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LA THÈSE A ÉTÉ
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BIOCHEMICAL AND GENETIC ANALYSES OF
VACCINIA VIRUS TEMPERATURE SENSITIVE MUTANTS
DEFECTIVE IN ENVELOPE SELF-ASSEMBLY

by

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Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

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London, Ontario, Canada

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ABSTRACT

To elucidate the genetic endowment of vaccinia virus, it is essential to identify the maximum number of biological functions, inter-relate these functions to specific polypeptides and finally to locate them to precise loci on the viral genome. In such studies mutants are vital in illustrating the fundamental biological phenomenon. Recent developments in microanalytical technology have permitted systematic biochemical and genetic analyses of a group of thermo-labile mutants, derived from the IHD-W strain of vaccinia, defective in envelope self-assembly.

The polypeptides of vaccinia virus were separated and analyzed by two-dimensional gel electrophoresis. Following labeling with [³⁵S]-methionine, [³³P]-phosphate, or [³H]-glucosamine, highly purified virions were dissociated and subjected to electrophoresis using either isoelectric focusing or non-equilibrium pH gradients in the first dimension and SDS-polyacrylamide slab gel in the second dimension. By this means at least 111 polypeptides, about 50% of which were basic proteins, were resolved in fluorograms. Authenticity of various single spots was established. This included a glycoprotein of molecular weight 34,000 (34K), a phosphorylated basic protein of 11K, isolated purified surface tubular elements of 58K, two major core polypeptides of 60 and 62K derived from larger precursors after proteolytic cleavage, a precursor polypeptide of 25K known from previous studies with a ts mutant 1085 to undergo cleavage, and an 18K polypeptide which appears in wild-type and ts 1085 infections under permissive conditions.

Another ts mutant (ts 6757) over-produces immature viral envelopes (IE) and thus provided an opportunity to isolate and partially characterize the IE. These IE, like the envelopes of normal immature virions, possess an external layer of spicules. Experimental evidence was obtained by several approaches suggesting that an early 65K polypeptide (p65E) constitutes the spicule. Evidence obtained by peptide mapping clearly differentiated p65E from another 65K polypeptide. Data obtained with an antiserum specific against p65E combined with biochemical and electron-microscopic observations on the development of several ts mutants, including 6757 are consistent with the view that spicules attached onto envelopes function to provide transitory scaffolding. During virion maturation these spicules are replaced by the surface tubular elements.

Among five assembly-defective mutants relegated to group E which mimic closely in morphogenesis the effects of the antibiotic rifampicin, four have previously been shown to be defective in cleavage of one or more polypeptides. The one evincing no cleavage defect (ts 9251) was found to possess a novel EcoRI restriction site. Analysis of spontaneous revertants derived from ts 9251, employing the restriction enzyme and two-dimensional electrophoresis, provided compelling evidence that the ts lesion resides at a single base pair constituting the EcoRI restriction site in the gene which codes for a 37K polypeptide.

A recombination map including 5 group E mutations, a DNA-minus mutation and the locus for rifampicin-resistance, was derived from the data obtained by two and three factor crosses. The group E mutations, though phenotypically identical, were found widely spread on the

genome. The morphogenetic basis of the envelope aberration, like polypeptide composition of the virion, therefore appears to be even more complex than it was thought before.

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LIST OF ABBREVIATIONS

ara-C	arabinosyl cytosine (cytosine-1- β -D-arabinofuraside)
BSA	bovine serum albumin
$^{\circ}$ C	degrees centigrade
cm	centimeters
cpm	counts per minutes
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOC	sodium deoxycholate
EDTA	ethylenediamine-tetracetic acid
FCS	fetal calf serum
g	gravity
hpi	hours post-infection
hr or hrs	hour or hours
IE	immature viral membrane
IF	isoelectric focusing
l	litre
m	molar
mA	milli-amp.
MEM	Eagle's minimum essential medium
min	minutes
ml	milli-litre
mm	millimeter
moi	multiplicity of infection
mRNA	messenger RNA
μ	millimicron

MW	molecular weight
N	nucleus
NEPHGE	nonequilibrium pH gradient electrophoresis
NM	nutrient medium (MEM + 10% FCS + 100ug/ml streptomycin + 100 units/ml penicillin)
p	polypeptide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFU or pfu	plaque forming units
R	rifampicin-resistant or resistance
Rev	revertant
RF	recombination frequency
RNA	ribonucleic acid
rpm	revolutions per minutes
SDS	sodium dodecylsulfate
sec	seconds
STE	surface tubular elements
SNM	solubilizing solution containing 2.3% SDS, 0.5% NP-40 and 5% 2-mercaptoethanol
TEMED	N, N, N', N'-tetramethylethylenediamine
ts	temperature sensitive
Vhr	voltage x hrs
μg	microgram
μl	microlitre

v voltage
 λ lambda
1-D one-dimensional
2-D two-dimensional

If you can see only what light reveals
and hear only what sound announces,
Then in truth you do not see nor do you hear

[Kahlil Gibran in 'Sand and Foam' 1926]

INTRODUCTION

Following the careful chemical analyses of purified elementary bodies by Joklik (1962a,b), it became apparent that poxviruses contain a genome of molecular weight approximating that of the T even bacteriophages. More recent electron microscopic measurements have established the molecular weight of vaccinia virus DNA to be $120-130 \times 10^6$ (Geshelin and Berns, 1974; Cabrera and Esteban, 1978; McCarron et al., 1978). Exhaustive genetic studies have identified over 130 separate functions controlling the biogenesis of T4 bacteriophage (Wood and Revel, 1976), but the genome capacity may be sufficient to code for 300 proteins of average molecular weight (cited in Luria et al., 1978). One might, therefore, anticipate that the DNA of vaccinia also encodes approximately 200-300 functions.

Numerous biochemical and electron microscopic studies have clearly established the complex nature of the poxviruses and their replicative cycles (reviewed in Moss 1974; Dales and Pogo, 1981). In addition to clearly identifiable structural polypeptides such as the surface tubule elements (Stern and Dales, 1976a), the virion core contains a multiplicity of enzymatic activities some of which have been shown to consist of several proteins (Moss, 1979; Dales and Pogo, 1981). During the past decade improvements in one dimension (1-D) polyacrylamide gel electrophoresis have led in time to the resolution of ever more polypeptide bands (Holowczak and Joklik, 1967a; Moss and Salzman, 1968; Sarov and Joklik, 1972, McCrae and Silagyi, 1975), until the most recent demonstration of 55-56 bands, evident in the studies of Stern and Dales (1976a). By analogy with

T4 the poxviruses might, therefore, be expected to contain more polypeptides than is apparent from the number bands evident even in the best 1-D gels.

To resolve the maximum number of virion proteins efforts were turned to the high-resolution separation methods employing a two-dimensional (2-D) system of O'Farrell (1975). As shown with prokaryotes this technique can readily resolve over 1000 proteins (O'Farrell, 1975). When 2-D analysis is applied to eukaryotic cells, it is possible to resolve even more polypeptides (O'Farrell et al., 1977). An additional, highly desirable feature of 2-D analysis concerns the identification of genetically altered or modified polypeptides separable by virtue of minor charge differences during isoelectric focusing in the first dimension (Milman et al., 1976). On this basis Luck and associates (Piperno et al., 1977; Luck et al., 1977), were able to relate the genetic variability in Chlamydomonas flagella to specific structural components of these organelles.

However, poxviruses have not been subjected to a systematic genetic probing that was carried out with bacteriophages of a high degree of complexity (Wood and Revel, 1976). In such studies mutants were vital in elucidating fundamental biological phenomenon related to prokaryotes. Recent developments in microanalytical technology permit a systemic genetic analysis of one of the most complex eukaryotic agents, referred to as poxviruses and often represented by the vaccinia virus.

In order to understand the genetic organization of vaccinia virus, it is essential to identify the maximum number of biological functions, inter-relate these functions to specific polypeptides and

finally to locate them to the precise loci on the viral genome. This thesis encompasses an attempt to illustrate the genetic endowment of a group of thermo-labile mutants defective in envelope self-assembly.

CHAPTER 2

HISTORICAL REVIEW

The vaccinia virus has been classified as an orthopoxvirus in the family Poxviridae, which is characterized by complex morphology, large double stranded DNA and the cytoplasmic site of replication (Mathews, 1979). Poxviruses therefore relocated the functional center of the cell from the nucleus to the cytoplasm (Luria et al., 1978), which may require a large number of biological functions. This ability is reflected in the complexity associated with their structure and replicative cycle, and has broadened the interest in these agents as models of morphogenesis and assembly of organized biological structures, and gene expression in eukaryotic cells (Dales and Pogo, 1981).

Even before the introduction of animal cell culture technology, production of infectious virus particles, also referred to as elementary bodies (EBs), on a large scale was possible, and their etiologic role well established (Ledingham and Aberd, 1931; Craigie, 1932; Craigie and Wishart 1936a,b). Inauguration of tissue culture procedures in poxvirology (Joklik, 1962a,b) tremendously accelerated the pace of research eventually leading into the present day molecular biology of these complex eukaryotic agents. A number of reviews reflecting the status of developments in the biology and biochemistry of poxviruses have been published sporadically (Smadel and Hoagland, 1942; Joklik, 1966, 1968; Fenner, 1968; Woodson, 1968;

McAuslan, 1969; Moss, 1974; Moss, 1979) until the availability of the most recent comprehensive account on the biology of poxviruses (Dales and Pogo, 1981).

2.1 Purification of Virions:

Whole infected cells or tissue culture medium from such cells served as starting material for vaccinia virus purification. Infected cells have either been disrupted by repeated cycles of freezing and thawing (Green et al., 1942; Joklik, 1962b; Moss and Rosenblum, 1973), or by homogenization (Dales and Mosbach, 1968) and ultrasonic treatment (Stern and Dales, 1974). Fluorocarbon, to remove membraneous cellular debris from cell lysates, has been employed by various workers (Eptstein, 1958a,b; Pfau and McCrae, 1963). Differential and density-gradient centrifugation techniques have constituted the major procedures and have been proved highly effective in virion purification. Use of cesium chloride (Planterose et al., 1962), sucrose (Hoagland et al., 1940; Joklik, 1962c; Zwartouw et al. 1962), and potassium tartrate (McCrea et al., 1961; Stern and Dales, 1974; Moss et al., 1975) for density gradient centrifugation has been reported. Dales and Pogo (1981) have reviewed virus purification methodology and have observed that small fragments of adventitiously attached membranes of host origin are not completely removed from the virions, even following several cycles of centrifugation through sucrose gradients, while potassium tartrate gradients have yielded highly purified virus free from extraneous

membrane contaminants (Stern and Dales, 1974; Muller, 1976; Moss et al., 1975). It, therefore, appears necessary to critically evaluate the virus preparations prior to biochemical analysis.

