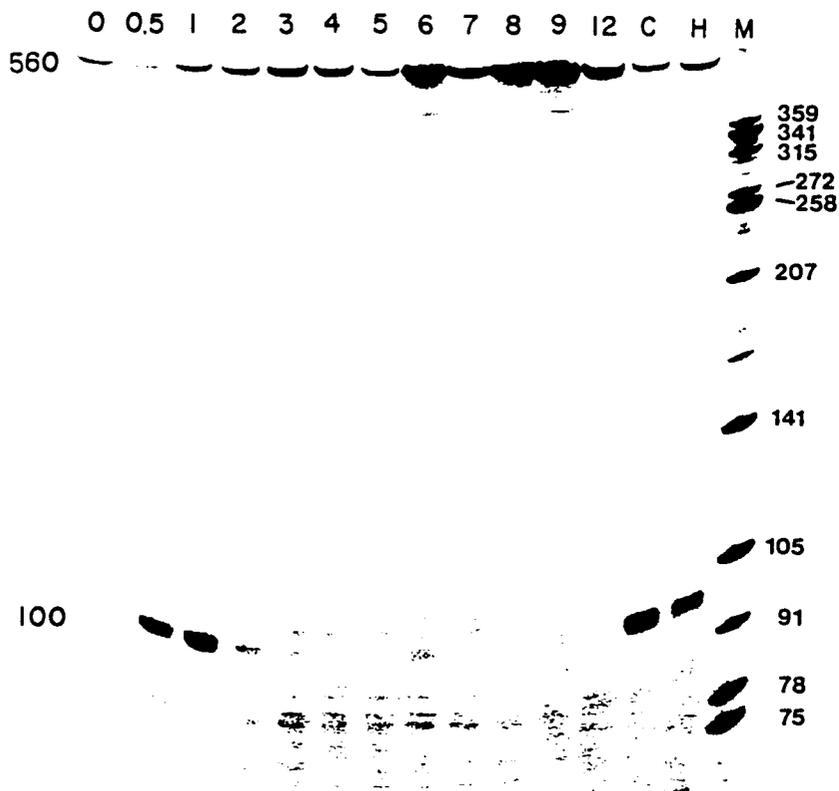


Figure II.5

gene, markedly different results were obtained (Figure 6). In contrast to the N2 gene, the M1 gene is expressed throughout infection from 0.5 - 12 hours. At early times a single 5'-end was detected approximately 560 bp to the right of the HindIII site. At late times (after 2 hours) two start sites were detected one of which corresponded to the early start and another approximately 50 bp farther upstream. Thus it would appear that the M1 gene can be added to the rapidly expanding group of VV genes, (Cochran et al., 1985; Weinrich and Hruby, 1986; Roseman and Hruby, 1987) that are expressed constitutively throughout VV infection. The M1 gene is unique in that unlike the previously reported constitutive VV genes which either switch start sites between early and late times or use the same start site throughout infection, the M1 gene uses two start sites at late times.

Because of the differences in their patterns of transcription, it was of interest to more precisely locate the transcriptional start sites of the N2 and M1 genes. To accomplish this, a combination of nuclease S1 mapping procedures and nucleic acid sequencing procedures were employed (Figure 7). Figure 7a shows the nuclease S1 data for N2 gene together with a Maxam and Gilbert sequencing ladder from the corresponding probe fragment and clearly shows that the start of transcription for N2 gene is 12 - 13 nucleotides upstream from the predicted

translational start site. Figure 7b shows the nuclease S1 protection data of MI-derived transcripts. This data maps the early/late start site for transcription of M1 gene to 567 bp to the right of HindIII site which agrees to our primer extension results (data not shown). The late start site of M1 gene is an additional 45 nucleotides upstream. The start sites detected by these analyses agreed with the data shown in Figures 5 and 6. The location of the MI and N2 transcriptional start sites used in vivo have been indicated in the context of the nucleotide sequence in Figure 2.

Fig. II.6. Nuclease S1 analysis of M1 gene transcription. RNA extracted from VV-infected cells at the indicated times was used in nuclease S1 protection assays with the single end-labeled, 1.5 kb HindIII M probe indicated at the bottom of the figure. Control lanes: Cyclo RNA and CL tRNA (calf liver transfer RNA) used in S1 assays; λ /pBR322, a mixture of ^{32}P -labeled λ /HindIII and pBR322/HinfI digests used as size markers.

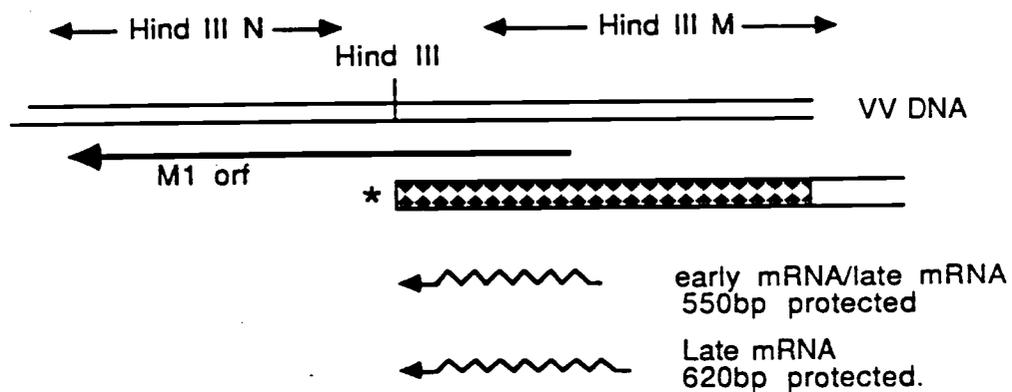
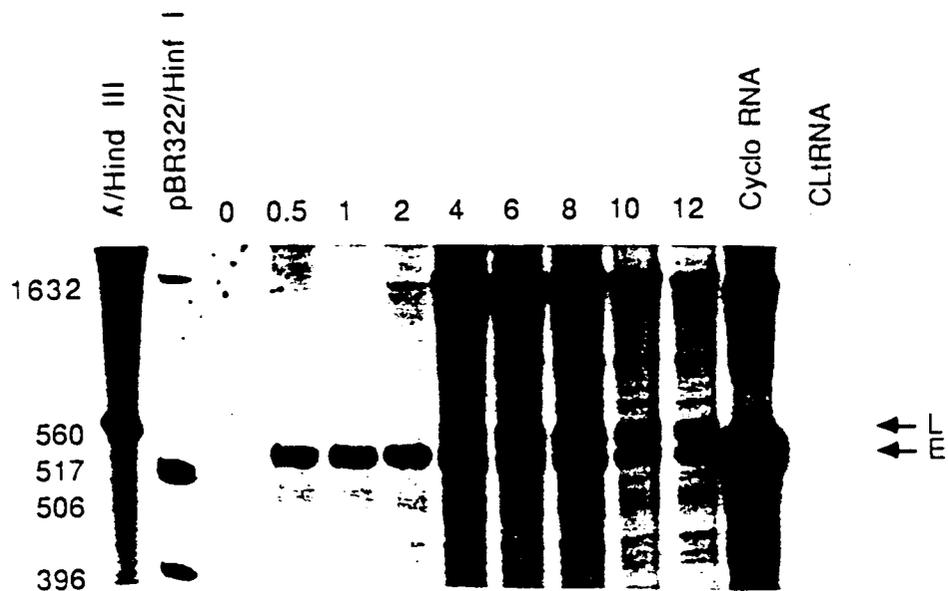


Figure II.6

Fig.II.7. Mapping of the N2 and M1 transcriptional start sites. The indicated probe fragments were asymmetrically end-labeled and used in nuclease S1 protection assays together with VV cyclo RNA, VV late RNA, or control tRNA. (A) The protected fragments were electrophoresed along side a A + G chemical sequencing reaction of input probe to localize the N2 start site. (B) The protected fragments were coelectrophoresed with radiolabeled pBR322/HinfI, pUC18/HaeIII digests. The positions of the markers are indicated at the left and the M1 start sites at right; E/L (proximal start site) and L (distal start site). [please note that in the HindIII M map, the corresponding probe and the M1-derived transcripts shown in (B) are drawn in opposite orientation relative to Fig. II.1]

Figure II.7 continued

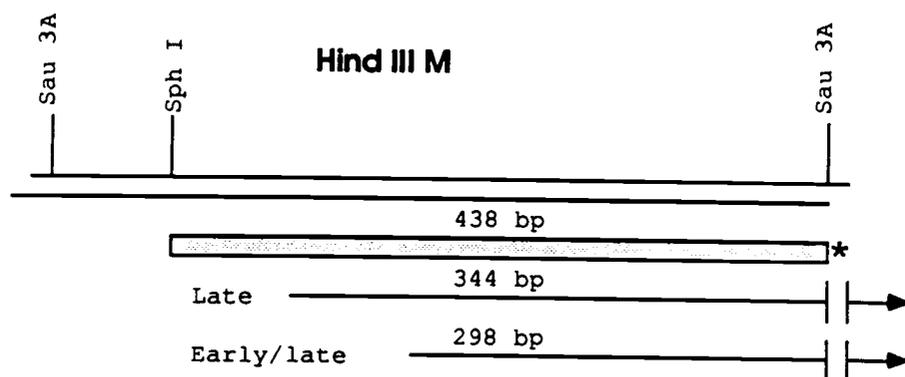
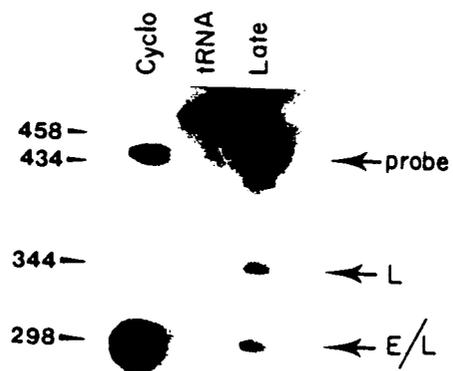
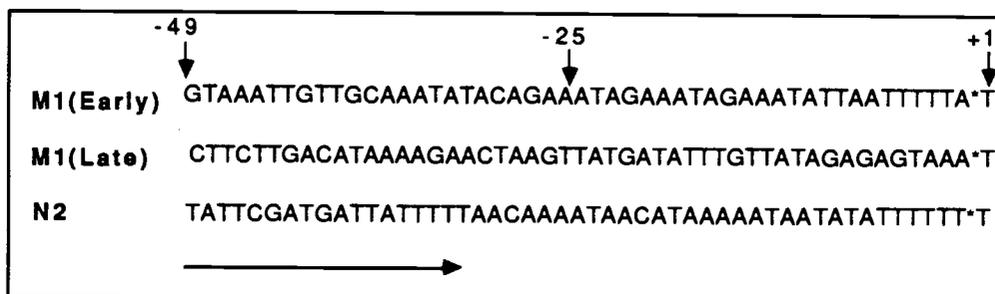
B

Fig. II.8. Potential regulatory signals from VV HindIII N/M transcriptional units. In the upper panel the 50 nucleotides upstream of the M1 (early and late) and N2 transcriptional start sites (+1) are shown. In the lower panel, the sequences downstream of the end (*TAA) of the M2, M1 and N2 open reading frames are shown. Putative termination signals are underlined.

5'-Proximal sequences



3'-Proximal sequences

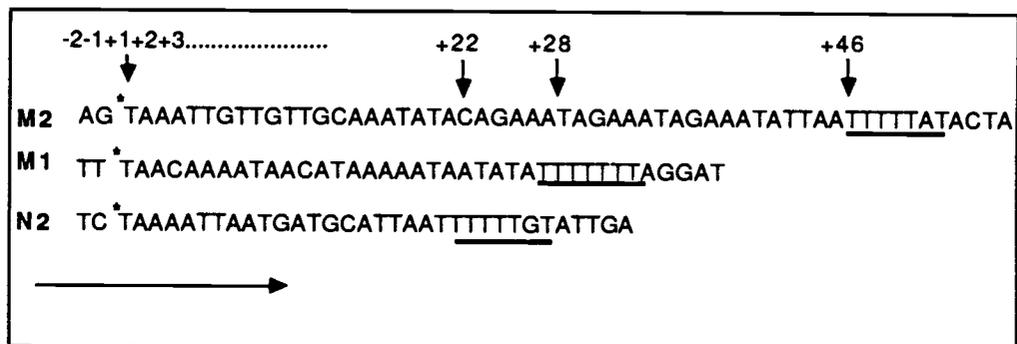


Figure II.8

Discussion

Vaccinia virus has recently been used as an eukaryotic cloning and expression system (Thomas et al., 1986) as well as a suitable vector for the construction of recombinant vaccines to be used against a variety of human and animal diseases (Panacali and Paoletti, 1982; Smith and Moss, 1983; Smith et al., 1983a; Smith et al., 1983b; Paoletti, 1984). In order to fully exploit the potential of VV for these purposes, it will be necessary to gain insights at the molecular level into both the biology of the virus and the nature of its interactions with the recipient host cell. With this perspective in mind, it was of interest to continue our studies regarding the nature and function of the host cell nuclear factors that are apparently required during the VV replicative cycle. We have previously isolated VV mutants capable of replicating independently of the host cell nucleus, biochemically characterized these mutants, and used marker transfer techniques to map them to the HindIII N/M region of the viral genome (Villarreal and Hruby, 1986). In this report we have presented the nucleotide sequence of this region plus information

regarding the structure and expression of the genes which reside there.

Morgan and Roberts (1984) have previously carried out translational mapping studies on the VV HindIII N/M region. The nucleotide sequence which we have determined has confirmed their preliminary map. This region encodes a series of VV early genes (N1, N2, M1, M2, K1, etc.) which are arranged in tandem, reading leftwards. The genes are non-overlapping, but tightly packed with 135 bp, or less, of sequence separating the individual ORFs. Transcripts representing the individual genes are apparently expressed with different kinetics implying that each is under the control of independent regulatory elements. In Figure 8, the proximal sequences immediately upstream and downstream of the genes have been aligned for comparative purposes. Rohrman et al. (1986) and Yuen and Moss, 1987 have suggested that the sequence element TTTT(N)T is important in the transcriptional termination of VV early gene transcription. As can be seen in Figure 8, this sequence or a close homolog to it, is found downstream of each of the ORFs from the HindIII N/M region. In contrast, no obvious homologies were detected when the sequences upstream of the N2 gene and M1 transcriptional start sites were aligned. As has been

noted with other VV early genes, the sequences upstream of the N2 and M1(early) start sites were very A-T rich. Also it was of interest to note that the M1 (late) start site which was expressed at late times during infection, sequence TAAAT characteristic of many VV late genes was present at the 5'-end determined by nuclease S1 mapping. In view of recent reports of discontinuously synthesized RNA present upstream of VV late transcripts (Bertholet et al., 1987; Schwer et al., 1987), it will be of interest to ascertain whether the M1 late, and perhaps early/late transcripts are similarly modified.

We have interpreted the data in Figure 6 as indicating that both the proximal (-85) and distal (-130) M1 transcriptional start sites are used at late times. There is an alternative hypothesis. It is possible that, coincident with the onset of VV late gene expression, the site of M1 transcriptional initiation switches from the proximal to the distal site, but that the shorter transcript is stable and remains detectable by nuclease S1 mapping. In the absence of any published data indicating differential stability of viral (or cellular) transcripts in VV-infected cells the latter explanation seems less likely but can not be excluded on the basis of the data presented here.

As with other regions of VV genome previously examined, the genetic information present in the HindIII N/M region is tightly-compressed. For example, the M2 ORF extends through the distal transcriptional start site of the downstream M1 gene. Similarly, the probable M1 transcriptional termination site overlaps the start site of the N2 transcript. While the biological consequences of potentially overlapping transcripts are not yet known, it is possible that *in vivo* regulation of this gene cluster may employ, in part, a "anti-message" strategy.

Two complete ORFs (N2 and M1) encoding 20 Kd and 48 Kd proteins were found within the sequenced area. Computer analysis of both proteins revealed them to possess characteristics typical of non-membrane proteins although both contain potential addition sites for N-linked sugar residues. Our previous studies have mapped the locus responsible for providing resistance to amanitin to the HindIII N fragment (Villarreal and Hruby, 1986). Thus, the 20 Kd (entirely encoded within HindIII N) and the 48 Kd (60 percent of ORF within HindIII N) proteins are the most probable candidates for this activity. It is possible that the N1 gene product might be involved, but the small amount of N-encoded N1 sequences (135 bp) make this unlikely. Also, previous

genetic analyses of α -amanitin temperature sensitive mutants have shown them to map exclusively to HindIII N and not to HindIII C. From the analyses carried out to date, it is not possible to establish which of the proteins is responsible for the drug resistant phenotype. However, there is circumstantial evidence to implicate the 48 Kd protein. The block in VV replication in enucleated cells or in the presence of α -amanitin, is observed only at late times during infection. The data in Figures 5 and 6 demonstrated that the N2 gene was transcribed only at early (0.5-1.0 hpi) in infection whereas the M1 gene was transcribed throughout infection. Although, there is no proof that the M1 transcript expressed at late times is translated into functional protein, it's kinetics of expression is consistent with the step at which sensitivity to drug inhibition is overcome.