2.2 Morphology and Gross Composition:

Following the purification of EBs, a number of extensive electron microscopic studies (Peters, 1956; Peters and Muller, 1963; Dales, 1963; Westwood et al., 1964); Easterbrook, 1966) revealed the structural complexity of the virion. The genome is enclosed in a core which is surrounded by a membrane to which two lateral bodies are attached. This whole structure is further surrounded by another membrane, called the envelope. Negative staining and freeze-etching studies on intact virions revealed numerous rod-like or tubular structures (Dales, 1962; Medzon and Bauer, 1970). Stern and Dales (1976a) using Esterbrook's (1966) procedure with some modification have isolated these surface tubules in purified form.

The genomic DNA has molecular mass approximating that of the T even bacteriophages (Joklik, 1962a). Recent electron microscopic measurements have established the molecular mass of vaccinia virus DNA to be $120-130 \times 10^6$ (Geshelin and Berns, 1974; Cabrera and Esteban, 1978; McCarron et al., 1978).

2.3 Protein Components of the Virion:

During the past decade improvements in one-dimensional polyacrylamide gel electrophoresis have led in time to the resolution of even more structural polypeptides, starting from 17 (Holowczak and Joklik, 1967a; Moss and Salzman, 1968; Holowczak, 1970; Katz and

Moss, 1970; Sarov and Joklik, 1972; Moss et al., 1973; Obijeski et al., 1973; McCrae and Szilagyi, 1975; Arita and Tagaya, 1977; Payne, 1978); until the demonstration of 55-56 bands evident in the studies of Stern and Dales (1976a). The polypeptide composition of the virus core and outer envelopes, based on SDS-PAGE, is well established (Holowczak and Joklik, 1967; Sarov and Joklik, 1972; Stern and Dales, 1976a). The purified surface tubular elements (STE) have been shown to consist of a single polypeptide of 58K molecular weight (Stern and Dales, 1976a).

Two-dimensional electrophoresis combining isoelectric focusing and SDS-PAGE (O'Farrell, 1975), as modified and applied to poxviruses (Essani and Dales, 1979), has proven even more informative regarding the number, modifications and genetic variability of vaccinia virus induced polypeptides. The vaccinia virions have been shown to contain twice as many polypeptides as resolved heretofore. Taking the limitations of technique into account, it has been suggested the present count must be an underestimate and with further technological improvements additional virion polypeptides will be demonstrated. Clearly more studies are needed to locate the position of structural polypeptides revealed by two-dimensional gels, in the virion.

Post-translational modifications of the viral structural polypeptides have been reported. These include cleavage of at least five polypeptides, phosphorylation and glycosylation. The two precursor-product relationships substantiated beyond doubt, heretofore, include the cleavage of 96K and 65K polypeptides into 62K and 60K polypeptides respectively (Moss and Rosenblum, 1973). The precursor-product relationship of remaining polypeptides has not yet

been established. The two-dimensional gel analysis further revealed that two 65K polypeptides are induced during vaccinia virus infection. They have been termed p65E and p65PC. The former has been found to be associated with immature viral envelopes, while the later has been shown to be the precursor of the 60K polypeptide (Essani et al., 1982). The number of phosphorylated and glycoproteins in the virion varies in different studies. Sarov and Joklik (1972) have reported the existence of 2 glycopolypeptides and 6 phosphorylated polypeptides in the WR strain of vaccinia virus. On the contrary, Garon and Moss (1971), using the same strain of vaccinia, have detected only one glycoprotein. Two-dimensional gel analyses of the IHD-W virion (Essani and Dales, 1979) have demonstrated the existence of only one glycopolypeptide of about the same molecular mass as reported by Garon and Moss (1971). Oie and Ichihashi (1981) have recently characterized the structural polypeptides of the IHD-J strain of vaccinia by O'Farrell's (1975) two-dimensional system, and have claimed that 17 out of a total of 84 polypeptides were glycosylated. Similar discrepancies among various reports are evident with regard to phosphoproteins. Six phosphoproteins have been reported by Sarov and Joklik (1972). Oie and Ichihashi (1981) have found ^{32}P incorporation in 9 spots on 2-dimensional gels, 5 of which coincided with [^{35}S]-methionine-labeled polypeptides. In the course of this investigation one phosphorylated protein has been detected in IHD-W virions. This phosphorylated proteins has about a 11K molecular mass and a basic isoelectric point. An identical phosphoprotein in the WR-strain of vaccinia has been reported by Rosemond and Moss (1973), and in the IHD-W strain of vaccinia by Pogo et al., (1975).

2.4 Enzymatic Activities Associated with the Virion:

In addition to structural polypeptides, the virion core contains a multiplicity of enzymatic activities. These enzymes include a DNA-dependent RNA polymerase (Nevins and Joklik, 1977; Baroudy and Moss, 1979), an endoribonuclease (Paoletti and Lipinkas, 1978), a polyadenylate polymerase (Moss et al., 1973), a polynucleotide 5'-triphosphatase (Tutas and Paoletti, 1977), an mRNA guanylyltransferase (Ensiger et al., 1975), an mRNA methyltransferase (Boone et al., 1977), an mRNA methylase (Barbosa and Moss, 1978), ssDNases (exonuclease and endonuclease) (Pogo and Dales, 1969), a nucleoside triphosphate phosphohydrolase (Gold and Dales, 1968), a DNA nicking-closing enzyme (Bauer et al., 1977), a protein kinase (Downer et al., 1973), a 5'-phosphate-polyribonucleotide kinase (Spencer et al., 1978), and an alkaline protease (Arzoglou, 1979). The significance of these enzymatic activities in the replicative strategy of vaccinia virus and other poxviruses has been extensively evaluated by Dales and Pogo (1981).

2.5 Protein Synthesis in Vaccinia Infected Cells:

Two unique features in the replicative cycle of vaccinia virus have greatly facilitated the biochemical studies related to transcription and translation. These include: (i) the rapid inhibition of host directed macromolecular synthesis (Moss, 1968; Esteban and Metz, 1973) and (ii) the autonomous cytoplasmic site of replication (Dales and Pogo, 1981). Biosynthesis of defined classes of mRNAs in the infected cells have evidently been related to the appearance of defined groups of authentic viral-directed polypeptides

(Bodo et al., 1972; Esteban and Metz, 1973; Baglioni et al., 1978). Some of these polypeptides have been detected either immunologically or by polyacrylamide gel electrophoresis (Loh and Rigs, 1961; Moss and Salzman, 1968; Moss and Katz, 1969; Katz and Moss, 1969; Pennington, 1974). Synthesis of early enzymes and some of the early proteins, in the normal course of infection has been reported to be completely ceased a few hours after infection (McAuslan, 1963; Jungwith and Joklik, 1965; Moss and Salzman, 1968; Esteban and Metz, 1973), while the biosynthesis of some early structural proteins has been shown to decrease gradually (Holowezak and Joklik, 1967b;). Some other proteins like p65E, an early structural protein not associated with the mature virion (Essani et al., 1982), is synthesized even late during the infection cycle. The phenomenon of switch-over from early (synthesized in the absence of DNA synthesis) to late (require DNA synthesis) can be obstructed by blocking DNA synthesis with drugs such as arabinosyl cytosine or hydroxyurea, resulting in prolonged expression of so called early functions (Pennigton, 1974). Although the basic mechanism of this switch-over is yet to be discovered, the existence of a tight transcriptional control has been postulated for the orderly synthesis of virus-coded polypeptides (Dales and Pogo, 1981).

An interesting approach to map the large number of viral coded polypeptides on the genome has been incepted by Moss and coworkers (Cooper and Moss, 1978; 1979b; Wittek et al., 1980; Isle et al., 1981). A preliminary translational map of about 50% of the genome has been constructed by hybridizing immediate early, early and late mRNAs to HindIII restriction fragments of vaccinia. The mRNAs thus

selected were translated in a cell-free translational system and approximately 75 early and 40 late polypeptides have been detected. Although the existence of artifacts created by either pre-mature termination or read-through of termination signals has not been ruled out in such a system, it seems to be an important step towards the mapping of polypeptides on the vaccinia genome (Isle et al., 1981)

2.6 Assembly and Morphogenesis:

The emergence of both early and late categories of virus-specified polypeptide and the chronology of the appearance of various morphological ultrastructures in the cytoplasm of infected cells can be inter-related (Moss and Salzman, 1968; Pennington, 1974; Essani et al., 1982). The assembly of mature virion occurs in the specific foci, termed "viroplasm" or "factories", located in the cytoplasm. Numerous coordinated and extensive structural, biochemical and genetic studies have contributed to the present understanding of the sequence of events in the process of morphogenesis (Moss, 1974; Dales and Pögo, 1981).

Viral envelopes are among the first recognizable structure to appear within the viroplasm (Morgan et al., 1954; Dales, 1963). These envelopes surround the immature virus particles, which then develop into mature particles. The availability of temperature sensitive mutants defective in various stages of morphogenesis (Dales et al., 1978) has significantly contributed to the understanding of the assembly process as a whole, and in some cases revealed various interdependent and integrative steps between major assembly events which otherwise are rarely captured by electron microscopy. For

example, removal of the spicule layer is an intermediate event during the conversion of the immature viral envelope into a mature viral envelope and has been visualized for the first time (Essani et al., 1982) during this study. Use of drugs to inhibit transcription, translation or DNA synthesis has been proved highly useful.

Employing arabinosyl cytosine, Dales and Mosbach (1968), Nagayama et al., (1970) and Grimley et al., (1970) have shown that the majority of polypeptides constituting immature viral envelopes are early functions which in turn has added to positive identification of the spicule polypeptide present in isolated immature viral envelopes and absent in envelopes stripped from mature virions (Essani et al., 1982). A variety of independent observations (Dales and Mosbach, 1968; Nagayama et al., 1971; Pogo and Dales, 1971; Dales et al., 1978; Essani et al., 1982) suggest that spicules attached onto immature viral envelopes function to provide a transitory scaffolding, or exoskeleton to provide the curvature required to generate a spherical envelope. Such observations include: (i) when two spicule-backed envelope segments become closely apposed by chance, the tendency to adopt curvature is counteracted (Dales and Pogo, 1981); (ii) immature viral envelopes lack spicules as a result of the antibiotic rifampicin action or in the case of group E mutants (Dales et al., 1978) envelopes display flexibility and pleomorphism (Dales et al., 1978; Lake et al., 1979). Using a conditional lethal mutant, it was possible to identify the constitutive polypeptide(s) of these spicules (Essani et al., 1982).

The next step in late maturation includes the removal of the spicule layer, allowing mature virus to assume a brick-shape appearance, and the addition of surface tubular elements (Stern and Dales, 1976a) replacing the spicules. At approximately the same time internal differentiation occurs so that the core and lateral bodies are distinguished (Stern and Dales, 1976b). The polypeptide composition and the biological function associated with lateral bodies is not known. However, Oie and Ichihashi (1981) on the basis of a circumstantial evidence, have suggested that a glycoprotein of 34-37000 molecular mass is a candidate for the composition of lateral bodies.

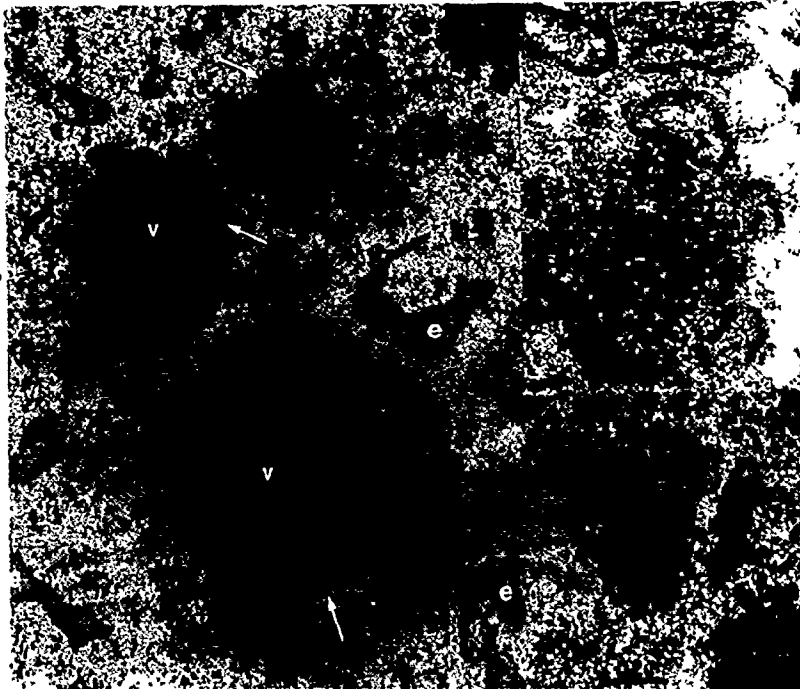
2.7 Genetics:

The large genome size and lack of genetically stable markers in the past has apparently been responsible for the slow development in poxvirus genetics. Restriction enzymes Hind III and Sma I maps of vaccinia and rabbit poxviruses (Wittek et al., 1977), R-loop mapping with vaccinia virus ts mutants (McFadden et al., 1980, Schumperli et al., 1980) two dimensional electrophoresis to monitor the products of such mutations (Essani and Dales, 1979, McFadden et al., 1980) and marker rescue of poxvirus thimosensitive mutants are among few important recent developments, which with technological improvement will gradually increase the pace of genetic studies with poxviruses, and seem to have a great potential to explore the organization of the vaccinia genome. Recombination and complementation analyses to determine the number of genes involved in the expression of a particular phenotype and their location on the viral chromosome have

been of great importance in the construction of genetic maps of various bacteriophages and have been applied to poxviruses (Padgett and Tompkins, 1968; Chernos et al., 1978; Lake et al., 1978; Condit and Motyczka, 1981). However, poxviruses have not been subjected to the extensive genetic analyses that was carried out with T even bacteriophages (Dales, Pogo, 1981) and no comprehensive genetic map is yet available.

The structural and functional complexity associated with the replicative cycle, morphogenetic pathway and virion itself (Moss, 1979; Dales and Pogo, 1981), together with a tightly regulated gene expression mechanism makes vaccinia an ideal model to understand the genetic and functional organization of the eukaryotic chromosome. A number of laboratories have been able to isolate a wide variety of ts mutants which are defective in assembly and maturation (Subak-Sharpe et al., 1969a,b; Nagayama et al., 1970; Dales et al., 1978; Condit and Motyczka, 1981). Using N, N', dimethylnitrosoguanidine as mutagen, Dales and coworkers (1978) were able to isolate a large number of ts mutants. Ninety of which have been grouped into 17 categories according to a scheme of ascending progression in maturation during the course of infection at restrictive temperature. Some of these mutants are defective in post-translational cleavage of major precursor polypeptides, mimicking the effect caused by the antibiotic rifampicin (Stern et al., 1977; Lake et al., 1979). These mutants have been classified in group "E" (Dales et al., 1978). The morphological defect in group E mutants is illustrated in Figure 1. The mechanism of rifampicin action in eukaryotic systems is not well understood, and therefore apart from the significance of these

Figure 1. Portions of the cytoplasm of a cell infected by ts 1085. Several areas of dens viroplasm, each surrounded by membrane sheets or vesicles (arrows) are shown; x49,100. The nature of defective virus membranes is illustrated at higher magnification in the inset; x106,200. e, endoplasmic reticulum; v, viroplasmic matrix. (from Dales et al., 1978, Virology 84: 403-428).



mutants in the process of vaccinia morphogenesis, they may also provide a better insight into the strategies of drug-resistance in poxviruses.

CHAPTER 3

MATERIALS AND METHODS3.1 Chemicals and Isotopes:

[³⁵S]-methionine sp. act. 509-1010 Ci/mmol, [³³P]- phosphate (carrier free), and [³H]-glucosamine 13 Ci/mmol were purchased from New England Nuclear Corporation; acrylamide and bisacrylamide of electrophoretic grade from Eastman; sodium dodecylsulfate (SDS), the ampholytes (pH 5/7 and 3/10), and N, N, N', N'-tetramethylethylenediamine (TEMED) from Bio-Rad Laboratories; ampholytes (pH 2/11) from Brinkmann; Ultrapure grade urea from Schwarz/Mann. Nonidet P-40 (NP-40) was a gift from Shell Oil Company. Restriction endonucleases were purchased from Boehringer Mannheim. The agarose (Seakem) was from Marine Colloids Div., FMC Corporation. Anti-rabbit gamma globulins conjugated with fluorescein isothiocyanate were obtained from Miles Laboratories. All tissue culture media, sera and antibiotics were from Flow Laboratories, Microbiological Associates, and GIBCO. All remaining chemicals and materials were purchased from FISHER unless otherwise indicated. Lambda plac5 DNA was a gift from Dr. George Mackie.

3.2 Viruses:

IHD-W strain of vaccinia, originally derived from IHD-J strain of vaccinia by Hanafusa et al., (1959) was selected for this study. This subtype, unlike its parental IHD-J strain, is characterized by its ability to induce polykaryocytosis in a variety of avian and mammalian cell cultures (Dales, 1963; Weintraub and Dales, 1974). The

prime reason for selection of this particular strain of vaccinia virus was the availability of a large variety of temperature sensitive (ts) mutants, which have been derived from it. All these mutants were initially obtained by Dales et al., (1978) using N, N'-dimethylnitrosoguanidine as a mutagen, and schematized into 17 categories (A-Q) based on the development stage reached at the restrictive temperature of 40°C. The mutants employed during these studies belong to categories C, F, E, and N. A brief phenotypic description of each mutant has been presented in Table I. The term wild-type (wt), in this study refers to the parental IHD-W strain of vaccinia virus.

3.3 Cells:

L₂ substrain of mouse L 929 fibroblasts (Rothfels et al., 1959) was used for most purposes during this investigation. Occasionally, HeLa cells were employed, as indicated. The nutrient medium (NM) consisted of Eagle's (1959) minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin.

3.4 Propagation of Viruses:

L₂ cell monolayers were infected with 1 plaque forming unit (pfu)/cell. The virus was allowed to adsorb for 1 hour (hr.) at 4°C, unadsorbed virus was removed by washing the monolayers with nutrient medium, fresh NM was added and virus was allowed to replicate at the appropriate temperature in an incubator with 5% CO₂, for 18-24 hrs. Infected cells were scraped with a rubber policeman and centrifuged at

Table I

Selected temperature sensitive mutants^a and their phenotypic characterization^b.

Category	Description	No.	Plaque ^c	
			Type	Relative ^d PFU/ml
			33°/40°	33°/40°
C	Rudimentary virus membranes with spicules in foci of viroplasm and defective in DNA synthesis.	6389	S/VS	2,100:1
F	Aberrant membranes with spicules and viroplasm.	6757	S/VS	7,000:1
E	Foci of viroplasm, aberrant membranes without spicules and DNA paracrystals.	1085	S/S	100:1
		7743	M/VS	100,000:1
		9203	S/S	1,800:1
		9251	S/VS	81,000:1
		9383	S/VS	100,000:1

a, derived from IHD-W strain of vaccinia virus.

b, adopted from Dales *et al.*, 1978.

c, diameter observed at 48 hpi under overlay medium.

d, based on two or more independent titrations.

M, medium (0.5-1.0 mm).

S, small (0.2-0.4 mm).

VS, very small (<0.1 mm).

160g for 10 minutes. The pellet was resuspended in nutrient medium diluted 1:1 by sterile deionized water, kept on ice for 30 minutes to swell the cells, and homogenized in a tightly fitted Dounce homogenizer. The rupture of cells was monitored by a microscope equipped with phase contrast optics. Usually 20-30 strokes were sufficient to rupture about 90% of the infected cells. Large cytoplasmic debris and nuclei were removed by centrifugation at 400g for 5 minutes. The supernatant was pooled with NM recovered from infected cells and centrifuged at 2000g for 30 min in a Sorval high speed centrifuge using a HB-4 rotor. The supernatant was centrifuged at 50,000g for 60 min. The pellet obtained was resuspended in a small volume of NM and considered as crude virus preparation, for further infections. The crude virus was stored frozen at -70°C and was sonicated for 30 seconds in sonicator (type Sonicup SE 101, Sorensen, Zurich) to break the clumps of virus particles just before its titration.

3.5 Titration of Viruses:

The sonicated crude virus preparation was serially diluted 10 fold in NM, and 0.2 ml of each dilution was inoculated in duplicate into a multidish (Falcon Plastics) well containing 1×10^6 cells in monolayer. The multidish was placed onto a slow shaker and the virus was allowed to adsorb at 4°C for 1 hr. Unadsorbed virus was removed, monolayers were washed twice with NM and 3.0 ml of overlay medium consisting of Eagle's MEM supplemented with 10% fetal bovine serum and 0.5% methyl cellulose (4000 cps) was added. The inoculated cultures were incubated at appropriate temperatures in a CO_2 -incubator, for 36

hours. Following incubation the overlay medium was removed carefully and 2.0 ml of fixing-staining solution containing 10% formaldehyde and 0.1% crystal violet was added and monolayers were allowed to stain for 30 minutes. The fixing-staining solution was then removed and the monolayers were washed in running tap water and dried. The plaques were counted using an illuminated magnifying glass.