Armed with the nucleotide sequence of this region and some knowledge of what gene products are encoded, it should now be possible to determine which protein(s) are essential for the required interaction with the host cell nucleus and what the function of these protein(s) may be. First, site-directed mutagenesis procedures can be employed to selectively inactivate either the 20 Kd or

48Kd gene to determine which is required. Second, bacterial plasmids expressing fusion proteins with either 20 Kd or 48 Kd epitopes can be constructed to produce the antigens necessary to produce monospecific antisera directed against each of the two individual proteins. These antisera will be invaluable in detecting the subcellular location of these proteins and what cellular factors that they interact with. Such experiments are currently in progress.

CHAPTER III

Molecular Genetic Mapping of the Vaccinia Virus Gene
Which Confers Resistance to Alpha-amanitin

Authors: Azaibi Tamin and Dennis E. Hruby

Summary

The location of the VV gene locus that confers resistance to α -amanitin was mapped previously to the HindIII N fragment of the VV genome (Elcira Villarreal and Dennis Hruby, 1986). This was achieved by marker rescue techniques utilizing a VV dual mutant α^{Rts7} which is capable of replicating in the presence of α -amanitin and is temperature sensitive. We showed that both phenotypes of α^{Rts7} are due most probably to a single mutation.

We sequenced the entire 1.5 kb HindIII N and part of the neighboring HindIII M and found two major open reading frames (ORFs), N2 and M1 (Azaibi Tamin et al., 1988). The data derived from transcriptional analysis suggest that N2 is an immediate early gene, whereas, M1 is constitutively expressed during VV infection in susceptible host cells. In order to determine which one of these two ORFs is the one responsible for α -amanitin resistant phenotype we have carried out high resolution marker rescue analysis on α^{Rts7} using various small fragments derived from WT VV HindIII N and M which represent either parts of N2, M1 or both ORFs. In the course of this work, we found that there is some cross-hybridization between HindIII J, M and N fragments of VV

genome possibly suggesting gene duplication events during viral evolution.

We demonstrated that the fragments of HindIII N that rescued $\alpha^{\text{I}ts7}$ VV to temperature insensitivity and α -amanitin sensitivity are not present elsewhere in the VV genome. The marker rescue data obtained, strongly suggest that N2 ORF is the gene responsible for conferring resistance to the drug α -amanitin and thus the gene that interacts with the host-cell nuclear factor(s) during infection.

Introduction

One approach to elucidate the molecular mechanism of the involvement of the host cell nuclear function in VV replication, is to identify the viral gene responsible and analyze it in detail. Other workers have utilized the selection of drug-resistant VV mutants as a procedure for mapping the gene of interest within the VV genome (Raczynski and Condit, 1983; Jones and Moss, 1984; Traktman et al., 1984).

In a similar concept, in order to locate the gene for this host-viral interaction, Elcira Villarreal and co-workers (1985) isolated a VV mutant (α -27) that was resistance to α -amanitin, a drug that is inhibitory to cellular RNA Pol II (Cochet-Meilhac and Chambon, 1974) but not to VV transcriptional system (Hruby et al., 1979; Silver and Dales, 1982; Silver et al., 1979). A double mutant was isolated, $\alpha^{\text{r}}\text{ts7}$ which is both resistant to α -amanitin and temperature sensitive. It grows normally at 31°C but not at 37°C or 40°C (Elcia Villarreal and Dennis Hruby., 1986). Both mutations were shown to be the result of one or two very closely linked mutation.

Marker rescue experiments on this double mutant $\alpha^{\text{r}}\text{ts7}$ VV were carried out with a battery of different WT HindIII fragments in order to discern the location of the

mutation. The results of the marker rescue indicated the mutation is within VV HindIII N fragment. Subsequently we sequenced the entire 1.5 kb of WT HindIII N and part of the neighboring HindIII M (Tamin et al., 1988).

VV gene expression is regulated temporally and divided into two major groups: early or late genes which are separated by their dependency of DNA replication. Those genes expressed before DNA synthesis belong to an early class, whereas, those expressed after or concomitant with DNA replication belong to the late gene class. However, there are genes that are expressed throughout infection (constitutive).

Computer analysis of the sequence coupled with the transcriptional works showed the presence of two major open reading frames (ORF) in this region of the genome, N2 and M1. N2 is a strict early gene whereas M1 is constitutively expressed during infection.

In this report we present the results obtained using a molecular genetic approach to pinpoint which one of the two ORFs is responsible actually for α -amanitin resistance phenotype, and the gene which encodes the protein product that is taking part in biological interaction with the host-cell nucleus during infection.

Methods and Materials

Cells and viruses. BSC-40 (monkey kidney cells), were grown in monolayers and maintained in Eagle minimum essential medium (Gibco Laboratories) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories), 2 mM L-glutamine and 50 μ g of gentamicin sulfate per ml. VV (WR strain), designated as WT in this work, was obtained from the American Type Culture Collection in 1977 and has been propagated by procedures described by Hruby et al., 1979. The isolation of the dual mutant $\alpha^{\text{r}}\text{ts7}$ VV, which is both temperature sensitive and resistant to inhibitory concentration of α -amanitin has been described previously by Villarreal and Hruby, (1986). Revertants of $\alpha^{\text{r}}\text{ts7}$ were obtained by serial passages of $\alpha^{\text{r}}\text{ts7}$ VV at a non-permissive temperature of 40°C followed by subsequent plaque purification.

Alpha-amanitin resistance analysis. Plates containing confluent BSC-40 cells monolayers were treated separately with media that contained different final concentration of α -amanitin (up to 10 μ g/ml), left for 18 hours at 31°C before infection to establish the effective drug dosage (Hruby et al., 1979). A correspondingly similar concentration of α -amanitin was maintained during

viral adsorption and throughout infection. The virus crude stocks obtained were titrated by plaque assay method as described previously (Hruby et al., 1979). The percentage survival of WT, α^{Fts7} and revertant VV at each concentration of α -amanitin was calculated and graphically presented in figures III.1 and III.4.

Recombinant plasmids. Initially the WT HindIII M and N fragments are cloned separately in the pBR322 plasmid vector (Tamin et al., 1988). In order to have overlapping fragments which correspond to certain parts of the N2 or M1 ORFs, the WT genomic HindIII N DNA fragment was subcloned subsequently in pUC plasmid vectors. *E. coli* strain JM83 was used to propagate the plasmids and large scale alkaline plasmid preparation were employed according to earlier reported methods (Micard et al., 1985). The cloned HindIII N subfragments used for marker rescue purposes are shown in figure III.2. All molecular cloning was performed according to the established methodology (Maniatis et al., 1982).

Marker rescue analysis. The detailed methods of marker rescue performed were similar to those reported by Villarreal and Hruby, (1986), and Thomson and Condit, (1986). Briefly, 10 μg of the corresponding sterile recombinant plasmids containing WT HindIII subfragment was coprecipitated with equal amount of salmon sperm DNA (ssDNA) by the calcium phosphate method (Graham and Van

der Eb, 1973). Confluent BSC-40 cells in 60 mm tissue culture plates were infected with the α^{Rts7} VV at multiplicity of infection equal to 0.1. After 45 minutes of adsorption, the inoculum was aspirated and fresh complete medium was added. The plates were incubated at permissive temperature (31°C) for 3 hours to allow early event of viral infection to be initiated. The medium was removed and the corresponding coprecipitated plasmid and ssDNA was layered gently onto the infected cells monolayer and incubated at nonpermissive temperature (40°C) for 3-4 hours. Then the cells were treated with 15% glycerol in phosphate buffered saline for 40 seconds, followed by the addition of fresh medium and the plates were incubated at 40°C incubator for 48 hours. For single cycle marker rescue, at the end of the incubation period the plaques were visualized by staining with 0.5% methylene blue in 50% methanol. For two steps marker rescue, the viral progeny was harvested as crude stock and titrated by standard plaque assay protocols at both permissive and non-permissive temperatures.

DNA analysis. The genomic DNA of WT and revertant VV was isolated by procedures described elsewhere (Rice et al., 1985). In order to obtain α^{Rts7} VV DNA, a similar methodology was utilized with some modifications; the virus was grown at permissive temperature (31°C) for 72 hours, and a sample of the

crude stock was tested for α -amanitin resistance phenotype as described above. Each DNA sample obtained was cleaved separately with HindIII restriction enzyme and the cleavage products were separated by electrophoresis in 0.5% agarose gel with 1X TAE (tris-acetate EDTA) running buffer, stained with ethidium bromide for visualization, and subsequently immobilized on a nitrocellulose filters. Different probes were isolated separately and purified from agarose gels by freeze-phenol methods (Benson, 1984). These fragments were labeled independently by nick-translation (Rigby et al., 1977) with [α - 32 P]dATP (New England Nuclear). Hybridization procedures were done according to methods described by Southern (1975). Autoradiography was done using Kodak XAR-5 X-ray film with an intensifying screen at -70°C .

Fig. III.1. The effect of different concentrations of the drug α -amanitin on single step growth of WT, α ^{Fts7} and revertant VV. The results are presented as a percentage of surviving virus grown at different concentrations of α -amanitin to that of the virus yield grown in the absence of the drug.

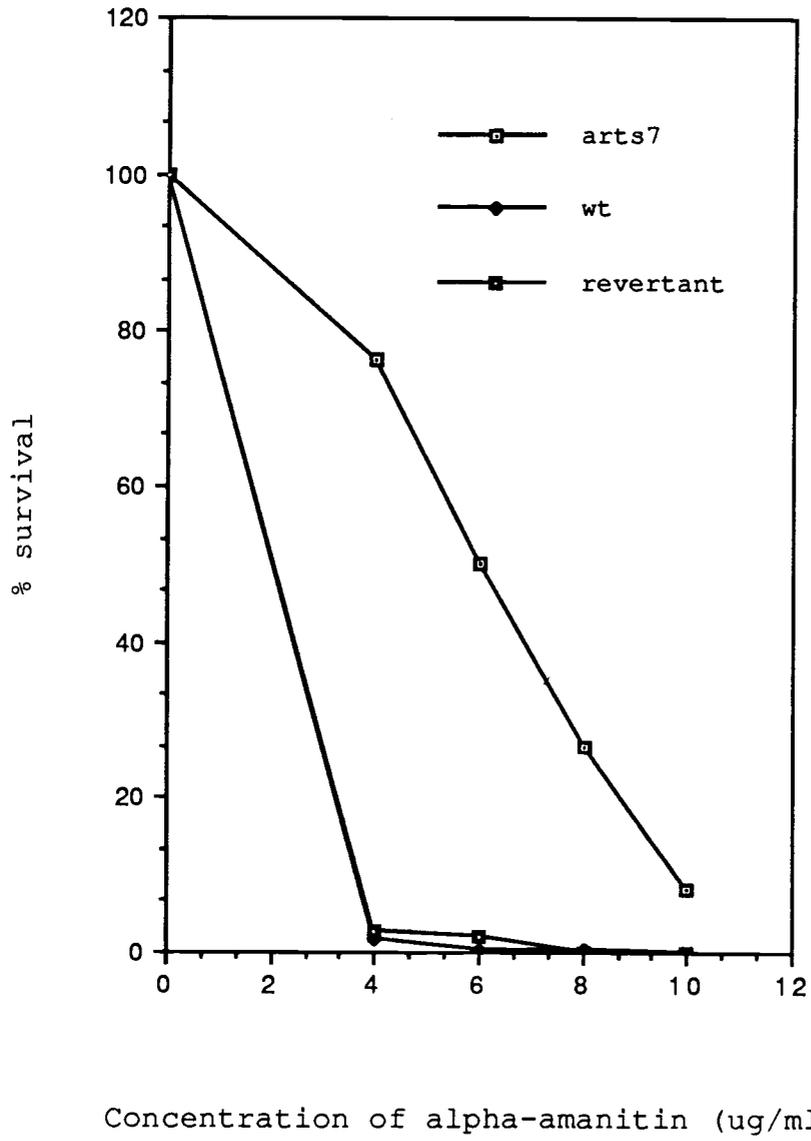


Figure III.1

Results

Comparison between WT, α^r ts7 and revertant

VV. The isolation α^r ts7 VV, which has both *ts* and α -amanitin resistance phenotypes, has been reported (Villarreal and Hruby, 1986). In this work we obtained revertant VV by serially propagating and titrating α^r ts7 VV at the non-permissive temperature of 40°C. In order to see any structural changes in their genomic DNA, we extracted viral DNA from WT, α^r ts7 and revertant virus independently. We digested each with HindIII restriction enzyme and separated the restriction cleavage products by agarose gel electrophoresis.

By comparing WT, α^r ts7 and revertant DNA, we note that each of the HindIII fragments produced has a similar migration (data not shown). This suggests that there are no major structural differences between the three viruses and thus implies that the dissimilar phenotypes between α^r ts7 and revertant VV are due to point mutations.

To determine whether the revertant VV still retained its α -amanitin resistance phenotype, we grew it in the presence of increasing concentration of α -amanitin along with WT and α^r ts7 VV as experimental controls. The data obtained are shown in Fig. III.1. Two important points could be formulated. First, the data gave further supporting evidence that the mutation in α^r ts7 is a point

Fig. III.2. Marker rescue analysis strategy. The top of the figure shows the location of HindIII N/M fragments, relative to the neighboring HindIII C and HindIII K at the left terminus of the VV genome. The HindIII N/M region has been sequenced entirely and shown with expanded restriction endonuclease enzyme cleavage sites in expanded form below. The arrows beneath the HindIII N/M map show the relative size and orientation of the coding sequences of N2 and M1 ORFs predicted by computer analysis. The lines at the bottom of the figure refer to the size and map location of different cloned WT HindIII N/M DNA fragments used in the marker rescue experiments on α^{Fts7} . Note that pUC19/454 is comprised of only sequences from the 3' end of the M1 ORF coding sequence and 95 bp fragment contains only sequences within the coding sequence of the N2 ORF.