3.6 Isotopic Labelling and Virus Purification:

Highly purified virus was isolated from cells infected at a multiplicity of 5 pfu/cell. Cells were infected and crude virus was prepared as described earlier. Virus purification was according to the methods of Stern and Dales (1974 and 1976a) with some minor modifications. The crude virus preparation was centrifuged through 2 ml. of 1.46 M sucrose solution prepared in 10 mM Tris-HCl (pH 8.1) in a Beckman ultracentrifuge using an SW-56 rotor for 1 hr. at 50,000g. The pellet was collected, resuspended in a small volume of Tris-HCl (10 mM, pH 8.1), sonicated for 30 sec. and subjected to gradient centrifugation through a 20-60% sucrose solution prepared in 10 mM Tris-HCl (pH 8.1), using an SW-56 rotor at 10,000 x g for 60 min. The virus band was collected, diluted in Tris-HCl and centrifuged in an SW-65 rotor at 50,000 g for 1 hr. The resulting pellet was resuspended and centrifuged through a 20-50% potassium tartrate solution prepared in Tris-HCl (10 mM, pH 8.1). The virus band was collected, diluted in Tris-HCl, and centrifuged to pellet the virus. The purity of each virus preparation was confirmed by electron-microscopy and slab gel electrophoresis as published earlier (Essani and Dales, 1979).

For labelling the pure virus with [^{35}S]-methionine, the isotope was added either soon after adsorption or at 2 hours post infection (hpi). In this case the infected cells were starved for 1 hr. in methionine free medium (MFM) consisting of Eagle's MEM devoid of methionine. The MFM was then replaced with MFM supplemented with 20 $\mu\text{Ci/ml}$ of [^{35}S]-methionine and 0.5 mM of cold methionine. Virus replication proceeded for 36 hrs. at 37°C. Vaccinia virus labelled with ^{33}P was prepared according to the method described by Pogo et al., (1975) using phosphate free medium supplemented with 10 $\mu\text{Ci/ml}$ of [^{33}P]-phosphate and 2% dialyzed fetal calf serum. For growing [^3H]-glucosamine labelled virus MEM was supplemented with 15 $\mu\text{Ci/ml}$ of [^3H]-glucosamine.

3.7. Purification of Surface Tubular Elements (STE):

STE were isolated and purified according to Stern and Dales (1976a). Briefly, a suspension of purified vaccinia virus in 10 mM Tris-HCl (pH 7.3) labelled with [^{35}S]-methionine was adjusted to contain 1% NP-40 and 1 mg/ml viral protein, as determined by the procedure of Lowry et al., (1951). The virus-detergent mixture was incubated in a water bath at 37°C for 15 min. Following incubation 0.01 ml of freshly prepared 1.22 M 2-mercaptoethanol was added to each 0.1 ml of the mixture, which was again incubated at 37°C for 10 min. Treated virus was carefully layered over 0.5 ml of 1.17 M sucrose solution in a 0.8 ml capacity tube and centrifuged at 100,000g for 15 min. using an SW 65 rotor in a Beckman ultracentrifuge. The material above the sucrose cushion and at the interface was collected, diluted

with 5 ml. of deionized water and again centrifuged at 100,000g for 18 hrs. The resulting pellet was monitored by electron microscopy and slab gel electrophoresis for purity.

3.8 Isolation of Mature Virus Envelope:

To analyze the polypeptides in the envelope of mature virion, the [³⁵S]-methionine labelled virus was treated in an identical manner as described above with the following exception. The material above the sucrose cushion and at the interface was collected and 1 vol. of SNM buffer was added to solubilize the envelope polypeptides for electrophoretic purpose.

3.9 Isolation of Immature Viral Envelopes (IE):

Monolayers of HeLa cells in 60 mm plastic dishes (Falcon Plastics) were infected with 20 pfu/cell of ts 6757 and the virus was allowed to replicate at the non-permissive temperature of 40°C for 16-18 hrs. All procedures for isolating envelopes were carried out at 4°C. The infected cells were released by scraping, collected into pellets by centrifugation at 160 x g for 10 min, washed at least twice with NM without serum, then resuspended to bring about swelling in 1 part MEM and 7 parts of deionized water, adjusted to a volume of $1 \text{ ml}/2 \times 10^6$ cells. Swelling was allowed to proceed on ice for 25-30 min prior to homogenization, using 20-30 strokes of a tight-fitting Dounce homogenizer. Cell rupture was monitored under phase-contrast optics. After removing nuclei by centrifugation at 400 x g for 5 min, large cytoplasmic particulates in the supernatant were sedimented at 2000 x g for 35 min in a Sorval centrifuge using the HB-4 rotor.

Finally, the smaller particulates in the supernatant were sedimented at 65,000 x g for 1 hr. in a Ti-60 rotor of a Beckman ultracentrifuge. The resulting pellets, resuspended in 0.5 ml MEM, contained free viral envelopes as well as membraneous cell components including mitochondrial fragments and elements of the endoplasmic reticulum. The material, designated immature envelope (IE) preparation was used for immunization.

IMMUNOLOGICAL METHODS

3.10 Preparation of Antisera to the IE Fraction:

The IE, mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories) was injected intracutaneously into male albino rabbits at multiple sites. Booster injections of IE were given 4 times at weekly intervals. The anti-IE serum was collected, inactivated at 56°C for 30 min and adsorbed extensively with acetone powder made from L₂ cells, to remove the bulk of host directed immunoglobulins. It was stored at -70°C and after thawing was centrifuged at 4000 x g for 30 min, prior to use.

3.11 Further Concentration of Immature Viral Envelopes by Means of Immunoagglutination:

The IE material was obtained from L₂ cells by a modification of the above procedure. Initially, viral proteins were labelled by incorporating into NM [³⁵S]-methionine (50 µCi/ml) as described earlier. Isotope was present commencing 10 hpi until the termination of the experiment at 16 hrs. After cell disruption, removal of nuclei

and large cytoplasmic particulates, each 0.5 ml of IE preparation was mixed with 30 μ l of anti-IE serum, incubated at 37°C for 60 min. in a water bath and allowed to stand overnight at 4°C. The agglutinates formed were sedimented at 1500 x g for 30 min. As the control identical samples were prepared with pre-immune serum. An aliquot of immunoagglutinate was used for electron microscopy and the remainder solubilized in a solution 2.3% SDS, 0.5% Nonidet NP-40, 5% 2mercaptoethanol and 10% glycerol in deionized water (SNM buffer), in preparation for electrophoresis.

3.12 Antisera to 65K Polypeptides:

The molecular mass (MW) of individual polypeptides is designated as follows: 10,000 as 10K, 100,000 as 100K etc. Areas from 1 or 2-D gels where 65K polypeptides appeared were excised, collected in 5 ml plastic syringes and mixed with 1.5 ml 0.85% NaCl solution to rehydrate the gel pieces. Then the gel material was homogenized, mixed with an equal volume of Freund's complete adjuvant for injection as described above. Material for six weekly booster injections was isolated as the 65K bands from the preparative 1-D SDS-polyacrylamide gels or from 65K spots in the 2-D gels. In the latter case spots from 15-20 gels were pooled to provide sufficient antigen for each injection. Sera were collected from the rabbits one week after the last inoculation and designated as anti-envelope 65K sera, anti-p65E (1-D) and (2-D) respectively. Following heating at 56°C for 30 min and storage at -70°C, the sera were centrifuged for 30 min, prior to use as described earlier.

3.13 Immunoprecipitation of Solubilized Envelopes:

Monolayers of L₂ cells were infected, labelled with [³⁵S]-methionine and IE isolated as described above. Material in pellets containing IE was solubilized in immunoprecipitation buffer (10 mM Na₂HPO₄, pH 7.4, 0.15 M NaCl, 1% DCC, 1% Triton X-100) by heating to 100°C for 3 min. The treated samples were centrifuged at 100,000 x g for 10 min and an aliquot from each resulting pellet and supernatant fraction was taken for determination of radioactivity (cpm). Each 50 µl sample of supernatant was mixed with 10 µl of antiserum, incubated at 37°C for 60 min, then kept at 4° overnight. In some cases, the size of agglutinates was increased by addition of killed Staphylococcus aureus cells. The resulting immunoprecipitates were collected by centrifugation at 12,000 x g for 30 min, in an Eppendorf microcentrifuge and the pellet obtained was dissolved in 20-30 µl of SNM buffer, prior to separation of virus-specified polypeptides by means of 1- or 2-D gel Electrophoresis systems.

CYTOLOGICAL METHODS

3.14 Localization of Cell Associated Antigens by Fluorescent Antibody Staining:

Coverslip cultures of cells were infected for 18 hrs. at permissive (33°C) or restrictive temperatures (40°C) with either ts-6757 or IHD-W wild type vaccinia virus at a multiplicity of 10 pfu/cell. After fixation at 0°C for 10 min. with freshly prepared 4% (W/V) formaldehyde buffered with PBS (0.015 M sodium phosphate, 0.15 M

sodium chloride, pH 7.3), cultures were exposed for 20-30 sec. to 95% ethanol precooled to -20°C , and washed 3 times with PBS (Herman and Pollard, 1981). Then cultures were inundated with diluted antiserum, or pre-immune serum, incubated for 30 min at 37°C , extensively washed 3-5 times and reacted with diluted fluorescein conjugates for 30 min. Following further extensive washings, the coverslips were mounted in 10% glycerol.

3.15 Electron Microscopy:

Preparation of materials for electron microscopy of intact virus particles, surface tubular elements (STE), immunoagglutinates and infected cells, was conducted as described by Dales (1962). Briefly, for negative staining purposes 2% phosphotungstic acid prepared in PBS (pH 7.2) was used. Infected cells were fixed in situ with 2% glutaraldehyde in PBS (pH 7.2), post fixed with 1% osmium tetroxide and washed with PBS prior to dehydration with graded ethanol solutions. Final dehydration was achieved with 2 washes of 100% ethanol, followed by embedding in epoxy resin (Epon). The sedimented cells and immunoagglutinates were processed in a similar manner with the exception that final dehydration was achieved in propylene oxide, followed by embedding in epoxy resin (Epon). The representative samples were thin sectioned (60-100 nm) and stained with uranyl acetate followed by Reynold's (1963) lead citrate. The Philips EM 300 operated at 80 KV was used for observations.