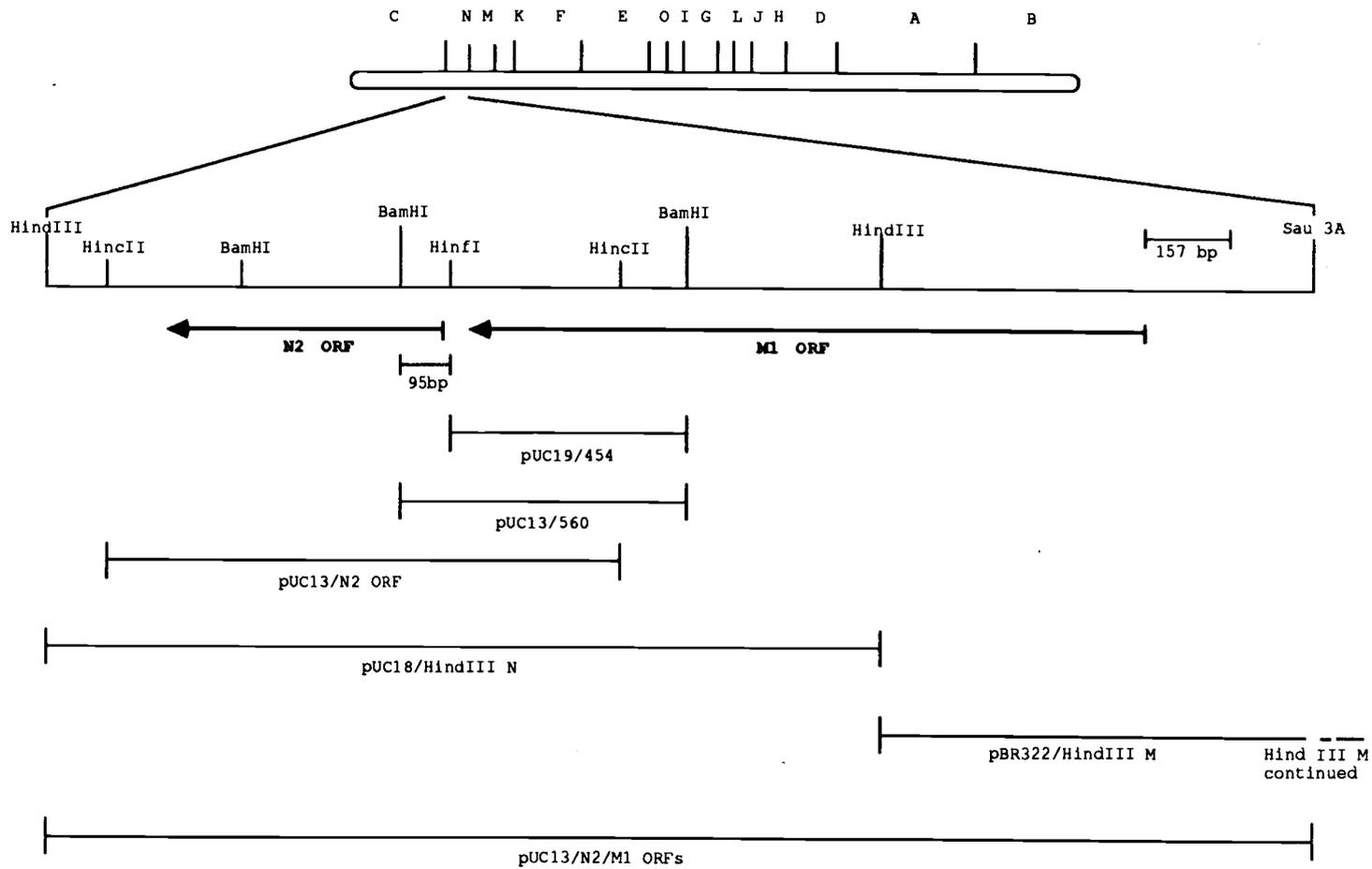


Figure III.2

mutation due to a single nucleotide substitution, deletion or insertion. Second, we isolated the revertant VV by employing only a single type of selection pressure (i.e. temperature insensitivity). This resulted in virus that lost both of its phenotypes (ts and α -amanitin resistance). This strongly implies that both phenotypes of $\alpha^{\text{r}}\text{ts7}$ are most probably the result of a single mutation.

Marker rescue. The putative location of the $\alpha^{\text{r}}\text{ts7}$ mutation, which is responsible for the temperature sensitive and α -amanitin resistance, has been mapped to HindIII N/M fragments (Villarreal and Hruby, 1986). However, we found there were two complete ORFs, N2 and M1 in this region (Tamin et al, 1988). To decide whether N2 or M1 is the gene involved in this mutation, we constructed a battery of recombinant plasmids containing different portions of the N2 and M1 WT coding sequences. The use of these plasmids in marker rescue strategies is shown in Fig. III.2.

The pUC13/560 contained some 3' of the coding sequences of M1 as well as 5' coding sequence of N2 and also the intergenic sequences between the two ORFs. The pUC19/454 contained only 3' coding sequence of M1 and the intergenic region between M1 and N2 ORFs. Cleavage of the pUC13/560 plasmid with Hinf I gives rise to a 95 bp fragment which corresponds exclusively to the 5' region

Table III.1. Two steps marker rescue of
VV arts7 by different fragments of WT
HindIII N in BSC-40 cells.

<u>HindIII N fragments</u>	<u>nos.plaques/plate</u>
95 bp **	208
pUC13/560	147
pUC18/HindIII N	85
pUC13/N2 ORF	105
pUC19/454	3
ss DNA	7

** Gel isolated fragment.

of N2 ORF. Table III.1 shows some of the results obtained from marker rescue with this WT 95 bp fragment analysis.

Fig. III.3 is a schematic of the WT DNA fragments used to marker rescue α^{Tts7} to temperature insensitivity. Other VV DNA fragments such as HindIII D and J did not rescue α^{Tts7} (data not shown). In each marker rescue experiments, we added 10 μ g of ssDNA as carrier to facilitate the transfection into eucaryotic cells. To show that the results obtained are due exclusively to the VV WT DNA fragments and not due to the presence of ssDNA, we carried out marker rescue experiments with ssDNA sample alone as negative control. The results revealed some leakiness (a very small percentage population of α^{Tts7} that are able to grow at non-permissive temperature) of α^{Tts7} VV and please note that the number of plaques observed on the ssDNA samples have been subtracted from each of the individual actual experiment's results i.e. background leakiness has been considered.

Roseman and Hruby, (1987) mapped the mutation of a mutant VV,ts17 which is DNA- and temperature-sensitive in HindIII D. In this experiment, we did marker rescue on ts17 VV using WT VV HindIII D fragment as positive control (data not shown). The data shown in table III.1 and fig. III.3 strongly suggests that the mutation of interest is located within the coding sequences of N2.

Fig. III.3. Map location of WT genomic DNA fragments which rescue temperature sensitive $\alpha^{r_{ts7}}$ VV to temperature insensitive. The text in figure III.2 legend is similarly applies here. Each bars correspond to the previously described DNA subfragments of WT HindIII N in figure III.2. Empty bar represent DNA fragment that do not rescued and the shaded bar indicate the DNA fragment that rescued temperature sensitive $\alpha^{r_{ts7}}$ VV to temperature insensitive VV. The black bar at the bottom most of the figure shows the map and size of the common region found in all the WT DNA fragments which rescued $\alpha^{r_{ts7}}$.

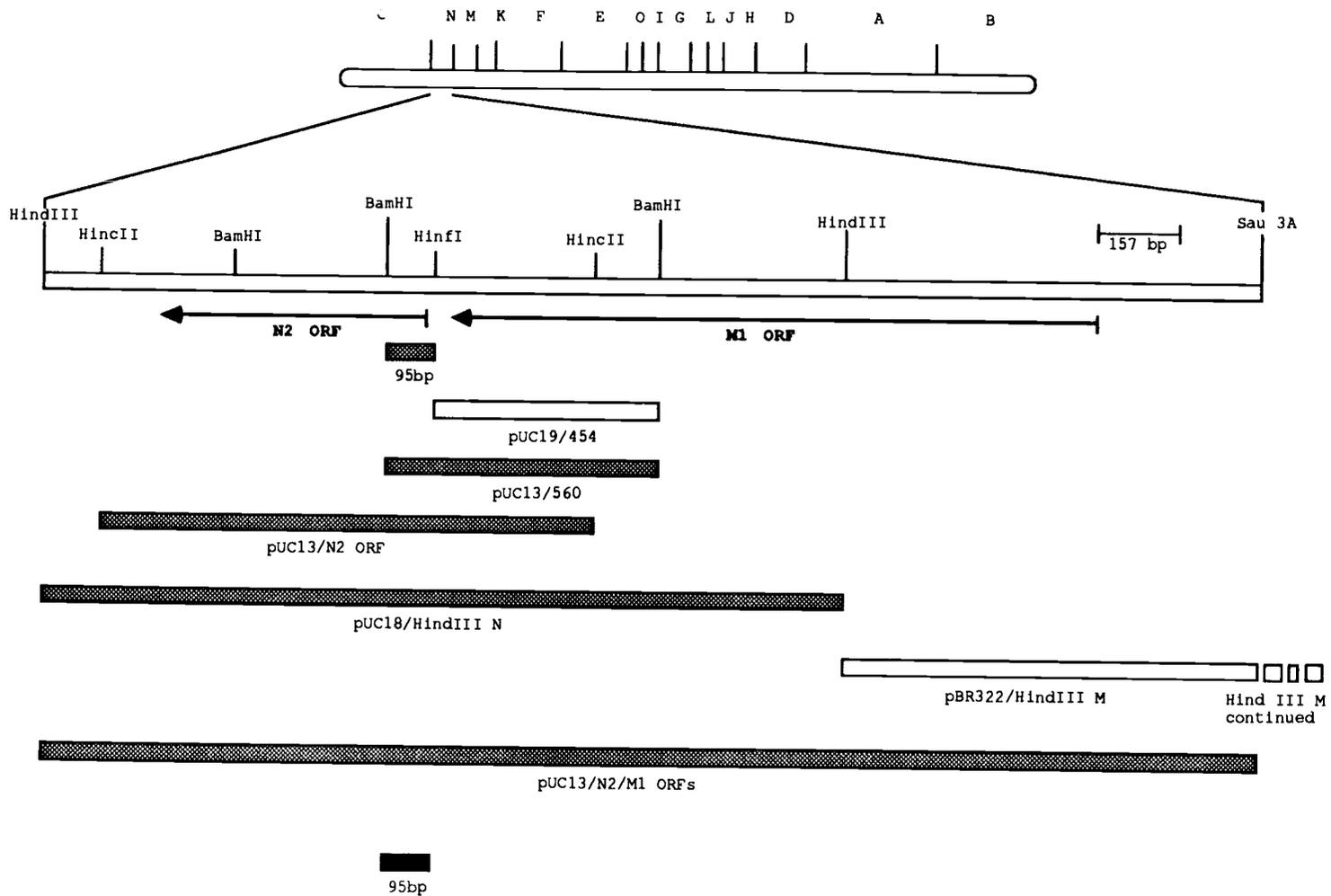


Figure III.3

This suggests that the N2 ORF is the gene that encodes the protein that interacts with host-nucleus in VV-infected host cells.

Alpha-amanitin resistance analysis of rescued $\alpha^{\text{r}}\text{ts7}$. We were interested in determining whether the rescued $\alpha^{\text{r}}\text{ts7}$ VV which is capable of growing at non-permissive temperatures, would show also wild type α -amanitin sensitivity. We grew one of the rescued $\alpha^{\text{r}}\text{ts7}$ crude stocks (rescued by WT Bam HI 560 bp fragment) at different concentrations of α -amanitin and the results are shown in fig. III.4. This information provided further evidence suggesting that both phenotypes are most probably the result of a single mutation.

Homology between VV HindIII fragments. We observed that the hybridization of VV DNA southern blot with a nick-translated HindIII N probe revealed homology between an additional fragment besides HindIII N (data not shown). This observation suggested the presence of a gene duplication within the middle portion of VV genomic DNA which traditionally is believed to be a unique sequence (Moss, 1985, and Wittek, 1984).

Recently, Kotwal and Moss, (1988) found a similar observation of gene duplication within the left-hand ITR. We repeated the experiments by digesting viral DNA from WT, $\alpha^{\text{r}}\text{ts7}$ and revertant with HindIII restriction enzyme and separated the cleavage products by agarose gel

Fig. III.4. To investigate the temperature insensitive rescued α^{Rts7} VV (560bp MRF) with respect to the drug α -amanitin resistant phenotype. The data is presented as percentage of survival of the virus grown at different concentration of α -amanitin to its growth yield in the absence of the drug. Wild type (Wt) and α^{Rts7} (arts7) viruses were utilized as controls.

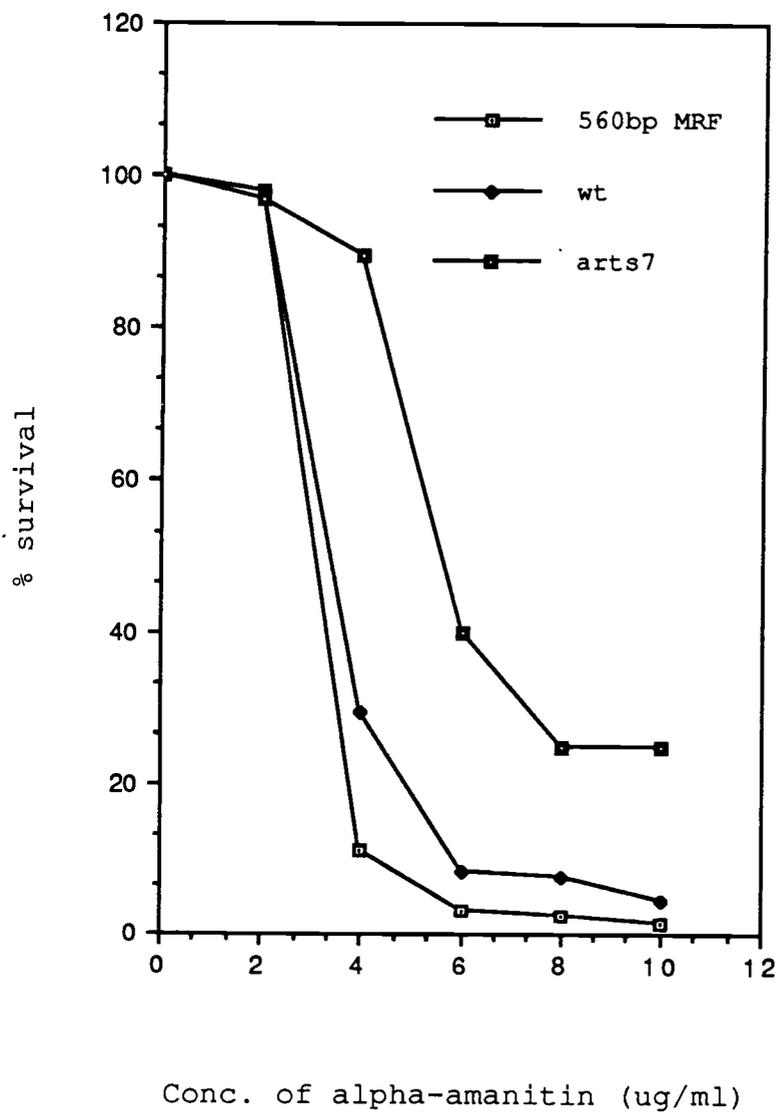


Figure III.4

electrophoresis, immobilized them on nitrocellulose, and hybridized separately with ^{32}P labeled HindIII N and J fragments as probes. The results of the cross-hybridization between HindIII N, M and J is shown in Fig. III.5.

The data suggest that some part of HindIII N is homologous to part of HindIII J and similarly some other part of HindIII J is homologous to part of HindIII M. This cross-hybridization phenomena could indicate that the WT DNA fragments that marker rescued $\alpha^{\text{r}}\text{ts7}$, actually were rescuing other genes in the genome i.e. not necessarily the N2 gene within the HindIII N fragment.

We decided to investigate whether the WT fragments that marker rescued $\alpha^{\text{r}}\text{ts7}$ were being duplicated elsewhere in the VV genome. We took one of the above filters, stripped it and rehybridized it with ^{32}P -labeled nick-translated Bam HI 560 bp fragment. The results are shown in fig. III.6.

The HindIII N Bam HI 560 bp fragment hybridized to only HindIII N fragment, which suggests strongly that 560 bp and 95 bp fragments are not duplicated elsewhere in the genome. These data give further support to the conclusion that N2 ORF is the VV gene responsible for conferring resistance to α -amanitin.

Fig. III.5. Cross-hybridization between different HindIII digests of DNA fragments of VV genome by Southern blot. The figure show the autoradiogram of hybridization experiments using three separate probes of VV HindIII DNA fragments on total VV genomic fragments. DNA extracted from WT, α^{Tts7} and revertant VV genome cleaved with HindIII restriction endonuclease separately and the cleavage products were separated by electrophoresis on 0.7% agarose gel along with DNA Molecular weight markers. The gel was stained with ethidium bromide to determine the extent of the migration of each fragment. The hybridization was performed using ^{32}P -dATP labeled nick-translated gel-isolated HindIII N and J independently.

(1) WT DNA (2) α^{Tts7} DNA (3) Revertant DNA.

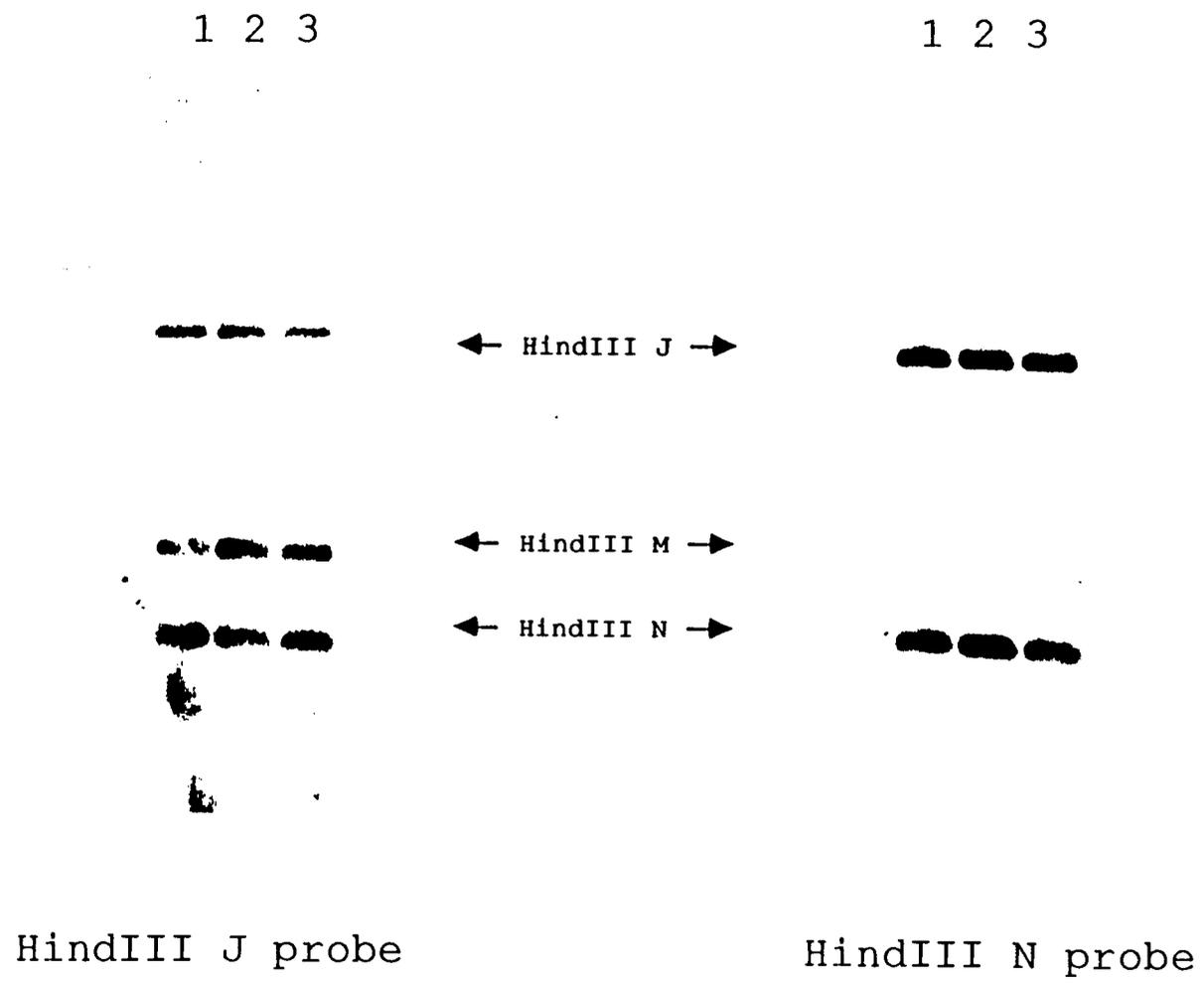


Figure III.5

Fig. III.6. The Bam HI 560 bp fragment that marker rescued temperature sensitive α^{Rts7} VV to temperature insensitivity, VV is a unique sequence within VV genome. The figure shows the autoradiogram of three separate independent lanes of HindIII digests of DNA genome from WT, α^{Rts7} and revertant VV, hybridized to ^{32}P -dATP labeled nick-translated Bam HI 560 bp probe. A similar experiment as described in Fig. III.5 was done. (1) WT DNA (2) α^{Rts7} DNA (3) revertant DNA.

1 2 3

← HindIII N

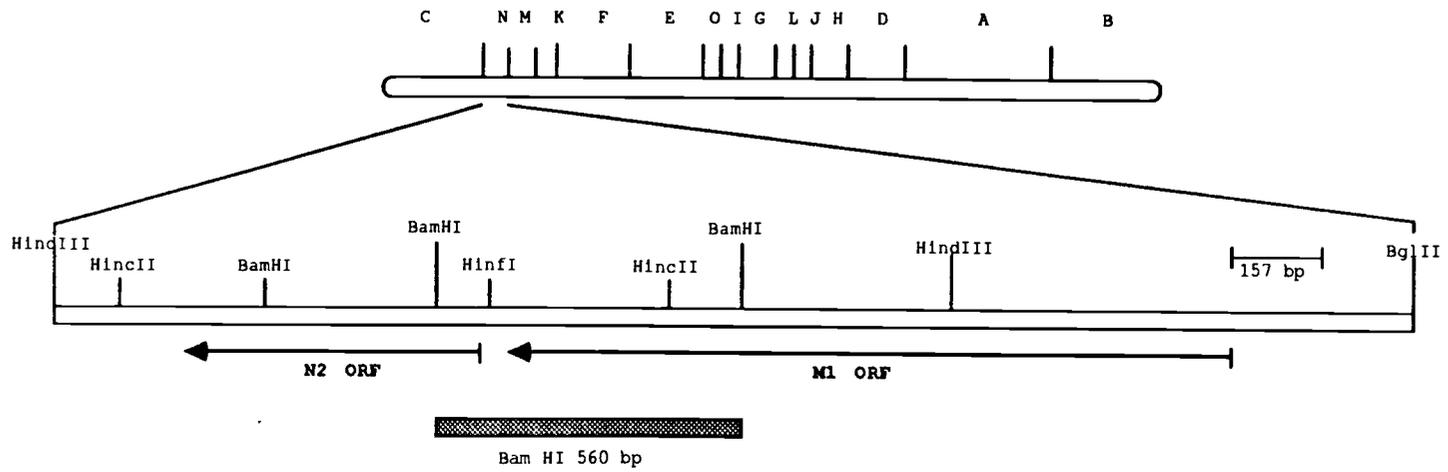


Figure III.6

Discussion

We isolated a revertant of α^{Rts7} by propagating it at the non-permissive temperature. It was interesting to know whether the revertant α^{Rts7} still retained its drug resistance phenotype. Drug resistance analysis determined its survival percentage at an increasing concentration of the drug α -amanitin. For experimental controls, we used WT and α^{Rts7} viruses under similar experiments separately. It has never been reported that a dual phenotypes mutant virus could revert back to both of its corresponding WT phenotypes by selection utilizing only one of the phenotypes involved. The ability of revertant α^{Rts7} virus to have both the corresponding WT phenotypes, suggests that it is highly likely both phenotypes of α^{Rts7} are the result of a single mutation. To map precisely the ORFs responsible for α -amanitin resistance, we used marker rescue analysis in a manner similar to Villarreal and Hruby, (1986).

Different sub-fragments of WT HindIII N which correspond to either part of the N2, the M1 or both N2 and M1 ORFs were used separately. The rationale of the experiment was that since we have shown that both phenotypes are most probably the result of a single mutation, any WT fragment that is able to rescue α^{Rts7} to temperature insensitivity suggests that the corresponding

genomic fragment of $\alpha^{\text{r}}\text{ts7}$ virus contains the mutation responsible to confer resistance to α -amanitin. The ORF where the mutation lies is the gene that we have been looking for.

The results from this high resolution marker rescue analysis are shown in fig. III.3 and table III.1. The marker rescue experiments closely narrowed the region of DNA suspected of containing the mutation after subtracting background from a negative control, and taking into account the observation that there is a difference in efficiency of rescue between the fragments that rescued $\alpha^{\text{r}}\text{ts7}$ to temperature insensitivity. A similar finding was observed by Thompson and Condit, (1986). They suggested that the efficiency may be affected by the map position of the mutation, relative to the end of the WT DNA fragments used.

The results show quite clearly that the *ts* mutation of $\alpha^{\text{r}}\text{ts7}$ VV lies within 5' coding sequence of N2 ORF, which strongly indicates that N2 is the gene which when mutated can confer resistance to α -amanitin. To confirm this, we carried out drug resistance analysis on the rescued $\alpha^{\text{r}}\text{ts7}$ and found that it has indeed lost its resistance to α -amanitin, in comparison to WT and $\alpha^{\text{r}}\text{ts7}$ as controls as shown in fig. III.4.1.

Previous work showed that the block in WT VV replication in the presence of α -amanitin occurred at a

late stage of replication (Villarreal et al., 1984). We showed that the N2 ORF is a strict early gene (Tamin et al, 1988). The kinetics of expression of N2 ORF is not consistent with the step at which the drug appears to operate. It is possible that the N2 protein is stable throughout infection or, even though the blockage is occurring late, it is possible that the late event(s) is dependent on processes that begin at early times during infection. Wilton and Dales, (1986) and Moyer, (1986) have postulated that the N2 protein may be interacting with cellular Pol II or its subunits and thus enable it to transcribe some portion of VV genome in the cytoplasm.

Another hypothesis is that the N2 protein is interacting instead with host factors which are the products of transcription of host genes by Pol II in the nucleus, an idea suggested originally by Hruby et al., (1979). To dissect either one of these possibilities, further investigation is required. The availability of anti-N2 protein antibody would be an excellent tool for this purpose.

In the course of these experiments we observed that the HindIII J fragment cross-hybridized with both HindIII N and M. The VV ds linear DNA genome is known traditionally to have inverted terminal repeats (ITR) at both termini, but it is believed the rest of the genome is unique (Moss, 1985). It is conceivable some gene or

sequence duplication has occurred and is responsible for the observed cross-hybridization phenomena. Kotwal and Moss, (1988) have a similar finding, where part of VV HindIII B fragment is duplicated within the deleted left ITR.

In our case, since this cross-hybridization phenomena was observed in WT, $\alpha^{\text{r}}\text{ts7}$ and revertant viruses and they seem to be normal, it is probable that the duplication involved non-essential genes, at least in tissue culture. The popular belief that most genes residing in the middle of the genome are essential must be taken into consideration. We interpreted the data in fig. III.6 to indicate that Bam HI 560 bp and thus the 95 bp fragment is present only within HindIII N fragment which provide further support for contention that N2 is in fact the locus which interacts with the host nucleus.

Currently we are sequencing the HindIII N fragment of WT, $\alpha^{\text{r}}\text{ts7}$ and revertant viruses to ascertain the specific mutation responsible for $\alpha^{\text{r}}\text{ts7}$'s phenotypes. We are in the process of selectively inactivating either N2 or M1 genes by site specific insertion inactivation procedures. With the gene that encodes a factor that has the potential to interact with host-cell nucleus, we have a tool to further our understanding of the molecular aspects of the viral-host cell relationship.

CHAPTER IV

Identification of Vaccinia Virus Regulatory
Elements Governing Expression of the N2 and M1
Genes Residing Within the Locus Which Confers
Resistance to Alpha-amanitin.

Authors: Azaibi Tamin and Dennis E. Hruby

Summary

Previous work demonstrated the presence of two open reading frames (ORFs) within the vaccinia virus (VV) locus responsible for resistance to α -amanitin (Tamin et al., 1988). The ORFs are designated N2 and M1 and they have sufficient coding capacity for 20 kd and 48 kd polypeptides respectively. Though these ORFs are arranged tandemly and closely packed, each appears to have different kinetics of expression. Transcriptional analyses show that the N2 ORF belongs to the immediate early gene subclass and the M1 ORF is constitutively expressed throughout infection. Typical of immediate early genes, transcription of the N2 ORF is not dependent on protein synthesis.

The M1 ORF transcriptional organization is unique in that it has two RNA start sites approximately 35 nucleotides apart. The proximal start site is used immediately upon infection and also after DNA replication. The distal start site is used concomitant with, or after DNA replication and the transcription at the distal start site is dependent on DNA replication. It is not utilized if the DNA synthesis is blocked with hydroxyurea. In this report, we isolated fragments which appear to contain potential cis-acting regulatory sequences from upstream regions of both the N2 and M1

ORFs. These fragments contain the in vivo mapped RNA start site(s), but neither has translational start codon ATG. We have analysed for promoter activity by abutting these fragments upstream of the bacterial reporter gene chloramphenicol acetyl transferase (CAT). Promoter activity is analysed by both transient expression and by recombining the promoter:CAT gene constructs into the viral genome. We carried out transcriptional analyses on these recombinants and have compared transcript levels from both cis-acting promoter elements.

The data obtained in this investigation strongly suggest the following points; 1) The fragments isolated from 5' non-coding region of the N2 and M1 ORFs are both biologically active i.e. they are both functional promoter elements. 2) The cis-acting regulatory sequence of the N2 ORF is part of the 3' coding sequence of the upstream M1 ORF. 3) Despite the fact that both N2 and M1 ORFs are oriented tandemly and very closely packed to each other, each has a separate regulatory sequence, suggesting each ORF within this gene cluster is controlled monocistronically rather than polycistronically. 4) Sequence motifs similar to TTTT(N)T within CAT gene is recognized by VV enzymes as a transcriptional termination signal. 5) Expression of CAT gene by the M1 promoter element is kinetically similar to that of the bonafide M1 ORF. 6) Regulation of

expression from N2 promoter is less straight forward; suggesting either an altered promoter function in recombinant construct, or the possibility of differential RNA stability as alternative mode of gene regulation in VV.

Introduction

Vaccinia virus (VV), the prototypic member of the Poxvirus family is a large virus and is structurally very complex. VV possess a 185 kb linear double stranded DNA genome which has the capability theoretically to encode 150 to 200 average sized polypeptides (Moss, 1985). The mature virions packages all the enzymes necessary for immediate early genes transcription within the cytoplasm of infected cells. The entire life cycle of the virus including early transcription, DNA replication, late transcription and morphogenesis takes place in the host cytoplasm.

Like its eucaryotic hosts, VV utilizes a variety of post-translational modifications affecting the activities of the proteins that it encodes. These include, proteolytic cleavage of precursor polypeptides (Katz and Moss, 1970; Moss and Rosenblum, 1973; Pennington, 1974; Van Slyke et al., manuscript submitted), ADP-ribosylation (Child et al., 1988) and myristylation (Franke et al., manuscript submitted). These attributes combined to suggest that VV is an excellent candidate for various investigations pertaining to eucaryotic gene regulation.

We are interested in the association between the virus and host cell nucleus during infection. From previous other work (chapter III), we have determined

that the N2 ORF encodes a product which may interact with a host cell nuclear function during VV replication. This interaction seems to be essential for the successful assembly of infectious progeny virions. The actual mechanism involved is still unknown.

Two different hypotheses have been suggested. It has been shown that cellular RNA Polymerase II subunit is transported from the nucleus to the cytoplasm during infection. Several workers have suggested that the N2 gene product may interact with this subunit to transcribe particular VV genes which are in turn necessary for assembly (Wilton and Dales, 1986; Morrison and Moyer, 1986).

The other hypothesis is based on work with α -amanitin which suggest that Pol II transcription within the nucleus is necessary for assembly (Hruby et al, 1979). In this case, the VV N2 20 kd polypeptides play a role in effecting the nuclear transcription event. This latter idea is supported by the absence of homology between VV promoter elements and eucaryotic promoters (Hruby et al., 1983; Weir and Moss, 1983; Pickup et al., 1986), and because promoters recognized by Pol II are not active within recombinant VV constructs (Panicalli and Paoletti, 1982; Mackett et al., 1982; Cochran et al., 1985).

To this date, neither of these proposed mechanisms has been proven directly. As part of our long term effort to understand this complex interaction, we have investigated the regulation of expression from the N2 and M1 genes. Within HindIII N fragment of VV genome, M1 ORF is just upstream and tandemly oriented relative to the N2 ORF. The N2 ORF belongs to the immediate early gene class and the M1 ORF is constitutive. In this report we present our preliminary findings on the identification of promoter elements required for the regulation of expression of the N2 and M1 ORFs.

Methods and Materials

Cells and viruses. Vaccinia virus (WR strain), designated as WT VV in this work was grown and assayed as previously described (Hruby et al., 1979). African green monkey kidney BSC-40 cells and TK⁻ L cells (American Type Culture Collection) was maintained as monolayers on tissue culture plates at 37°C under 5% CO₂ in Eagle in Eagle minimum essential medium (Gibco Laboratories) supplemented with 2mM L-glutamine, 10% heat inactivated fetal calf serum, and 10 µg of gentamicin sulfate per ml. All work regarding cell culture, virus infection and titration, unless otherwise specified was carried out according to methods described by Hruby et al., (1979).

Recombinant DNA. Putative promoter elements from the N2 and M1 ORFs were isolated from parental plasmids of pBR322/N and pBR322/M respectively. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and *E. coli* DNA polymerase I were purchased from New England Biolabs; Klenow fragment was purchased from Bethesda Research Laboratories; and calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim Biochemicals. Plasmid DNA manipulations were performed essentially according to methods described by Maniatis et al., (1982). The scheme for recombinant

plasmids constructions and the corresponding VVN2PCAT, VVM1PCAT and VV-PCAT recombinant VV is found in Fig. IV.2.