BIOCHEMICAL METHODS

3.16 Inhibitors of Vaccinia Virus Replication:

1- β -D-Arabinofuranosylcytosine (ara-C from Sigma), an inhibitor of DNA synthesis which block the replication of DNA viruses (Ch'ien et al., 1973; Becker, 1976; McFadden and Dales, 1980) was used whenever necessary. The drug was solubilized in the NM at 20 μ g/ml concentration and was added to the infected cultures immediately after adsorption of the virus at 4°C. Rifampicin (Sigma), an antibiotic which blocks vaccinia virus maturation by interfering with the post-translational cleavage (Nagayama et al., 1970), was used in a similar way at a final concentration of 50-100 μ g/ml as indicated.

3.17 Extraction of DNA:

DNA was extracted from purified virus, ts 9251 and its revertants according to the procedure of McFadden and Dales (1979). L₂ cell monolayers were infected with 10 pfu/cell with each virus independently, and were labelled with 0.1 μ Ci/ml methyl-³H-thymidine soon after adsorption. [³H]-thymidine labelled virus was purified as described earlier. Purified virus was then layered onto 39.0 ml 5-20 sucrose gradient consisting of 2% 2-mercaptoethanol, 1% sodium dodecylsulfate, 100 mM NaCl, 1 mM EDTA and 10 mM Tris-HCl (pH 8.0) and subsequently with the same solution without sucrose. An incubation of 30 min was carried out at room temperature prior to centrifugation for 4-5 hr. at 26,000 rpm at 20°C in an SW-27 rotor. The fractions were collected from the bottom of the tube. Aliquots were monitored for radio-activity, and the fractions containing viral DNA were pooled and

diluted with 10 mM Tris-HCl (pH 8), and 1 mM EDTA. The extracted DNA was precipitated with 2 volumes isopropanol and 1/10 volume of 3 M sodium acetate (pH 5.5) and resuspended in 10 mM Tris-HCl (pH 8.0) at 100 $\mu\text{g}/\text{ml}$ concentration.

3.18. Restriction Enzyme Digestion:

The reaction mixture in 30 μl volume contained 5 μg viral DNA in EcoRI buffer (100 mM Tris-HCl pH 7.5, 50 mM NaCl and 10 mM MgCl_2) and 2-3 fold excess of EcoRI enzyme (i.e. 2-3 units of enzyme/1.0 μg DNA). Following incubation at 37°C in a water bath for 3 hrs, 2-3 units of enzyme/ μg of DNA were again added and incubation was continued overnight. The enzymatic digestion of DNA was stopped by adding 15 μl 50% sucrose, 50 mM EDTA, 10 mM Tris-HCl (pH 8.0), and 0.125% bromophenol blue. Digested viral DNA was either loaded onto an agarose gel immediately or stored at 4°C.

3.19 Agarose Gel Electrophoresis:

A horizontal gel apparatus was used for the analysis of restriction enzyme digested viral DNA fragments. Agarose (0.7%) was dissolved in electrophoresis buffer consisting of 40 mM Tris-acetate (pH 8.0), 20 mM sodium acetate and 1 mM EDTA, and autoclaved before pouring. About 2.0 μg of digested DNA was added to each well and electrophoresis was carried out at 50 V for 12 hrs. at room temperature. Following electrophoresis the gels were stained with 1 $\mu\text{g}/\text{ml}$ ethidium bromide and the DNA bands were visualized with a Black-Ray ultraviolet transilluminator (Ultra Violet Products) and photographed through a Kodak No. 23 yellow filter with Kodak high-

contrast copy film. Lambda plac5 DNA was digested with either EcoRI or Hind III and electrophoresed cocomitantly to provide markers (Robinson and Landy, 1977).

3.20 Preparation of Cytoplasmic Extracts for Polypeptide Analyses:

Confluent L₂ or HeLa cell monolayers in 60 mm petri dishes (Falcon Plastics) were infected with 20 pfu/cell of either IHD-W vaccinia or the temperature sensitive mutants derived therefrom. Following adsorption, cultures were incubated at 37°C in case of wild type (wt) or at 40°C with ts mutants in MEM plus 2% fetal bovine serum. At 8 hpi cultures were placed in MFM for 60 min, then for 60 min. in MFM supplemented with 30 µCi/ml of [³⁵S]-methionine. Pulse samples were collected immediately after labelling while chase samples were transferred to chase medium consisting of MEM with 2% fetal bovine serum (Stern et al., 1977) for an additional 8 hr. and were kept at either 40°C or 33°C, depending on the experiment. Comparison mock-infected cells were prepared and processed in an identical manner.

To prepare cytoplasmic extracts, cells were washed once with phosphate buffered saline (PBS) and then removed by scraping into 100 µl of a solution containing 1% NP-40 and 1 mM MgCl₂ in distilled water. The freed cells were placed in a centrifuge tube, kept on ice for 15 min, then agitated on a Vortex mixer to bring about cell rupture. Released nuclei and unruptured cells were removed by centrifugation at 800g for 5 min. Two volumes of supernatant material were mixed with 1 vol of 3x SNM buffer, then processed further in the manner used with virions, described above.

3.21 Preparation of Samples for Electrophoresis:

Several alternative approaches were tested. In all procedures pure virus containing 15-30 μg protein (100,000-300,000 cpm) was centrifuged for 1 hr. at 20,000 rpm into pellets in 0.8 ml tubes using an SW-65 rotor of an L-65 Beckman ultracentrifuge. Dissociation of intact virus was carried out by the following methods:

(1) Virus pellet was placed in lysis buffer (O'Farrell, 1975) and subjected to 6 cycles of freezing and thawing.

(2) Virus was added to lysis buffer containing 0.5% (w/v) SDS according to O'Farrell et al., (1977). After standing for 10 minutes at room temperature, the samples were mixed with an equal volume of lysis buffer containing NP-40 (O'Farrell, 1975).

(3) Virus was dissociated according to the procedures of Ames and Nikaido (1976), in lysis buffer containing different relative amounts of SDS, NP-40, and protein than in (2). Furthermore heating to 70°C was employed.

(4) The virus pellet was resuspended in 10-15 μl of a solution containing 1% NP-40 and 1 mM MgCl_2 . To this was added 5-7 μl dissociation solution consisting of 3.5 M 2-mercaptoethanol, 6.9% SDS, and 30% glycerol (3x SNM buffer). The mixture was either heated to 100°C for 2 min or subjected to six cycles of freezing and thawing or aspirated six times through a 25- μl microsyringe equipped with a fine bore needle to shear the DNA. The preparations were either loaded at once onto isoelectric focusing gels or stored at -70°C. Method (4) has been adopted for routine analyses.

Aspiration through the microsyringe not only reduced the high viscosity of some samples, but appeared to aid in breaking down the virion as an alternative to heating.

Pellets obtained by immunoagglutination were resuspended in 20-30 μ l of deionized water and solubilized by adding equal volumes of 2x SNM buffer. The same samples were used for separation of polypeptides on slab gels using the SDS-PAGE system of Laemmli (1970) and two-dimensional system of O'Farrell (1975) as modified during this study (Essani and Dales, 1979). Pure virions or viral envelopes stripped from purified virions, were included as markers whenever necessary. Autoradiograms from gels were prepared as described later.

3.22 SDS-Polyacrylamide Gel Electrophoresis:

Samples for electrophoresis were prepared as described earlier. Slab gels were prepared as described by Laemmli (1970). The stacking gel consisted of 4.5% acrylamide (acrylamide-bisacrylamide ratio = 30/0.8) in 0.125 M Tris-HCl and 0.1% SDS (pH 6.8). Lower or separating gel contained desired concentration of acrylamide (as indicated in figure legends) in 1.375 M Tris-HCl and 0.1% SDS (pH 8.8). The polymerization of gels was initiated by adding 200 μ l of 10% ammonium persulfate and 30 μ l of N, N, N', N'-tetramethylethylenediamine (TEMED) per gel. The concentration of ammonium persulfate was reduced to 100 μ l and 10 μ l TEMED per gel when gradient gels were poured. A vertical slab gel cell (Bio-Rad Laboratories, Model 200), was used throughout these studies and 22.0 ml of lower gel was poured to prepare each gel, unless otherwise indicated. The running buffer consisted of 0.025 M Tris-base,

0.192 M glycine and 0.1% SDS. A constant current of 30 mA was applied until dye marker reached the bottom of the gel (usually 3-4 hrs.)

Following electrophoresis gels were fixed and stained overnight in a solution consisting of 50% methanol, 7% acetic acid and 0.25% coomassie brilliant blue R, whenever necessary. Destaining of gels was carried out in a solution containing 50% methanol and 7% acetic acid. The gels were then either subjected to fluorography or left in water for 1 hr. before drying on a Whatman filter paper 3 employing a slab gel dryer (Model 224, Bio-Rad Laboratories).

3.23 Two-dimensional Electrophoresis:

Two different procedures, originally described by O'Farrell (1975) and O'Farrell et al., (1977) were employed throughout these studies with some modifications (Essani and Dales, 1979). Glass tubes (130 x 2.5 mm inside diameter) were soaked in nitric acid overnight, followed by 3 washings with hot running water and 3 washings with deionized water. Washed tubes were then dipped in 0.01% (v/v) column coat solution (Miles Laboratories) prepared in deionized water and finally washed with deionized water once prior to drying in an oven at 120°C. The bottom of the tubes was sealed with parafilm. Isoelectric focusing (IF) gel mixture was composed of 9.5 M ultra-pure urea, 4% acrylamide (acrylamide-bisacrylamide 28.38/1.62), 2% (v/v) Nonidet P-40, 2% ampholytes (pH 2/11:pH 5/7 = 2:3 or otherwise indicated). Urea was dissolved by swirling the flask and to each 5.0 ml of gel mixture 10 µl of 10% ammonium persulfate was added and the mixture was degassed under vacuum for

about 2 min. Then 7 μ l TEMED was added and the gel mixture was poured into tubes, overlaid with 8 M urea and allowed to polymerize for 1-2 hrs. The overlay solution was then replaced by lysis buffer consisting of 9.5 M urea, 2% NP-40, 2% ampholytes (1.6% pH 5/7 and 0.4% pH 3/10) and 5% 2-mercaptoethanol. The gels were allowed to set for 2 more hours, after which the parafilm was removed and gels were placed in a standard tube gel electrophoresis chamber. The lysis buffer was then removed, samples were loaded and overlaid with sample overlay solution (8 M urea, 1% ampholytes (0.8% pH 5/7 and 0.2% pH 3/10)). Tubes were carefully filled with 0.02 M NaOH which was degassed extensively. The upper chamber of the electrophoresis apparatus was filled with 0.02 M NaOH and the lower chamber was filled with 0.01 M H_3PO_4 . The IF gels were then run at 200 V for 5 min, at 300 V for 18 hrs., and finally the voltage was increased to 400 for 90 min. Once the run was complete, gels were removed from the tubes by means of a 5 ml syringe connected with small Tygon tubing. The IF gels were either stored at $-20^\circ C$ in SDS sample buffer consisting of 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, and 0.0625 M Tris-HCl pH 6.8, or equilibrated at $37^\circ C$ for 60 min prior to loading onto SDS slab gels. Once the IF gel was transferred to slab gel, about 2.0 ml of melted agarose solution (held at about $50^\circ C$) containing 1% (w/v) agarose in SDS sample buffer was poured into the notch in such a way that no air bubbles were trapped between IF gel and the SDS-slab gel. About 5-10 min were allowed to permit the agarose to solidify prior to electrophoresis at 25 mA constant current. About 0.1 ml of 0.1% bromophenol blue was

added to the upper reservoir of electrophoresis apparatus containing running buffer. The run was stopped when the dye front reached the bottom of the gel (about 3 - 4 hrs.).