DNA transfection and marker transfer. Unless otherwise noted DNA transfection, plaque hybridization, plaque purification and the isolation of recombinant VV were carried out as previously described (Rice et al., 1985; Graham and Van der Eb, 1973; Lapata et al., 1984, and Villarreal and Berg, 1977).

DNA analyses. All methods for extractions of viral DNA, southern hybridization blot, and preparation of the ^{32}P -labeled nick-translated probes have been described (Rice et al., 1985; Sharp et al., 1973, and Southern, 1975).

RNA analyses. The isolation of total VV-infected RNA was carried out as described by Weinrich et al., (1985). The CAT transcripts were detected by Northern hybridization blot (Maniatis et al, 1982) using specific nick-translated DNA probes. For S1 protection studies (Favaloro et al, 1980; Weaver and Weissman, 1979), 5'-or 3'-end-labeled specific DNA fragments were used in order to map the transcriptional start sites and transcriptional termination sites respectively.

CAT assays. Transient expression was carried out according to methods described by Cochran et al.,

(1985a). CAT activity assay was performed according to methods used by Hodges and Hruby, (1987).

Fig. IV.1. The map location of the promoter elements required for N2 and M1 genes expression. A) The top of the figure shows the HindIII map of VV genome. Below it is an extended view of HindIII N and part of HindIII M fragments of interest. The major restriction enzyme cleavage sites are shown. The two arrows indicate size, map and orientation of the coding sequences of N2 and M1, which are predicted by computer from sequence data of this region (Tamin et al., 1988). The empty bars preceding each of the ORFs correspond to the size and location of the promoter elements dissected in this study, relative to the ORFs. B) The line illustration indicates the extended view of the promoter elements studied. The vertical arrows show the transcriptional start sites, +1 refer to early/proximal start sites for N2 and M1 respectively.

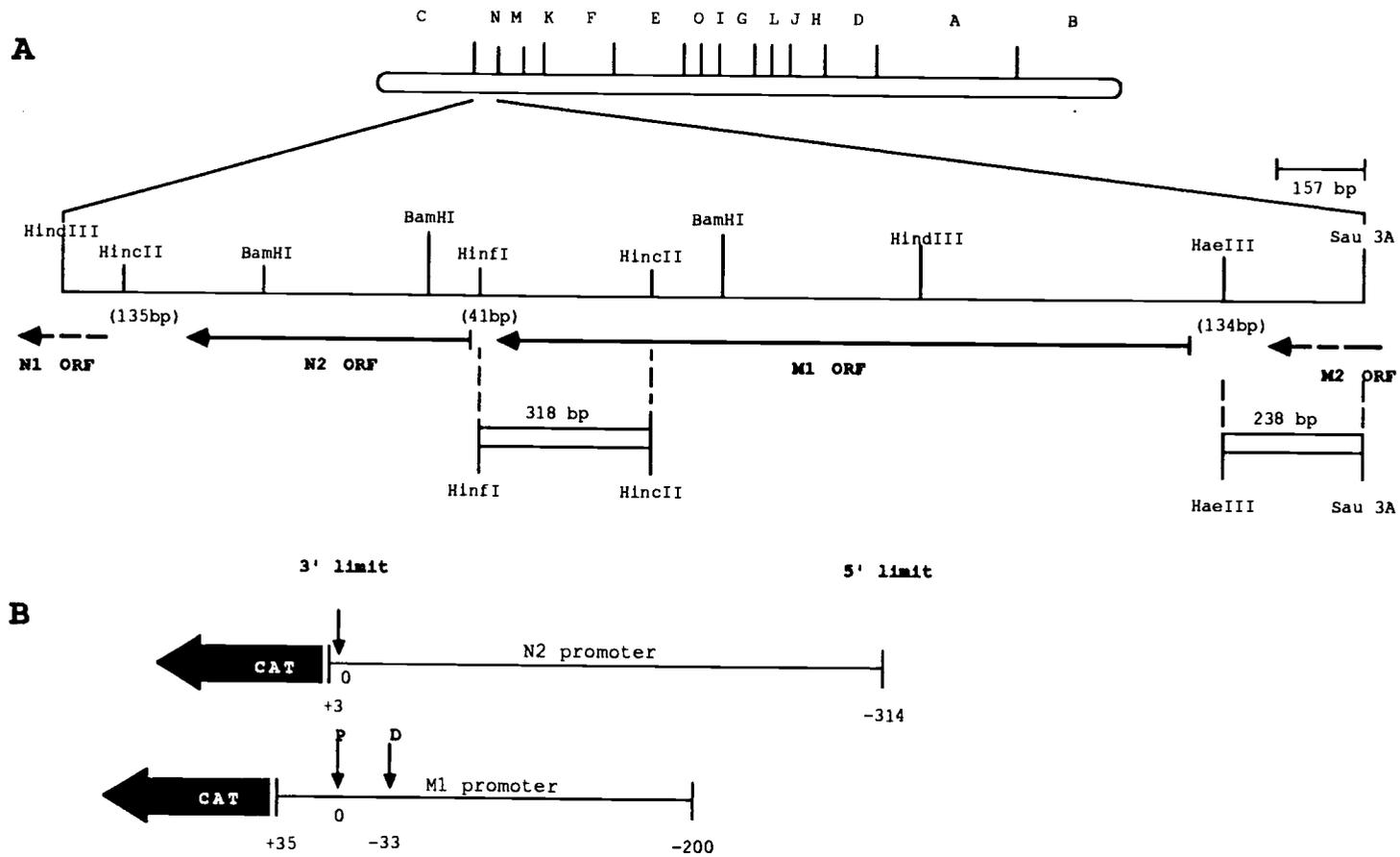


Figure IV.1

Results

Cloning of M1 and N2 promoter elements and construction of recombinant VV. The organization of M1 and N2 ORFs, relative to the neighboring HindIII fragments is shown in fig. IV.1. As has been observed by others, the ORFs within a VV gene cluster tend to be tightly packed (Morgan and Roberts, 1984; and Weinrich and Hruby, 1986). In the HindIII N/M region, there are only short intergenic spaces between the ORFs. The cis-acting regulatory elements for each of the ORFs may be part of the upstream gene coding sequence.

In another, larger gene cluster, Miner et al., (1988) demonstrated that each ORFs expression is regulated independently by an upstream cis-acting promoter element. We have previously shown that M1 ORF is expressed constitutively whereas N2 ORF is a member of an immediate early gene subclass (Tamin et al., 1988).

To determine whether sequences 5' to the RNA start site(s) of N2 and M1 ORFs are responsible for these different kinetics of expression, this region of the N2 and M1 ORFs was excised and abutted to the bacterial reporter gene, chloramphenicol acetyl transferase (CAT) in a plasmid vector pVVCAT. The sizes of the promoter fragments tested, relative to RNA start sites are shown at the bottom of fig. IV.1. The construction scheme for

Fig. IV.2. Construction strategy of recombinant VV for promoter activity analysis. The figure illustrates the construction scheme of VVN2PCAT and VVM1PCAT recombinant VV which has N2 or M1 promoter elements abutted to bacterial Chloramphenicol Acetyl Transferase (CAT) gene inserted within tk gene locus in VV DNA genome. For the purpose of negative experimental control, the identical CAT gene cassette is present in the similar HindIII J fragment within VV-PCAT recombinant VV, without any promoter element but upstream 5' tk sequence. The parental plasmids shown; pUC13/1.5kb M and pUC13/560bp were obtained from the corresponding WT HindIII M and N fragments. The putative promoter elements of M1 and N2 ORFs were excised separately from these plasmids using the available restriction enzymes cleavage sites as shown. The VV tk flanking sequence in pVV5.1CAT recombinant plasmid vector will facilitate the insertion of the Promoter:CAT cassette into the tk gene locus within HindIII J of VV DNA genome. The orientation of the promoter elements relative to CAT was determined by restriction enzymes digest and sequencing analyses.

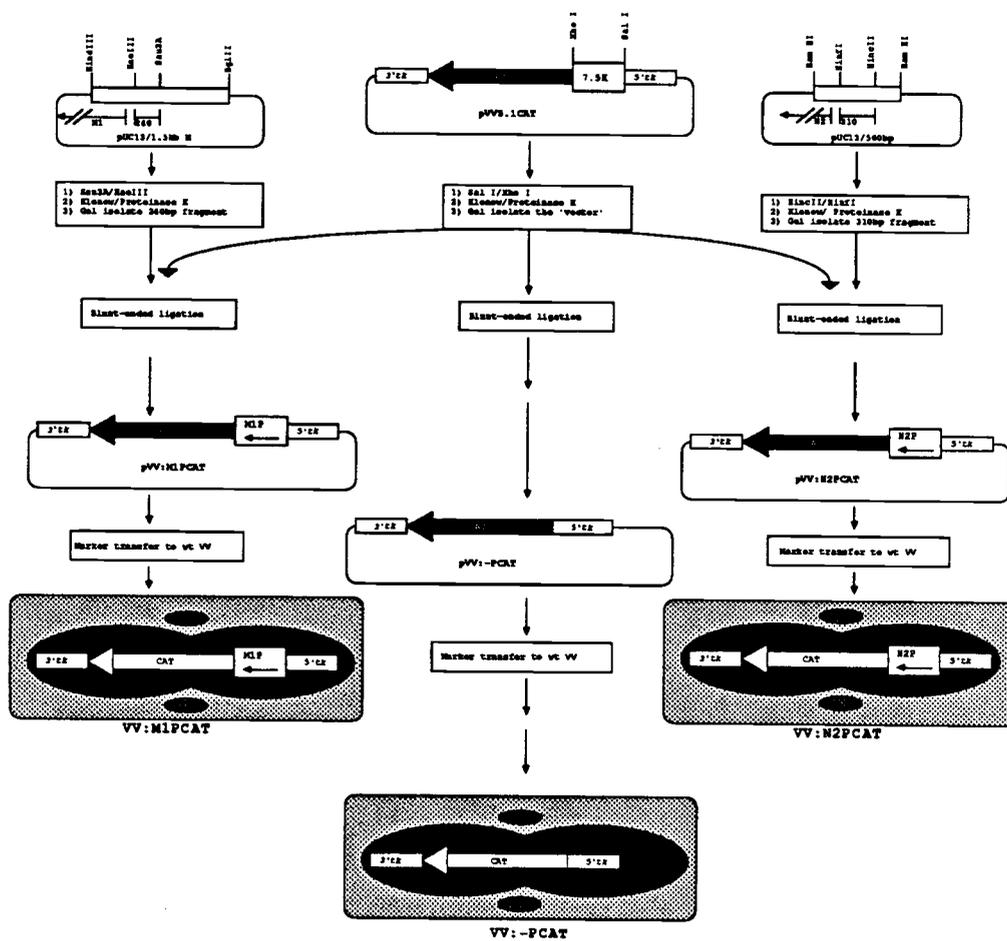


Figure IV.2

making the recombinant plasmid insertion vector and subsequent recombinant VV is illustrated in fig. IV.2. The VV thymidine kinase (tk) is non-essential in tissue culture (Mackett et al., 1982). Therefore, the tk gene serves as a suitable site for insertion of foreign genes.

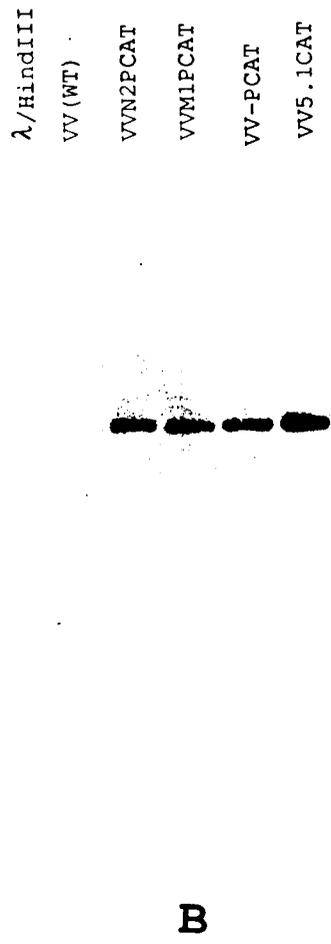
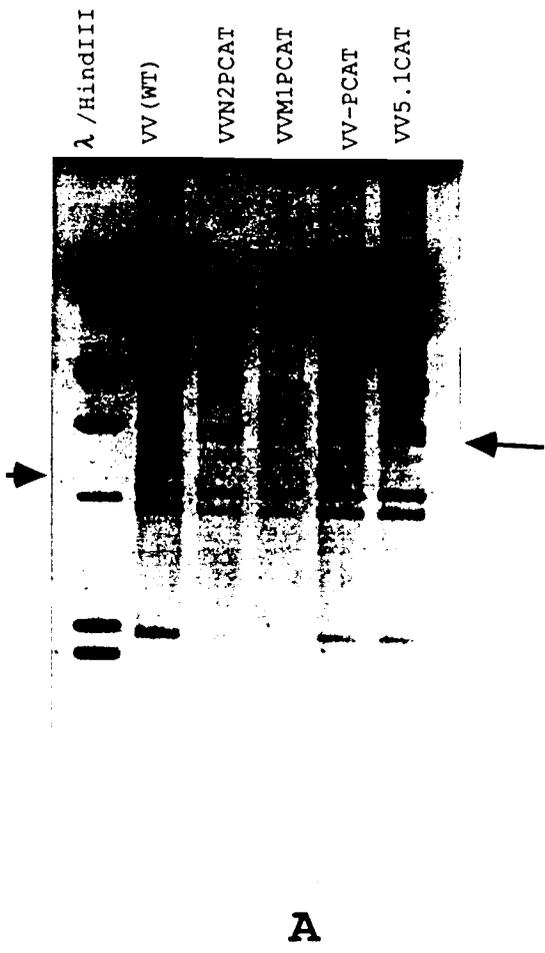
These plasmids are designed with tk sequence, flanking the promoter:CAT cassette in the insertion vector plasmid pVVCAT which will facilitate the insertion of promoter:CAT cassette into tk gene locus in WT VV DNA genome by homologous recombination. The pVV-PCAT and the corresponding VV-PCAT recombinant VV were constructed use as a negative control. The presence of the CAT cassette in the recombinant VV is confirmed by restriction analysis of wt and recombinant DNA as well as by southern blot hybridization analysis as shown in fig. IV.3. The higher migration of HindIII J fragment in recombinant VV in comparison to WT VV suggests it is due to the promoter:CAT cassette insertion in the tk locus. This is further supported by data obtained on hybridization with nick-translated CAT probe as shown in the same figure. The predicted promoter:CAT cassette orientation relative to VV genome is shown at the bottom of the fig. IV.3.

M1 and N2 ORFs cis-acting promoter elements are necessary and sufficient for CAT gene expression. Recombinant insertion plasmids; pVVM1PCAT, pVVN2PCAT and pVV-PCAT was analysed by VV

Fig. IV.3. South hybridization blot of recombinant VV.

A) Total viral DNA from each viruses (VVN2PCAT, VVM1PCAT, VV-PCAT, VV5.1CAT and WT VV) were cleaved with HindIII restriction enzyme and the cleavage products were electrophoresed in 0.5% agarose gel along with DNA markers of λ DNA digested with the same enzyme. The gel was stained with ethidium bromide and the migration of each fragments were measured. The arrowhead shows the location of the WT HindIII J fragment and the long arrow indicate the new migration of HindIII J fragment in recombinant viruses. Its slower migration is due to the insertion of Promoter:CAT gene cassette within the resident tk gene. B) The fragments were immobilized onto nitrocellulose filter and hybridization was performed using ^{32}P -labeled nick-translated CAT gene insert as probe.