3.24 Measurement of pH gradient:

The isoelectric gel was cut into 5 mm sections. Each section was placed in a tube containing 2 ml of freshly prepared 9.2 M urea in degassed deionized water which was then shaken for 15 min. at room temperature and the pH was measured on a pH meter. In latter experiments a microelectrode (Ingold Electrode Inc.) was used to measure the pH directly from the IF gel.

3.25 Peptide Mapping:

For a comparison of the peptide composition of two 65K polypeptides, one being p65E and the other the core precursor (p65PC) the relevant spots were separated by 2-D PAGE, excised from dried gels and subjected to peptide mapping using the limited proteolysis technique of Cleveland et al., (1977), which basically employs Laemmli's (1970) SDS-polyacrylamide gel electrophoresis as described earlier. The enzymes (as indicated) were solubilized in 1x running buffer containing 10% glycerol at a final concentration of 20 $\mu\text{g/ml}$. About 30-40 μl of the enzyme solution was directly added to the gel slice placed in a well, 30 min. prior to the electrophoresis.

3.26 Fluorography and Autoradiography:

To amplify latent image formation in autoradiograms, destained gels were dehydrated in dimethylsulfoxide (DMSO) and were impregnated in 20% (w/w) PPO in DMSO for 3 hrs. Following impregnation, the gels were soaked in water for 60 min, dried and exposed to Kodak X-Omate (RP/R) films at -70°C according to Bonner and Laskey (1974) and Laskey and Mills (1975).

GENETICS

3.26 Isolation of Rifampicin-resistant IHD-W: Confluent L_2 cell monolayers in 35 mm plastic dishes (Falcon Plastics) containing about 1×10^6 cells were infected with a multiplicity of infection (moi) of 1 pfu/cell. The virus was allowed to adsorb at 4°C for 1 hr., residual unadsorbed virus was removed by repeated washings with MEM without fetal bovine serum and NM containing $100 \mu\text{g/ml}$ of rifampicin (Sigma) was added. The infected cells were incubated at 37°C until some areas of limited cells fusion were observed. Well isolated plaques were picked by means of a Pasteur pipet and placed in a tube containing 1ml of NM. This rifampicin-resistant virus was enriched in the absence of rifampicin, and re-plaque purified prior to titration in the presence and absence of rifampicin. One such isolate demonstrating similar titers under conditions where rifampicin was either present or absent (wtR), was selected to generate recombinant mutants which retained temperature-sensitivity and were endowed with rifampicin resistance (tsR).

3.28 Isolation of ts Mutants Resistant to Rifampicin:

Confluent L₂ cell monolayers in 35 mm plastic dishes were infected with an moi of 5 pfu/cell of both IHD-W resistant to rifampicin (wtR) and one of the six ts mutants, as described earlier. The viruses were allowed to replicate at permissive temperature of 33°C for 18 hrs., following which infected cells were disrupted by 2-3 cycles of rapid freezing and thawing, and resulting recombinants (ts R) were plaque purified as described earlier. Each such isolate was screened for temperature sensitivity and resistance to rifampicin as described previously. Multiple recombinant isolates were obtained from each of the ts mutants.

3.29 Recombination Analyses:

Both two and three factor cross experiments were carried out in 35 mm plastic dishes. Cells were either mixedly infected with 10 pfu/cell of each of two mutants or singly infected with 10 pfu/cell of each ts mutant in case of two factor crosses, or recombinant (ts R) in case of 3 factor crosses as described earlier (Lake et al., 1979). Every possible cross in 2 factor crosses and a number of 3 factor crosses were carried out. A few crosses involving two double mutants or a double mutant and ts⁺ were also analyzed.

Viruses were adsorbed for 1 hr at 4°C, unadsorbed virus was removed by washing the infected monolayers, and 2.5 ml NM was added to each dish. Incubation was carried out at permissive temperature for different time intervals as indicated, or routinely for 18 hrs. The resulting virus was released by 3 rapid cycles of freezing and thawing and brief (30 sec) ultra-sonic treatment. The resulting

virus yields were titrated at 33°C and 40°C with or without 50 µg/ml of rifampicin as previously described (Lake *et al.*, 1979). Per cent recombination frequency (RF) was calculated by the following formula:

$$\text{Percentage RF} = \left\{ \frac{(A \times B)^{40}}{(A \times B)^{33}} - \left[\frac{A^{40}}{A^{33}} + \frac{B^{40}}{B^{33}} \right] \right\} \times 100 \times 2,$$

where A and B are mutants, $(A \times B)^{33}$ is the yield of mixed infection titrated at 33°C, and $(A \times B)^{40}$ is the yield titrated at 40°C; A^{33} , B^{33} , A^{40} and B^{40} are the single infection yields at 33°C or 40°C as indicated. The \underline{ts}^+ RF representing total \underline{ts}^+ was ascertained without regard to rifampicin selection and includes both \underline{ts}^+R^+ and \underline{ts}^+R^- recombinants. The \underline{ts}^+R % RF values measure the distance between the \underline{ts} defect of $\underline{ts}R$ and the rifampicin locus (R) and was used to calculate the position of R locus, as described by Lake *et al.*, (1979). Each cross was repeated 3-5 times under identical experimental protocol and average recombination values were used to plot the genetic maps.

3.30 Isolation of Revertants:

The double mutant (\underline{ts} 7743RIII) which possessed two different markers, namely temperature sensitivity and rifampicin resistance was used to generate revertants. Semi-confluent monolayers in 100 mm petri dishes were inoculated with 1 pfu/cell as described earlier. Following 3-4 days of incubation at 40°C, well isolated plaques were picked, plaque purified, and screened for rifampicin resistance. Revertants from \underline{ts} 9251 were obtained using a similar protocol.

3.31 Statistical Analysis:

The %RF values were transformed into arcsine values prior to analysis of variance. Analysis of variance of complete block design was performed on the data set using the formulae outlined in Steel and Torrie (1960). Briefly, the variability in total data set was partitioned into variability due to treatments (moi and hpi), blocks and residual (error). Subsequently, F ratios were computed between treatment and residuals. The significance of F ratio was checked in the table of F distribution. Least significant difference (LSD) was calculated at an arbitrary probability level of 0.05.

CHAPTER 4

RESULTS

4.1 Comparison of Results Obtained Using Different Methods for

Preparing Samples:

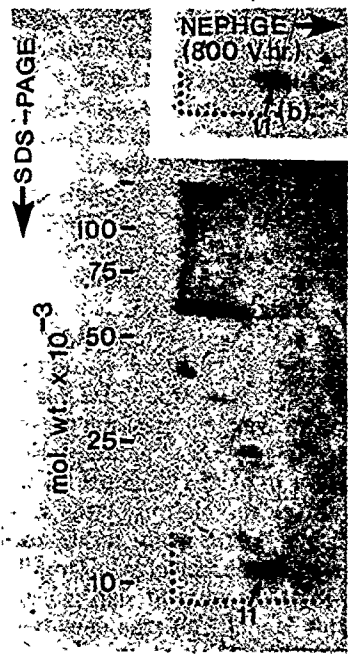
The optimum solubilization of polypeptides is a prerequisite for their detection and characterization in polyacrylamide gel electrophoretic systems. Vaccinia virus has been dissociated into a maximum of thirty polypeptides using SDS, 2-mercaptoethanol and urea in continuous phosphate buffer electrophoretic system (Holowczak and Joklik, 1967a; Holowczak, 1970; Katz and Moss, 1970; Sarov and Joklik, 1972; Moss et al., 1973). With the introduction of a discontinuous buffer system in polyacrylamide gel electrophoresis (Laemmli, 1970), the number of structural polypeptides almost doubled resolving upto 56 polypeptides, even though the urea in the solubilizing buffer was omitted (Obijeski et al., 1973; McCrae and Szilagyi, 1975; Stern and Dales, 1976a; Arita and Tagoya, 1977; Payne, 1978).

In exploring different approaches to solubilize vaccinia virions the aim was to achieve optimum separation of individual polypeptides while avoiding alterations in the isoelectric points of the proteins. When virus pellets were dissolved in lysis buffer containing 9.5 M urea, 2% (w/v) NP-40, and 5% (v/v) 2-mercaptoethanol plus 2% ampholine (pH 2/11), using six cycles of freezing and thawing to enhance the breakdown, several identifiable polypeptides, including two major core proteins 60 and 62K and less prominent ones of high molecular weight as well as the 58K STE component all remained at the origin of the IF

gels, causing them to migrate along the edge on the cathodal side of the second dimension SDS-slab gels. Use of increased or decreased amounts of protein in the samples ranging from 10-100 μ g did not improve the resolution. If freezing and thawing to improve dissociation of the virus material were followed by centrifugation at 100,000g for 60 min, according to Ames and Nikaido (1976), about 20-30% of the radioactive material could be sedimented into the pellets, indicating that separation into individual polypeptides was not complete under these conditions. Nor did the enzymatic hydrolysis of DNA from ruptured virus with pancreatic DNase improve the separation patterns. Heating the samples in dissociating buffer solution caused the formation of artifactual multiple spots in the second dimension slab gels, as shown previously (Wilson et al., 1977).