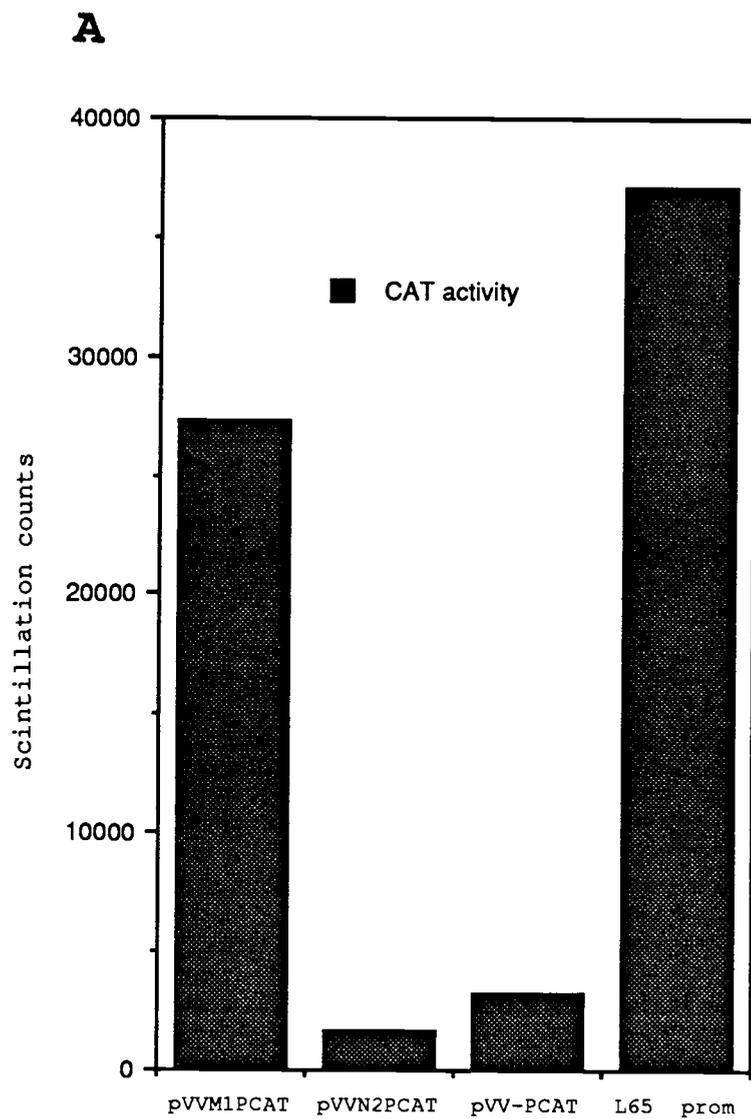
Figure IV.3



helper dependent transient expression assay and the data obtained is shown in fig. IV.4. The transient expression protocol preferentially measures activity from late promoters. The transient expression data suggest that a functional M1 ORF promoter element is in the isolated fragment. L65:CAT consists of the L65 (03) promoter driving the CAT gene and served as a positive control for the experiments (Miner et al., 1988).

In order to analyze the early promoter function of ORF N2 and to assay for correct regulation of the M1 promoter, we have prepared several recombinant VV. The results obtained by the construction of recombinant viruses and by the transient-expression assay have been shown to be similar (Bertholet et al., 1986; Hanggi et al., 1986; Weir and Moss, 1987b). Both recombinant virus, VVM1PCAT and VVN2PCAT showed CAT activity. This suggests that both putative fragments are biologically active promoter elements. This provides further support for the notion that both the bonafide M1 and N2 ORFs are regulated independently. The absence of CAT activity in VV-PCAT infected cells demonstrates that the CAT activity observed upon VVM1PCAT and VVN2PCAT infection is due to the presence of biologically active M1 or N2 cis-acting promoter element, rather than the activity of other promoter elements from upstream genes in the VV genome.

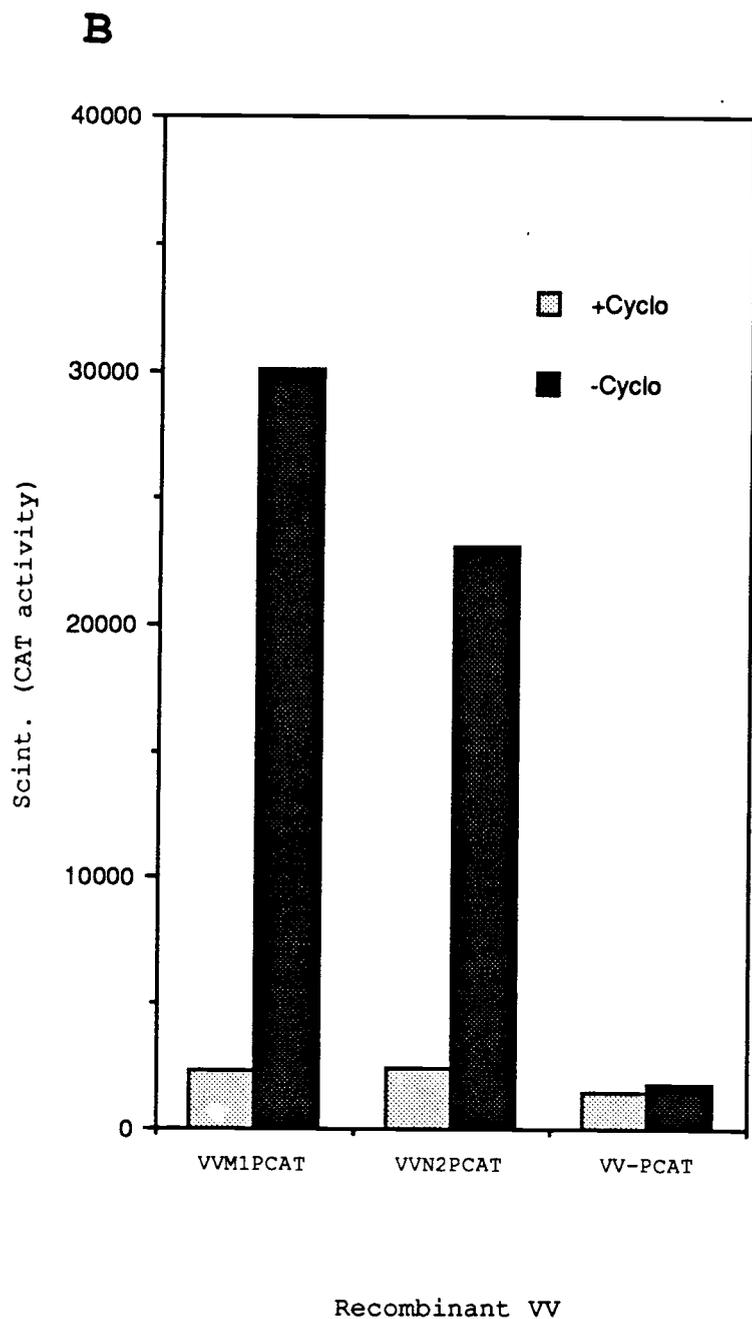
Fig. IV.4. Cis-acting elements derived from N2 or M1 genes is required for CAT expression. A) The figure shows the CAT activity by WT VV-aided transient expression on recombinant plasmids shown on fig. IV.2. The L65 promoter plasmid has strong VV late promoter (L65) abutted in right orientation to CAT which has already been described (Miner et al, 1987). B) The results of CAT activity assay on recombinant VV constructed in this study as shown in fig. IV.2. Cyclo refers to the CAT extracts obtained from recombinant VV infected cells in the presence of cycloheximide (protein synthesis inhibitor).



Plasmid/Promoter:CAT

Figure IV.4

Figure IV.4 continued



Transcription from M1 promoter. To establish that the expression of the CAT gene corresponds to correct RNA initiation, we carried out nuclease S1 protection assays using the recombinants virus. The results are shown in fig. IV.5. The protected probe fragments expected are illustrated at the bottom of the figure. The data suggest that the regulation of transcription of CAT gene by M1 promoter element in VVM1PCAT recombinant VV is similar if not identical to the bonafide M1 ORF. At early time post infection (before DNA replication), only the proximal RNA start site is used. After DNA replication or concomitant with DNA replication the distal RNA start site is utilized. In addition, a signal from the proximal start site is seen at late times.

Transcriptional regulation of N2 ORF is complex. We have shown that N2 ORF belongs to an immediate early gene subclass and by time-course nuclease S1 analysis, we could not detect any of its transcript at late times post infection after DNA replication (Tamin et al., 1988). We analysed total RNA extracted from VVN2PCAT recombinant infected cells with a similar nuclease S1 protection assay. Fig. IV.6 shows the results.

A start site is present at both early and late times, in contrast to the situation with the wild type N2 ORF. The results suggest that the kinetic of expression

Fig. IV.5. Nuclease S1 analysis of M1PCAT expression in recombinant VV. Total RNA extracted from VVM1PCAT recombinant virus-infected cells at late time of infection in the presence or absence of cycloheximide, was used in nuclease S1 protection assay with 5' single end labeled probe indicated in the bottom of the figure. Control lane: CLtRNA (calf liver tRNA). The ^{32}P -labeled DNA used as size markers are indicated.

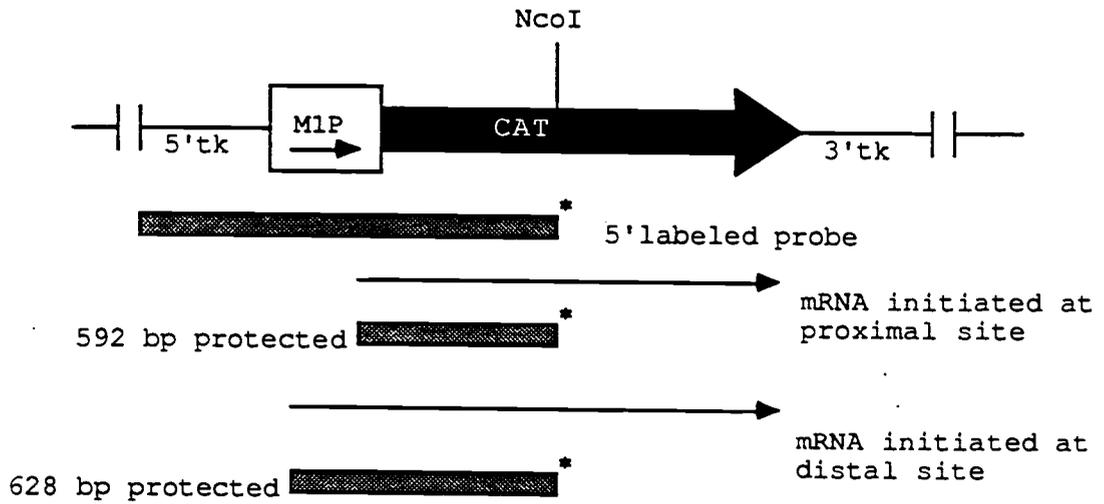
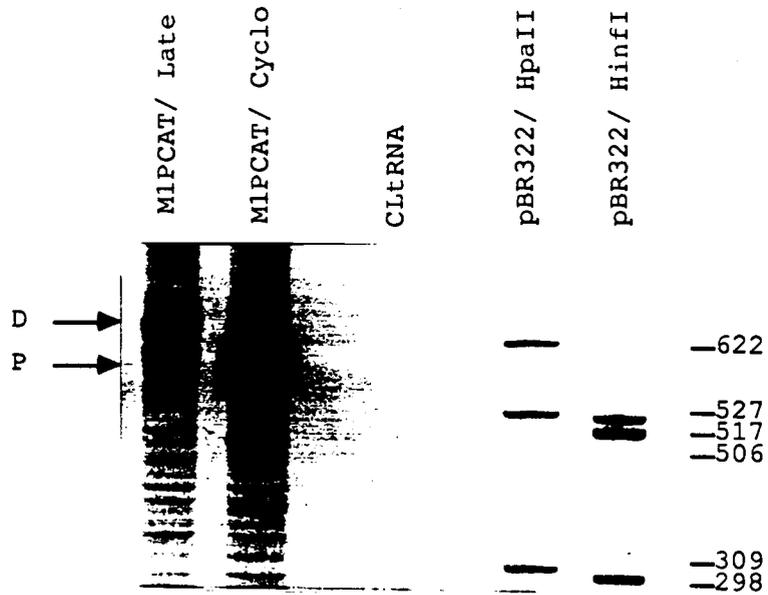


Figure IV.5

of the N2 promoter abutted to CAT reporter gene is different from the kinetic of expression of bonafide N2 ORF. To show that this difference in regulation of the recombinant N2 promoter does not reflect global change in regulation within VVN2PCAT, we monitored simultaneously transcription from the N2 locus and from the translocated N2 promoter:CAT locus using a mixture of radiolabeled Bam HI 560 bp fragment as probe in the same reaction with CAT probe. Bam 560 probe will detect transcription from the authentic N2 promoter. Fig. IV.7 shows these results.

The arrow corresponding to the size of 100 nucleotides shows the protected authentic N2 transcript, present only prior to DNA replication i.e. in the presence of cycloheximide or hydroxyurea. CAT transcript was present at both early and late time post infection. Please note that the presence of protected fragment equivalent to the size of protected CAT in VV-PCAT lanes are parental Bam HI 560 bp probes. The data shown in fig. IV.7 support the notion that the N2 promoter fragment used in this study do not have or it is missing an element that is required in order to have similar transcriptional kinetic as the bonafide N2 ORF.

Northern blot hybridization and 3' S1 protection assay. In order to further characterize transcription from the translocated N2 promoter, we carried out northern hybridization experiments. The

Fig. IV.6. 5' transcriptional mapping of N2PCAT in recombinant VV. Total RNA extracted from VVN2PCAT recombinant virus-infected cells at late time of infection (7 hpi) or in the presence of cycloheximide was used separately in S1 protection assay with 5' single end labeled N2PCAT probe as indicated at the bottom of the figure. The ^{32}P -labeled DNA markers used are indicated.

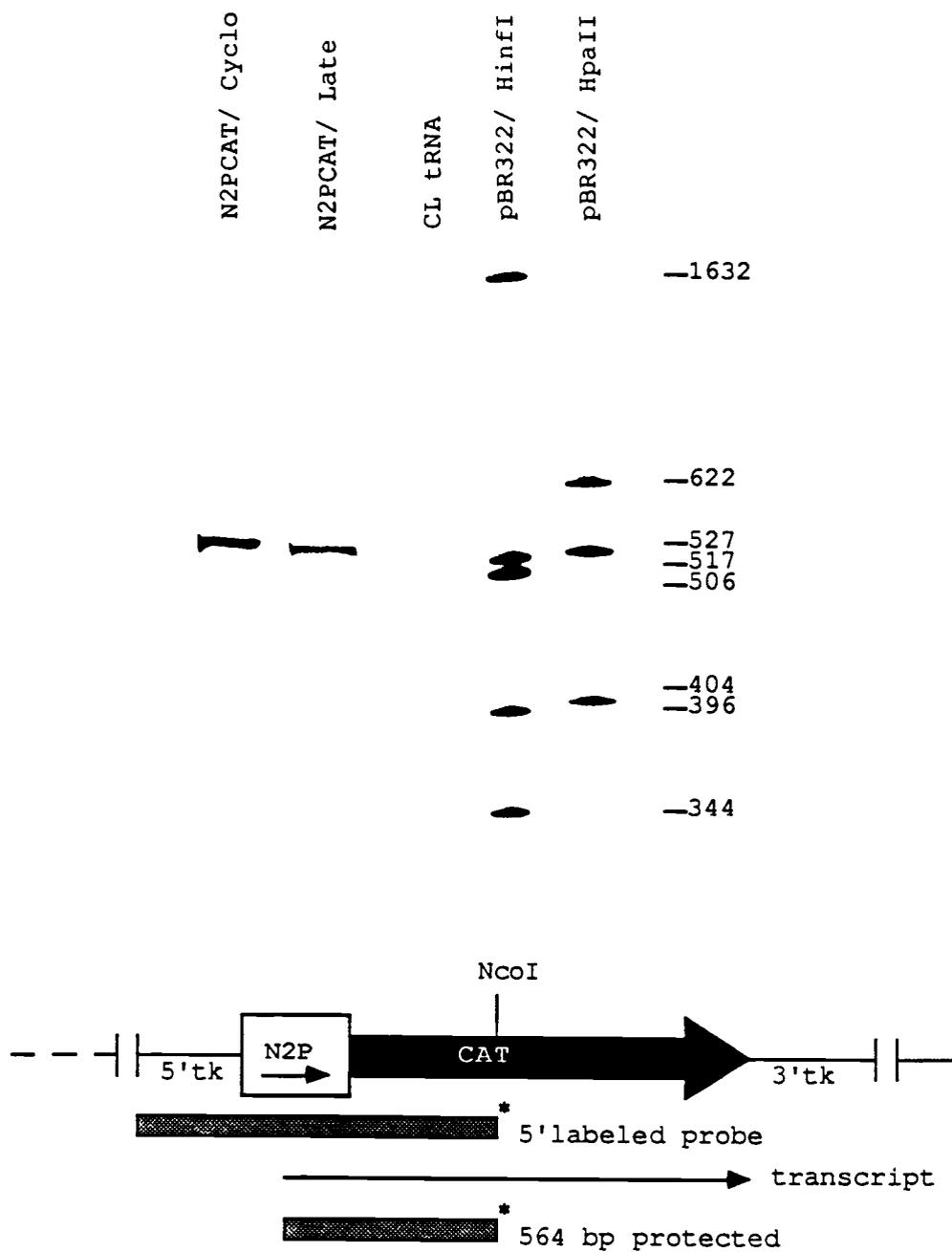


Figure IV.6

results are shown in fig. IV.9. The data suggest that we have two populations of CAT transcripts: one of which terminates at the CAT termination signal and the other terminate at the tk termination signal. This occurs with both the M1:CAT and N2:CAT recombinant. Late transcription is characterized by heterogeneous large transcripts caused by readthrough transcription from late promoters. We carried out 3' S1 protection assay on the same stock RNAs used in the northern blot referred to fig. IV.9. The results are shown in fig. IV.10A. The data agree with our prediction that a portion of the CAT transcripts terminate within the tk sequence. The shorter transcript is produced due to termination within the 3' CAT gene at a TTTTAA sequence.