In the second procedure, the virus pellets containing 15-26 μ g of protein were dissolved in lysis buffer containing 0.5% SDS (O'Farrell et al., 1977). In this case satisfactory separation of basic proteins was obtained if samples were loaded at the anode, and nonequilibrium pH gradient electrophoresis (NEPHGE) was carried out for 2 hr at 400 V (800 Vhr.). As shown in Fig. 2 this made possible the identification of one major virion polypeptide, known from previous studies to be an arginine and lysine-rich phosphoprotein of 11K (Pogo et al., 1975). By extending the time of NEPHGE to 4-5 hr for a total of 1200-1500 Vhr, the resolution deteriorated. More prolonged electrophoresis for 12-13 hr, so as to establish an equilibrated pH gradient but of reversed polarity, also failed to improve the separation of spots. Under these circumstances the two core polypeptides 60 and 62K became distributed in the IF gel so as to

FIGURE 2: Autoradiogram resolving basic polypeptides, of which one is the arginine and lysine-rich phosphorylated protein. Electrophoresis in the first dimension for 800 Vhr NEPHGE (O'Farrell et al., 1977) and in the second dimension SDS-PAGE. Rate of protein migration in the first dimension is also a time of electrophoresis. (a) [³⁵S]-methionine labeled virus polypeptides were separated by NEPHGE, then in 10-20% polyacrylamide. (b) Virus labeled with [³³P]-PO₄ was subjected to identical electrophoresis. The measured distances of migration of the phosphoprotein and the 11 K basic polypeptides in NEPHGE and SDS-PAGE were identical in the comparative gels. After fluorography, the exposure to produce latent images in the X-ray film was for three days. The numbers are the molecular weights $\times 10^{-3}$ (K) of some marker polypeptides in this and other gels shown. In both cases the amount of viral protein loaded on NEPHGE gels was 21 μ g.



produce streaks in the slab gel (Fig. 3). This implies that these major polypeptides had not been adequately solubilized prior to electrophoresis. In this regard increasing the SDS concentration and application of heat were helpful, as judged by the migration of 60 and 62 K into the IF gel, because these polypeptides became evident as discrete spots in the second dimension. However, appearance of numerous artifacts such as formation of clusters of spots, where only a single one should have occurred, precluded the use of this technique.

The solubilization method of Ames and Nikaido (1976), failed to produce satisfactory electrophorograms of vaccinia proteins despite various modifications made with respect to the ratio of SDS/ protein in the samples. Although increased solubilization of virion polypeptides was achieved, judging by the presence of more numerous spots in the slab gel autoradiograms, the majority of these spots appeared as reduplicated clusters. After storage of samples at -20 C for 1 week or longer additional chains of reduplicated spots were found on the acidic side of the gel, caused perhaps by modification of the charge (O'Farrell, 1975), as cited also by Wilson et al., (1977). Application of heat with urea being present or when added after heating, resulted in the formation of similar artifactual multiple spots. It seemed therefore desirable to leave out urea entirely from the dissociating solution. Advantage was taken of the possibility suggested by O'Farrell (1975), that any SDS present in the samples perhaps combines with NP-40, so as to form micelles which, upon electrophoresis, could be expected to migrate toward the acidic end of the IF gel. Furthermore, urea per se may not enhance the

FIGURE 3: Autoradiogram demonstrating artifactual "streaking" of virion core major polypeptides 60 and 62 K following electrophoresis, when the sample was loaded at the anode of the IF gel. The sample contained 90,000 cpm. Electrophoresis in IF and SDS-slab gels exactly as in Figure 5. The basic phosphorylated protein of 11 K (see Figure 2) appeared at the basic end of IF gel as a single spot (arrow). Two major capsid proteins, 60 and 62 K did not migrate to the cathode end, therefore, are not basic.



solubilization of vaccinia virions when SDS is also present. In any case after release from virions and introduction into IF gels these proteins become exposed to 9.5 M urea, while the SDS combined with NP-40 is removed away from proteins so, as not to influence the charge on them. Evidence for this notion was obtained by comparing the pH gradient in IF gels with and without SDS addition (Fig. 4).

Finally, using the dissociation scheme (4) described under Materials and Methods, the two-dimensional electrophoresis made it possible to resolve, at least 104 polypeptides (Fig. 5). However, the polypeptides which were basic appeared as streaks at the cathodal region of the IF gels. Some of these could, however, be resolved using as the first dimension NEPHGE at 800 Vhr. (Fig. 2).

When purified dihydrofolate from CHO cells (Essani and Flintoff, unpublished results), commercially available bovine serum albumin, E. coli cells, L₂ mouse fibroblasts, cytoplasmic fractions of CHO cells, and purified rabbit IgM molecules were solubilized according to the dissociation scheme (4) described in Materials and Methods, highly satisfactory and reproduceable results were obtained. These observations clearly revealed that use of SDS increases the solubility of proteins from complex biological structures. Whenever, SDS was omitted in parallel experiments relatively fewer spots were resolved. Concomitantly, these experiments further supported our earlier conclusion that the presence of SDS in the solubilization buffer, under conditions used in this study, does not apparently alter the isoelectric points of proteins (results not shown), thus broadening the applicability of the technique to other biological systems.

FIGURE 4: Determination of pH gradient in IF gels used in experiments of Figure 5. Note that presence of SDS in the solubilizing solution altered slightly the shape of the pH gradient, although the gels were of identical composition. Lysis buffer of O'Farrell (1975) without SDS (●); solubilizing solution with SDS (▲).

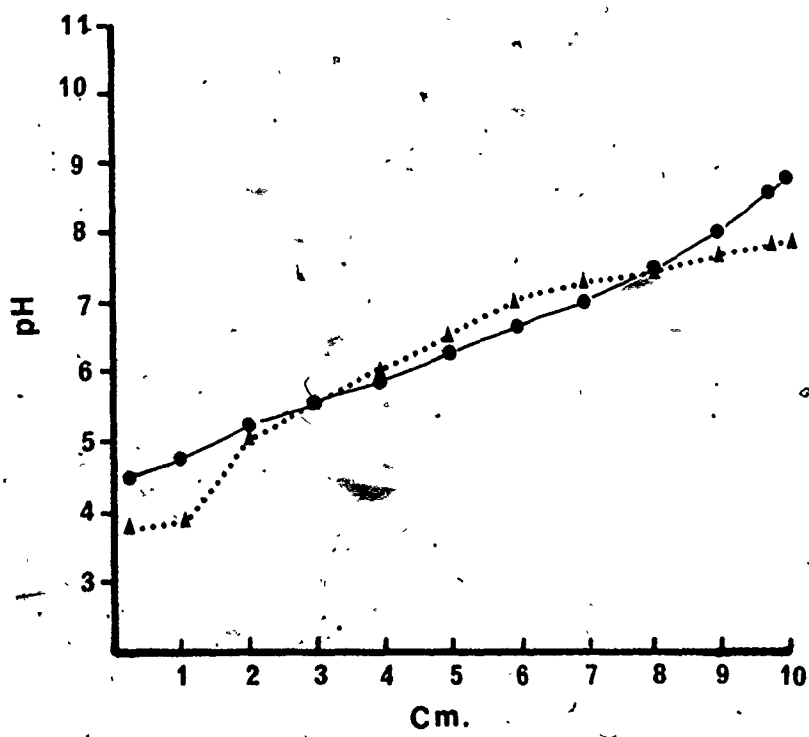
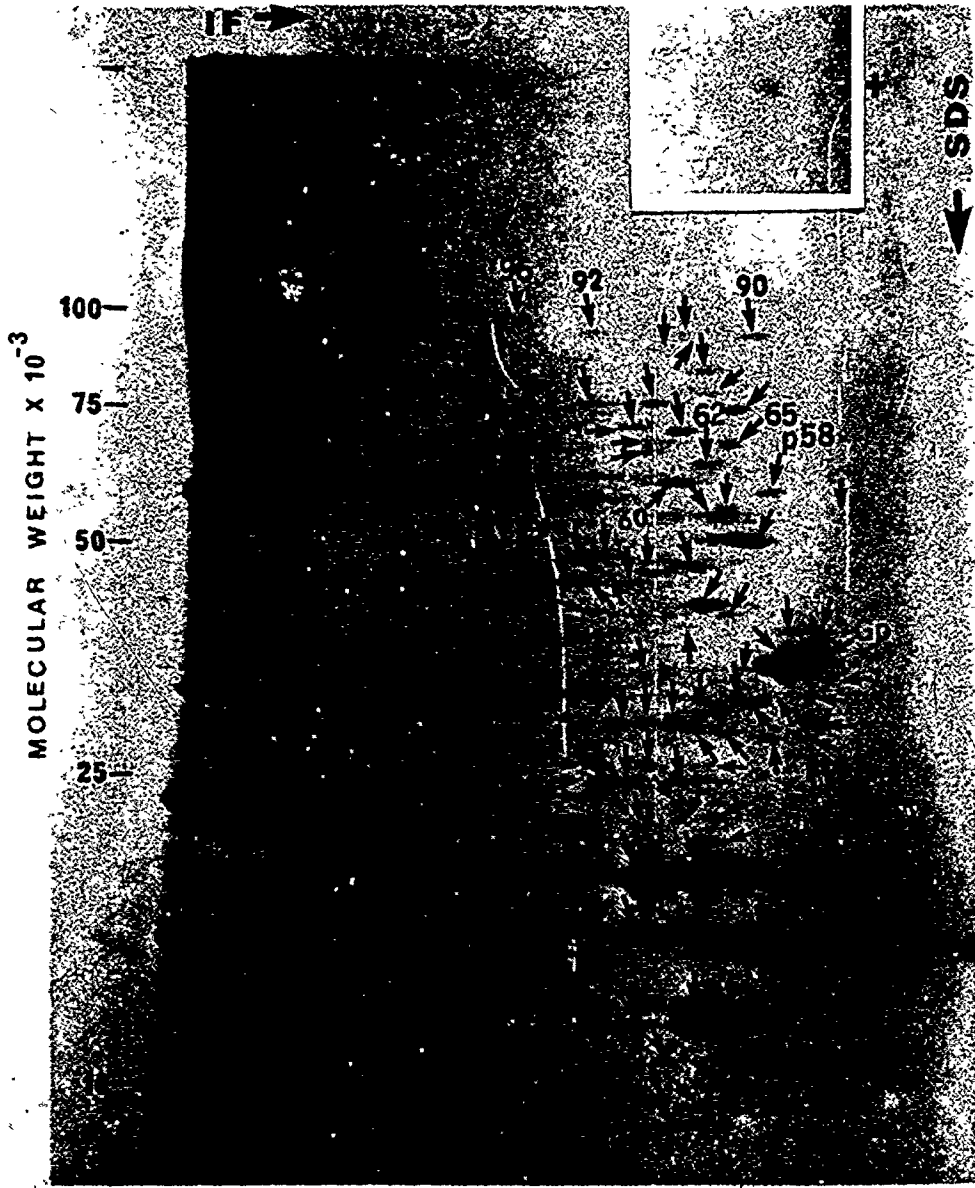


Figure 5: Autoradiogram of two-dimensional separation of vaccinia polypeptides; and in the inset, the 58 K purified surface tubular elements (STE). Pure [³⁵S]-methionine labeled virus was dissociated according to procedures under Materials and Methods. Aliquots of 15 μ l each containing 15 μ g protein with 170,000 cpm were introduced onto the IF gels at the cathode. Electrophoresis was conducted for 6000 Vhr. following warming to 37° C for 60 min to equilibrate the system. The IF gels were placed onto slab gels and electrophoresis in the second dimension was carried out for 5 hr at 25 mA. In the case of [³⁵S]-methionine labeled STE the conditions used were identical except that the slab gel in the second dimension was made with 10% acrylamide instead of 7-18% acrylamide gradient gel employed with whole virus. Exposure was 30 days. GP is the glycoprotein of about 34 K.