Fig. IV.7. The expression of bonafide N2 gene versus N2PCAT in VVN2PCAT recombinant VV. A) Total RNA extracted independently from VVN2PCAT and VV-PCAT recombinant VV-infected cells at late time post infection in the presence or absence of cycloheximide was used in 5' S1 nuclease protection assay. Control lane: CLtRNA (calf lever tRNA). B) Two separate 5' end labeled probes used are indicated, relative to their corresponding location within recombinant VV DNA genome.

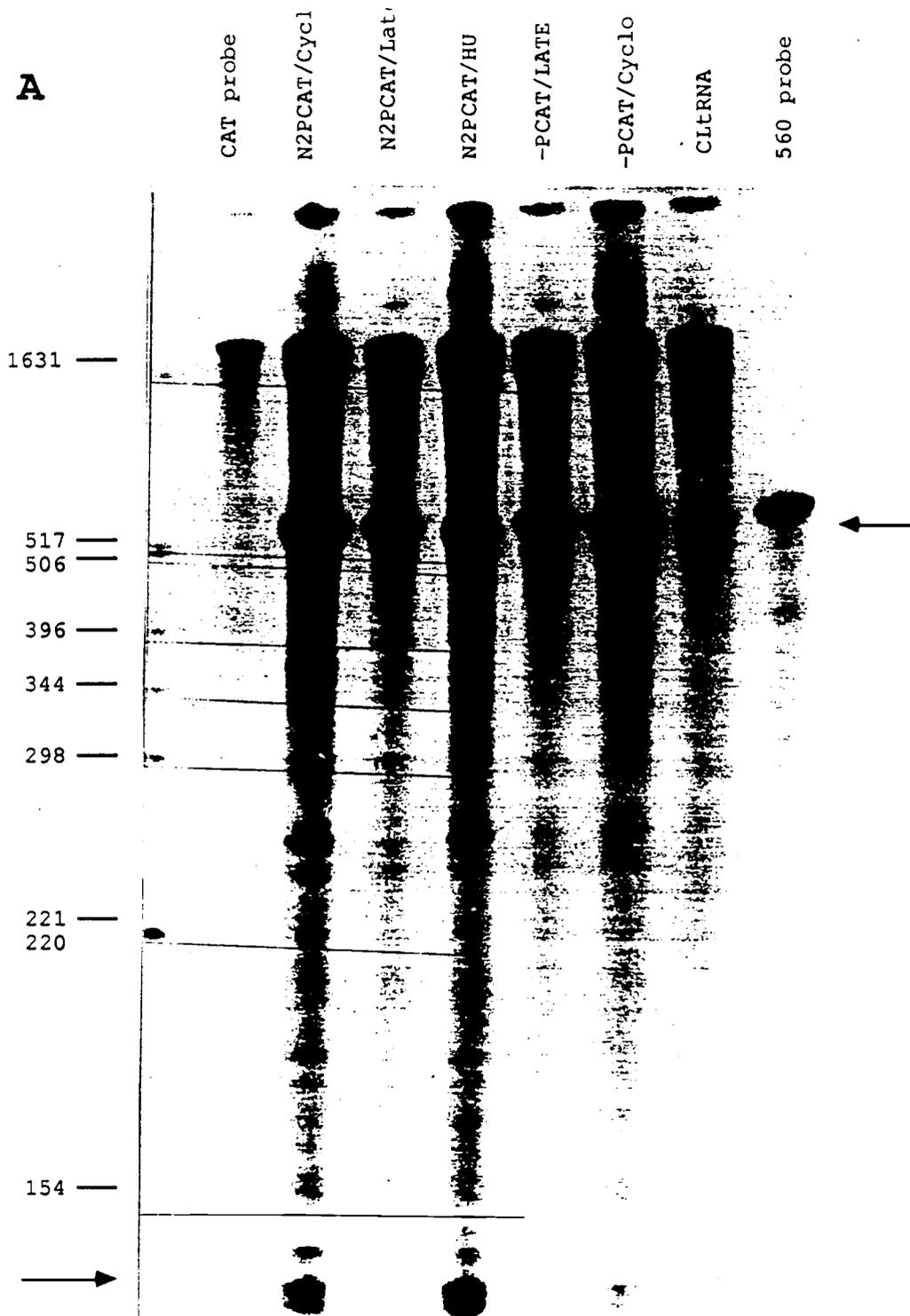


Figure IV.7

Figure IV.7 continued

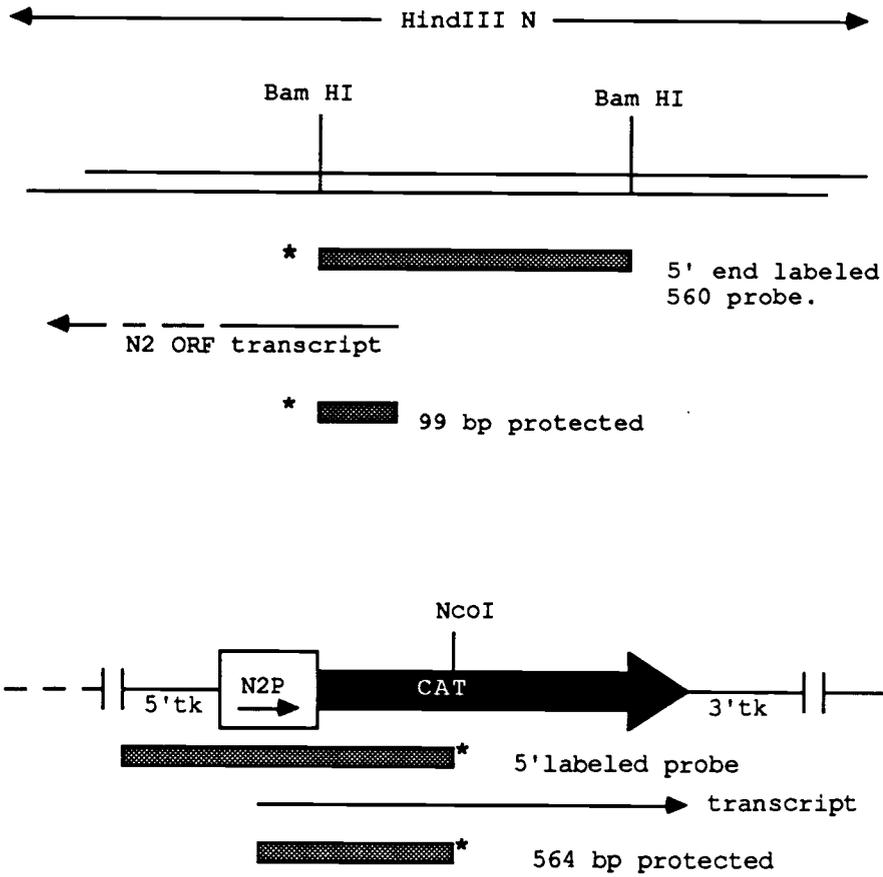
B

Fig. IV.8. Primer extension analysis on N2PCAT transcription in recombinant VV. Total RNA extracted from VVN2PCAT recombinant virus-infected cell at late time post infection in the presence or absence of cycloheximide was used in primer extension analysis experiments. The primer used was 5'single end labeled CAT oligo (17 mer). The illustration at the bottom of the figure indicates the map and orientation of the primer used, relative to the promoter element under study. Control lanes: CLtRNA (calf lever tRNA). The fragments obtained from the primer extension was electrophoresed along side the sequence ladder of the corresponding pVVN2PCAT plasmid primed with the same CAT oligonucleotide primer.

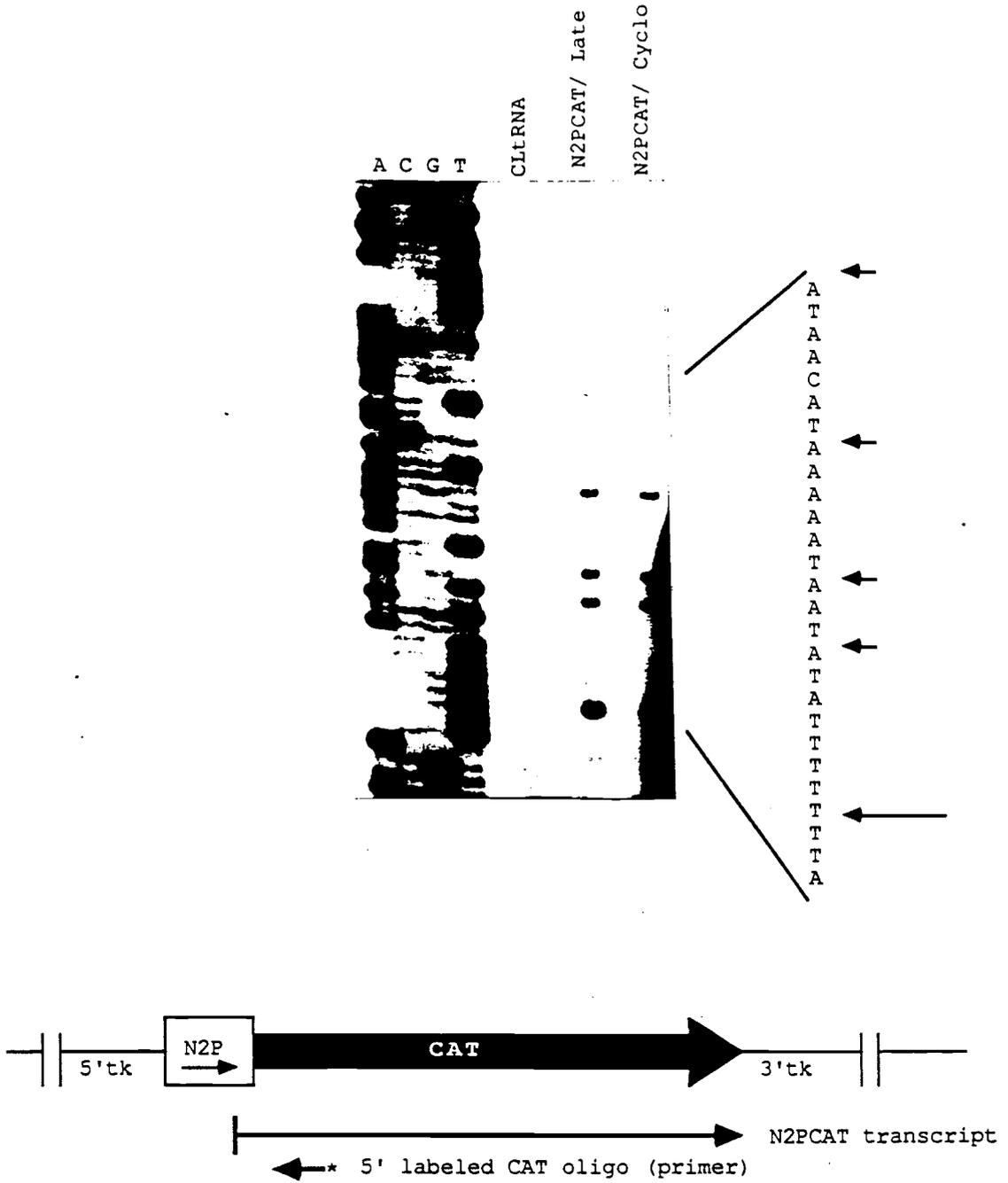


Figure IV.8

Fig. IV.9. Northern blot hybridization analysis on total RNA from recombinant virus infected cells. Total RNA extracted independently from VVN2PCAT and VVM1PCAT recombinant VV-infected cells at late time post infection in the presence or absence of cycloheximide. These were electrophoresed separately in duplicate under denaturing conditions and subsequently were transferred onto nitrocellulose filters. The filters were hybridized separately to either ^{32}P -labeled nick-translated probes of CAT or tk gene. The predicted structures of two different size populations of CAT transcripts are shown at the bottom of the figure. The indicated size markers were derived from the migration of the corresponding RNA ladder used.

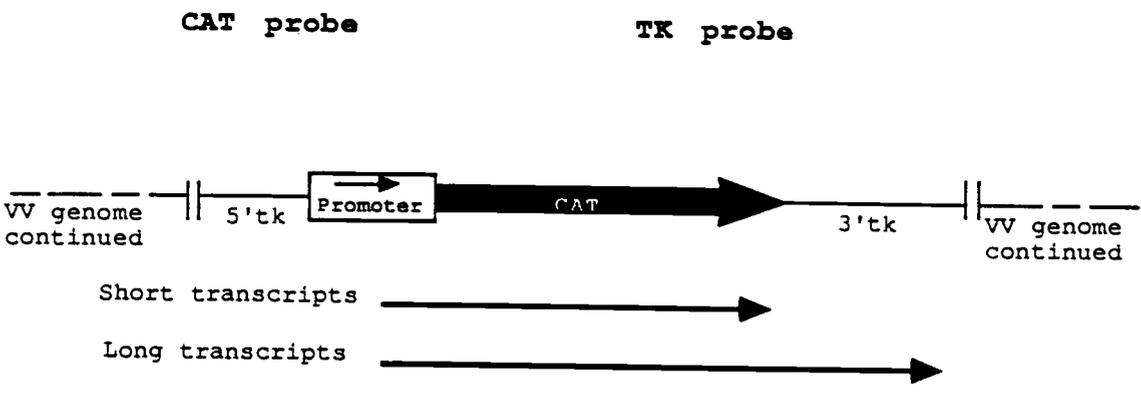
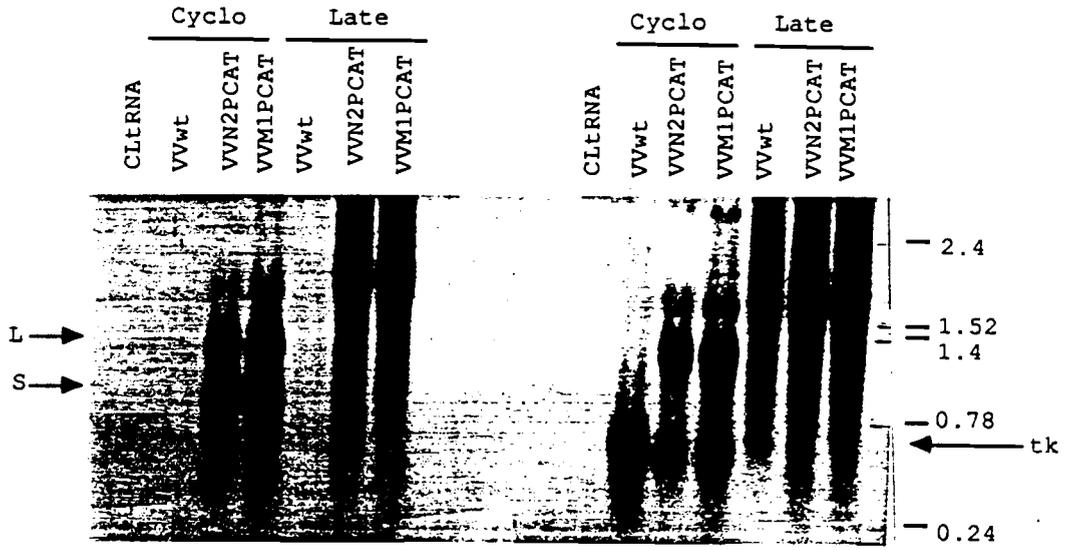


Figure IV.9

Fig. IV.10. 3' nuclease S1 analysis on N2PCAT and M1PCAT transcripts from recombinant VV. A) The same total RNA stocks as described in fig. IV.9 was used in 3' S1 nuclease protection assay. The probe used was 3' single end labeled fragment as indicated in panel B. Control lane: CLtRNA (calf lever tRNA). ³²P-labeled pBR322 plasmid digested with HpaII or HinfI restriction enzymes are used as size markers. B) The location of putative potential termination signal of VV early genes are indicated at the 3' regions of CAT and tk flanking sequence in recombinant VV genome.

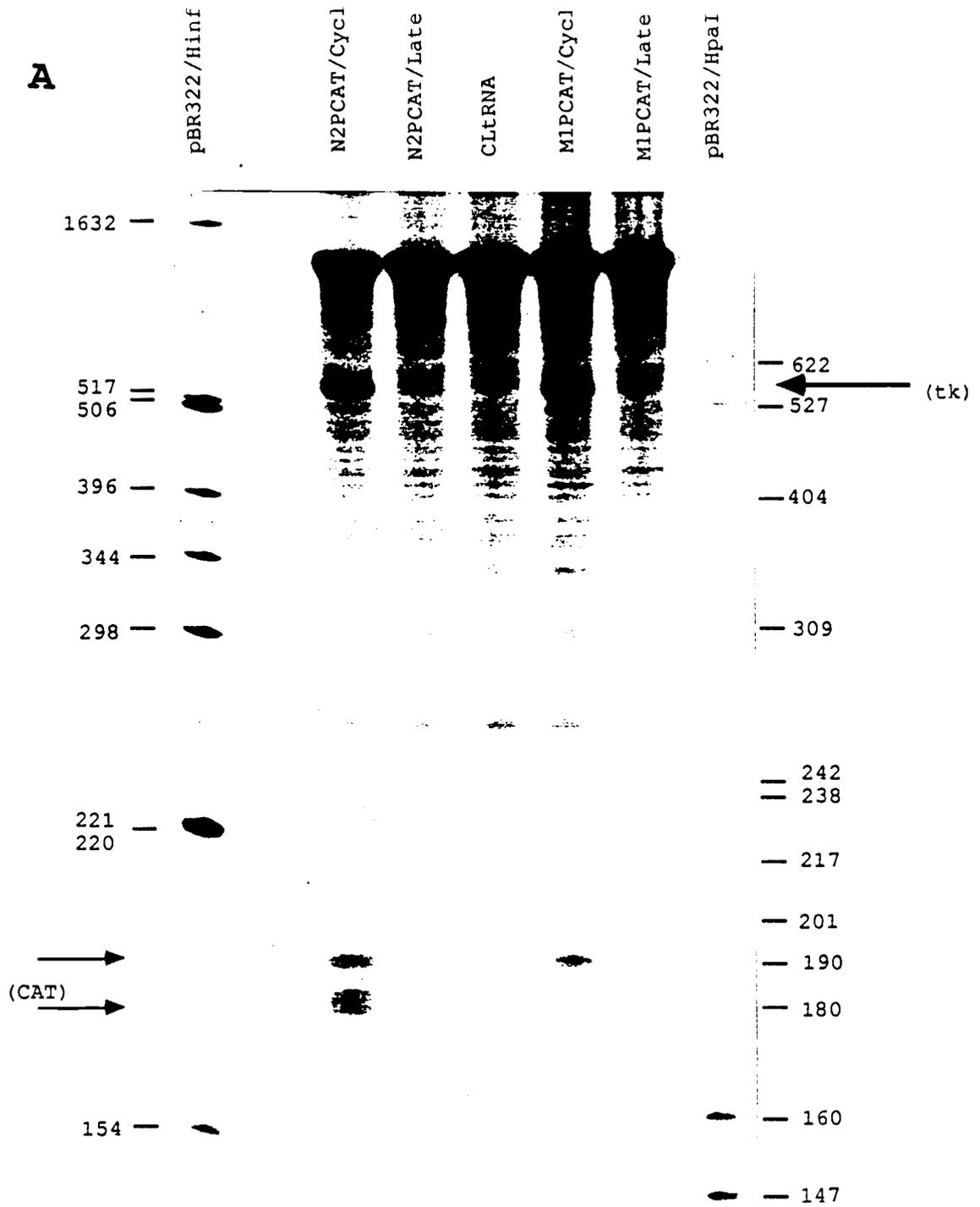
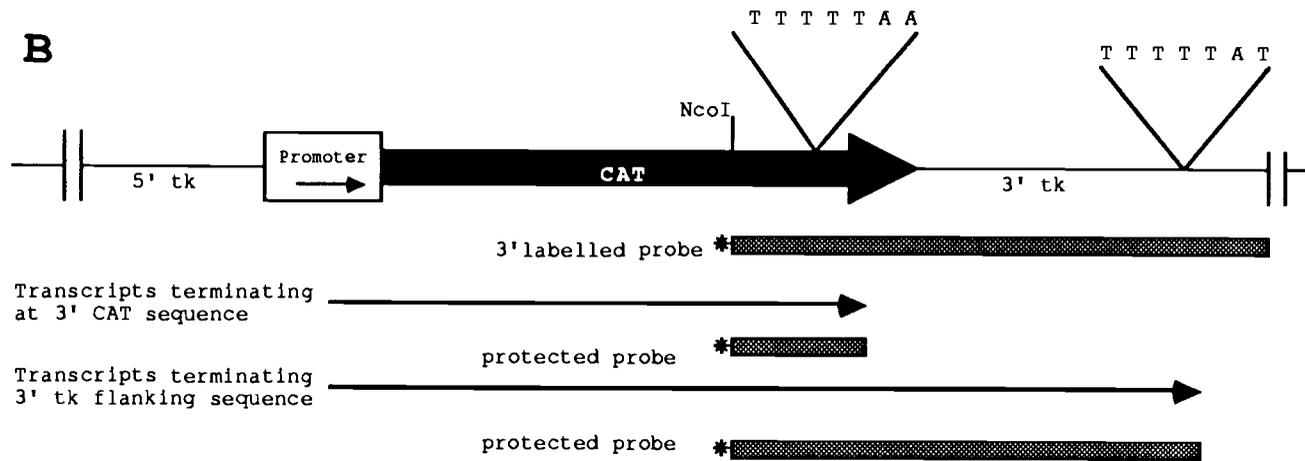


Figure IV.10

Figure IV.10 continued



Discussion

There are several reasons why we have used tk locus in VV DNA genome as an insertion site for our promoter:CAT cassette. Thymidine kinase is not essential at least in tissue culture work and thus can be manipulated. We can use tk functional inactivation to select recombinant virus (Mackett et al., 1984). Its sequence is known. More important is the availability of tk insertional vectors in our laboratory such as pVVCAT used in this work. Several workers have used this site for similar work in promoter analysis as well as for expressing foreign proteins. In each case they have been expressed with appropriate kinetics (Cochran et al., 1985; Vaseff et al., 1986; for review Hruby, 1988).

The insertion of the N2 and M1 prom:CAT cassette in tk is confirmed by southern blot hybridization (fig. IV.3) and also Northern blot hybridization (fig. IV.9). Its insertion is visualized by the increased in size for HindIII J, relative to WT VV. The loss of the tk transcript in all the three recombinant VV clearly provides more evidence that the insertion is within tk gene locus in the HindIII J fragment of VV DNA genome.

The data derived from the CAT assays (fig. IV.4) shows that the CAT activity is due to the presence of the

corresponding cis-acting regulatory element of N2 and M1 ORFs. VV late transcripts are known to have 3' heterogeneity i.e. termination occurs at random and often continues to transcribe throughout the downstream genes. However, the absence of activity in VV-PCAT recombinant VV-infected cell extracts shows that the CAT activity observed is not due to readthrough transcription from upstream genes in the VV genome but rather reflects the presence of active promoter elements driving expression from the M1 and N2 ORFs fragments isolated. Since the Multiplicity of infection (M.O.I.) of VVM1PCAT, VVN2PCAT and VV-PCAT recombinant VV were the same during infection, and their corresponding extracts were suspended in identical volumes of CAT assay buffer, the higher activity of CAT for VVM1PCAT suggest M1 promoter is 'stronger' than N2 promoter.

The results suggest; 1) the putative cis-acting regulatory sequence isolated from the upstream coding sequence of N2 and M1 ORFs are biologically active. 2) That these two tandemly oriented ORFs in this tightly packed cluster have separate regulatory elements suggesting that each is controlled monocistronically (Miner et al., 1988). 3) These promoters are within the coding portion of the upstream gene.

Transcriptional analysis by a 5' S1 protection assay indicate that the translocated M1 ORF promoter has the

same kinetics of expression as that of the bona fide M1 ORF. The proximal RNA start site signal is present both at early and late time post infection, and the distal start site is switched on only after DNA synthesis (fig. IV.11)

Transcriptional analysis on recombinant N2 promoter give surprisingly different results when compared to the bonafide N2 ORF expression. Instead of being expressed only at early time, CAT transcripts are present at both before and after DNA replication i.e. its kinetic of expression is somewhat altered. The CAT mRNA seem to have the same 5' ends at both early and late time post infection (fig. IV.8).

Two possible hypotheses could explain this phenomena. First, possibly it is due to N2 promoter activity at late times. If this notion were true we would expect the pVVN2PCAT insertion plasmid to have CAT activity by transient expression assay. The results of this experiment shown in fig. IV.4 do not support the idea of a constitutive N2 promoter. Unlike any other DNA viruses, VV late mRNA has a 5' poly(A) leader sequence. A TAAAT motif upstream from RNA start site(s) is believed to be critical in order to produce 5' leader sequence (Schwer et al., 1987; Bertholet et al., 1987).

There are several TAAAT motif upstream from RNA start site(s) within the N2 promoter fragment studied. To

Fig. IV.11. The effect of hydroxyurea on N2PCAT and M1PCAT transcription in recombinant VV. Total RNA extracted independently from VVN2PCAT and VVM1PCAT recombinant virus-infected cells at late time post infection in the presence of cycloheximide or hydroxyurea was used in S1 protection assay. The bottom of the figure indicates the corresponding 5' single end labeled probes used. Control lane: CLtRNA (calf liver tRNA). ³²P-labeled pBR322 digested with HpaII or HinfI restriction enzymes was used as size markers.

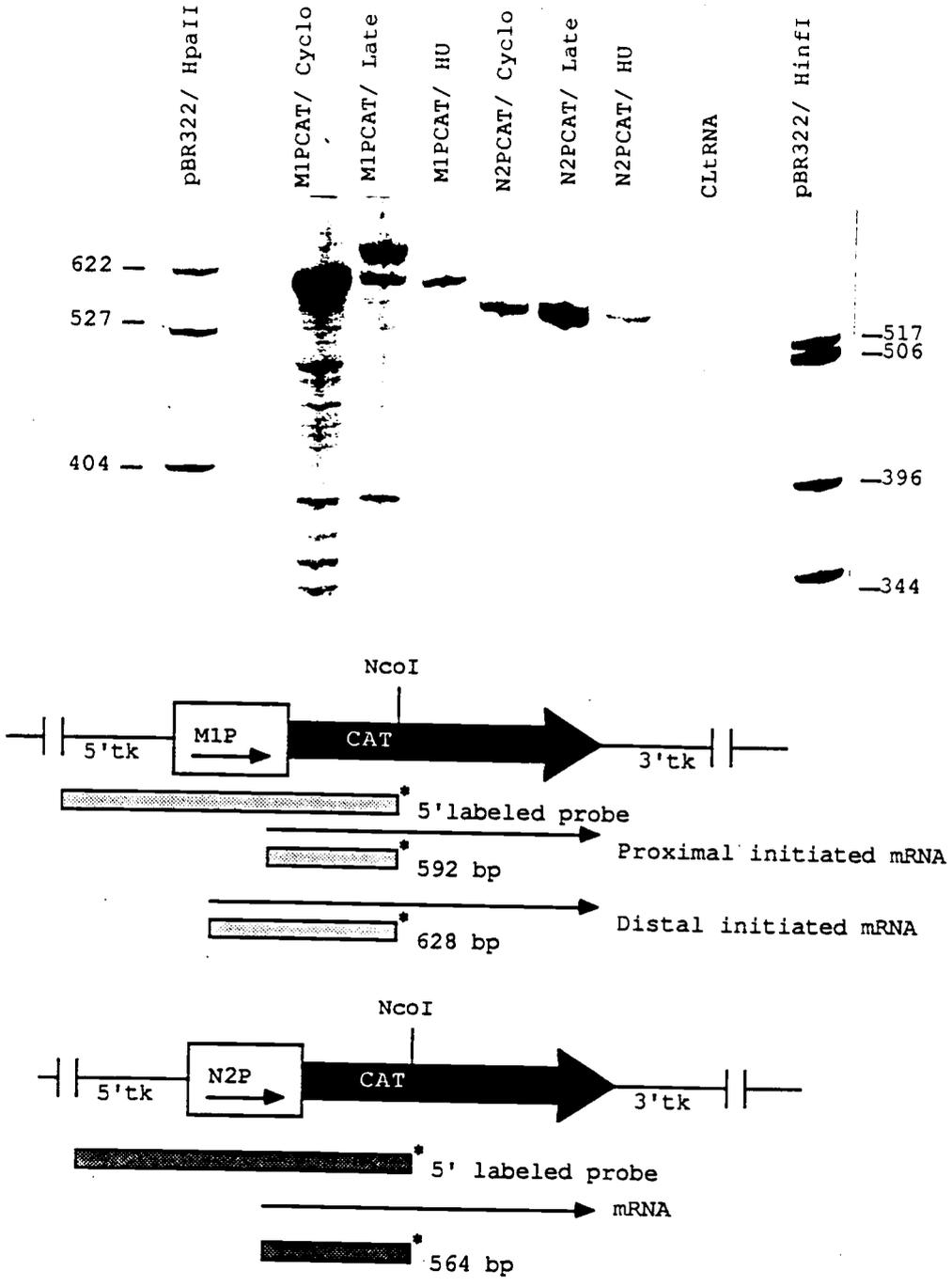


Figure IV.11

determine whether these late CAT transcripts were products of transcription from these TAAAT motif, we carried out primer extension experiments on CAT mRNA using a 5' end labeled CAT specific primer. The results are presented in fig. IV.8. The primer extension data suggest that the majority of the transcripts map to the same site as the S1 signal. The light higher bands probably represent readthrough transcription. The data shows clearly that the late CAT mRNA does not have the predicted poly(A) leader structure and by comparing between early and late primer extension products, it is clear that they have similar 5' ends, which means that either this promoter is unique and functions at late time without poly(A) leader addition, or the RNA is stable.

Alternatively, the structure of CAT transcript would probably give us some clues regarding this altered expression of recombinant N2 promoter. The result of northern blot hybridization analyses shown in fig. IV.9 indicates an interesting regulation of CAT gene expression. There are two populations of CAT mRNAs differing in terms of size, irrespective which promoter it used. From previous 5' S1 protection assay and also primer extension data, we were convinced that each transcript has the same 5' end. We predict that the difference in size ought to be due to a difference in their 3' ends. Therefore we carried out a 3' S1

protection assay. The data shown in fig. IV.10A seem to agree with the prediction.

The larger CAT transcripts are due to termination within tk and the short CAT transcripts terminate within CAT. The location of sequence motif TTTT(N)T [where N is any nucleotide] which has been proposed as an early genes transcriptional termination signal (Yuen et al., 1987) in 3' tk flanking sequence and in the 3' region of the CAT gene is schematically illustrated in fig. IV.10B. This motif appears to be sufficient for termination, even in the context of the procaryotic CAT gene. It is possible that the recombinant N2 promoter actually behaves in an identical manner to the parental N2 ORF with transcription occurring only at early times post infection, but the CAT/tk containing transcript is stable at late times and the authentic N2 ORF transcripts are not. This difference in stability is due probably to the sequence differences between the N2 ORF and the CAT gene. This could be the result of an "instability signal" in the N2 ORF coding sequence, or a "stability" signal in the CAT gene. The lack of a distinct transcript on northern blot argues against the possibility of stable CAT RNA from early times. From these data regarding N2 promoter expression, it is clear that the isolated N2 promoter fragment used in these analyses has the necessary element for promoting expression. However, its

regulation appears to be modified in the context of tk locus abutted to the CAT gene and whether this is due to differential promoter activity or RNA stability remains to be seen.

We are currently testing the stability/instability hypothesis by creating fusions of the N2 and CAT genes, making chimeric RNAs which will be assayed for a particular stability phenotype. Work is also in progress to determine the limits of the 5' upstream regulatory sequence driving the M1 RNA start site(s) [early and late]. Both transient expression and recombinant VV approach will be employed in this investigation. The data obtained in the present investigation gives us an interesting set of observations which undoubtedly will benefit future analyses.

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