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4.2 Analysis of Structural Polypeptides of Vaccinia in the Virion:

Following dissociation and PAGE in SDS-gels according to Stern and Dales (1976a), the [^{35}S] methionine-labeled polypeptides were resolved into 55-56 identifiable bands (Fig. 6). The prominent virion polypeptides of interest in subsequent analyses included 60 and 62 K core proteins, the 58 K STE, a glycosylated component about 34 K, and the highly basic phosphoprotein 11 K. When the samples were introduced at the cathode of IF gels and separation of proteins was carried out by the two-dimensional system, it was found that at an optimum resolution, over 104 individual spots were resolved on the autoradiogram (Fig. 5)

The authenticity of individual spots was substantiated by demonstrating that isolated ^{35}S -labeled pure STE migrated as a single component to the same position as a single polypeptide of 58 K from the dissociated virions (Fig. 5). Another component amenable to identification as a specific virion protein is the 34 K polyglucosamine glycopeptide (Garon and Moss, 1971). A comparison of data from 2-D electrophoresis of pure virus labeled with [^3H]-glucosamine showed that a single ^3H -labeled spot of approximately 34 K was resolved at the same position as a ^{35}S -labeled polypeptide (Fig. 7).

The quantity of isotopically labeled protein which migrated into IF gels from the loading zone was estimated by determining the total counts per minute applied and the amount remaining at the origin. These analyses revealed that 90% of the added material entered the IF gels.

FIGURE 6: Autoradiogram of one-dimensional SDS-PAGE of [³⁵S]-methionine labeled pure vaccinia virus. All identifiable bands are numbered on the left and molecular weight scale is shown on the right. Preparation as in Materials and Methods. About 20 µg of virus protein containing 20,000 cpm was applied to 11% polyacryamide slab gel. The exposure time was 10 days.

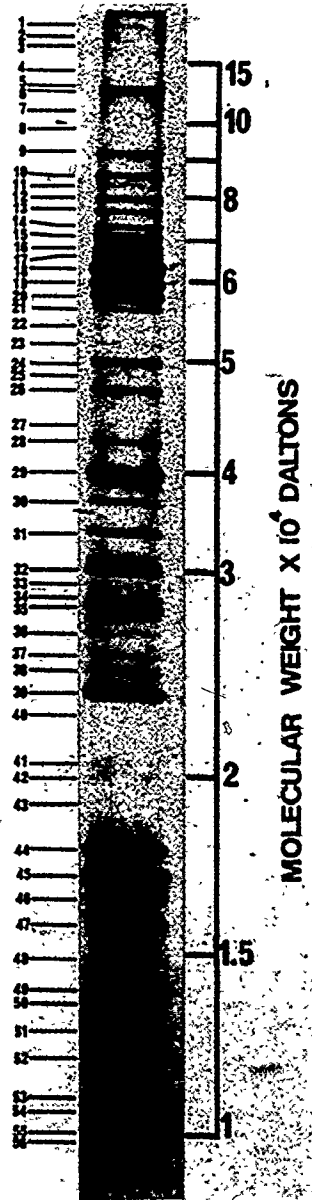
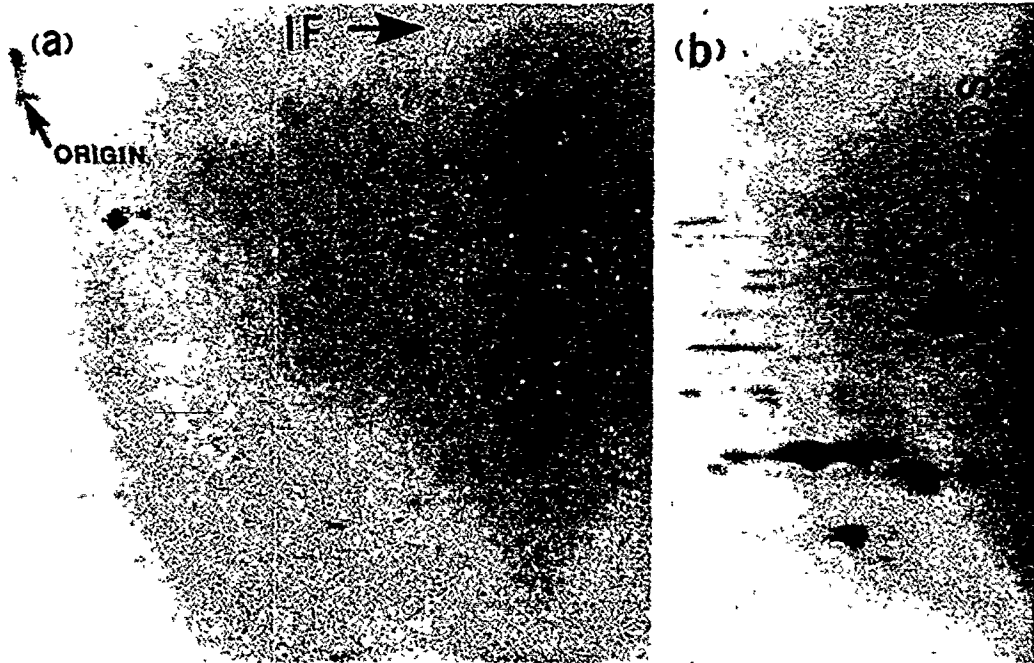


FIGURE 7: Autoradiogram of (a) [^3H]-glucosamine and (b) [^{35}S]-methionine labeled virion glycoprotein and polypeptides. Conditions of electrophoresis were like those employed in Figure 5 except that in the second dimension the gel was 10-20% acrylamide. In (b) selected area of the autoradiogram, the sample contained 90,000 cpm, in (a) the entire area, 21,000 cpm was applied. Exposure was 15 days. Arrows in (a) and (b) indicate the position of the single glycoprotein, about 34 K.



NEPHGE of shorter duration in the first dimension revealed at least 7 separate basic polypeptides, one of which by virtue of being labeled with ^{33}P , was recognized as the arginine-lysine-rich phosphoprotein of vaccinia (Fig. 2). Thus, pure vaccinia appears to be constituted from 111 or more proteins.

4.3 Virus Polypeptides in Cell Extracts:

Previous work has established that two major core proteins of 60 and 62 K are derived from higher molecular weight precursors of 94 and 65 K, respectively (Moss and Rosenblum, 1973). These and other precursor proteins of a conditional lethal mutant of IHD-W vaccinia, designated ts 1085, are processed inefficiently at the nonpermissive temperature of 40°C . As a means of identifying additional virus polypeptides, cytoplasmic extracts of infected [^{35}S]-labeled cells were analyzed by 2-D electrophoresis. The comparison involved material from cells which were pulse-labeled then incubated in chase medium following infection with either wild-type vaccinia or ts 1085 maintained at 40°C . The results, illustrated in Fig. 8 revealed that after infection with either agent, clusters of spots were evident in the autoradiograms at the site of protein(s) of about 65 K. With ts 1085 the amount of this polypeptide which had accumulated at 40°C was evidently greater. By contrast the spots presumed to be 60 and 62 K product proteins were present in lesser amount in the ts 1085 infection than in the wild type (compare Figs. 8a and b). Furthermore some of the 60 and 62 K polypeptide material in case of the wild type remained at the position of the origin of the IF gel (large arrow in Fig. 8a) indicating that the disparity in amounts of 60 and 62 K in

FIGURE 8 : Autoradiograms of cytoplasmic extracts of (a) wild-type and (b) ts 1085 infected L₂ cells. Eight hours after inoculation cells were kept in methionine starvation medium for 80 min, then labeled with [³⁵S]-methionine for 60 min and incubated in chase medium for 240 min. In case of ts 1085 labelling and chase were carried out at 40° C. The extract placed in each gel contained 80,000 cpm of virus specified protein. Electrophoresis in the second dimension was through 10% polyacrylamide gels. Autoradiograms were exposed for 15 days. In (a) and (b) arrows point to 65 K polypeptide spots which are presumably the precursors of the 60 K polypeptide. This is indicated by the greater area occupied by the precursor and smaller spot of the product in (b), as anticipated if there was an interruption of cleavage with ts 1085 (Stern et al., 1977). The 60 and 62 K products may be even more abundant in wild-type infection than indicated in the autoradiogram in (a), since not all of these proteins entered the IF gel, but instead migrated downward from the origin of the IF gel and produced streaking in the second dimension in (a). Note that with ts 1085 all of 60 and 62 K entered the IF gel. in (a) the 18 K polypeptide is probably the cleavage product of 25 K, since in (b) 25 K occurred in larger amount, but 18 K was missing.



Fig. 8a vs b' was even greater than evident in the autoradiograms. A smaller 25 K spot in (a) could be matched by an equivalent more massive one in (b), but an 18 K spot in (a) was completely absent from (b). This suggested that 25 K is a precursor of a lower molecular weight product and that polypeptide cleavage of the 25 K component fails to occur during ts 1085 infection, as already shown by one-dimensional analysis (Stern et al., 1977):

The above experiments with extracts of infected cells and purified virions have enabled us to identify certain specific vaccinia polypeptides, shown to be resolved into single spots. Extrapolating from this one can assume with some degree of assurance that all or most of the 111 spots resolved by 2-D gel analysis represent individual, authentic virion polypeptides.

4.4 Phenotype of ts 6757 Observed by Electron Microscopy:

This mutant is characterized by overproduction of immature virus envelopes and designated ts 6757 (Figures 9 and 10). Such envelopes form independently from the material pooled within viroplasmic matrices or factories, as indicated by the presence of lucent centers inside the envelopes. Assembly into what appears as multilayered spheres in some orientation, is evident from Figures 10 and 11 and previous observations (Dales et al., 1978). Regular quasi-crystalline arrangement of spicules is indicated in both the side view and tangential sections, as illustrated in Figures 10 and 11. The spicule layer frequently appeared to be disconnected from the subjacent bilayer 'unit' membrane (Figures 10 and 11), revealing the autonomy of spicules. The evident independent existence of spicules

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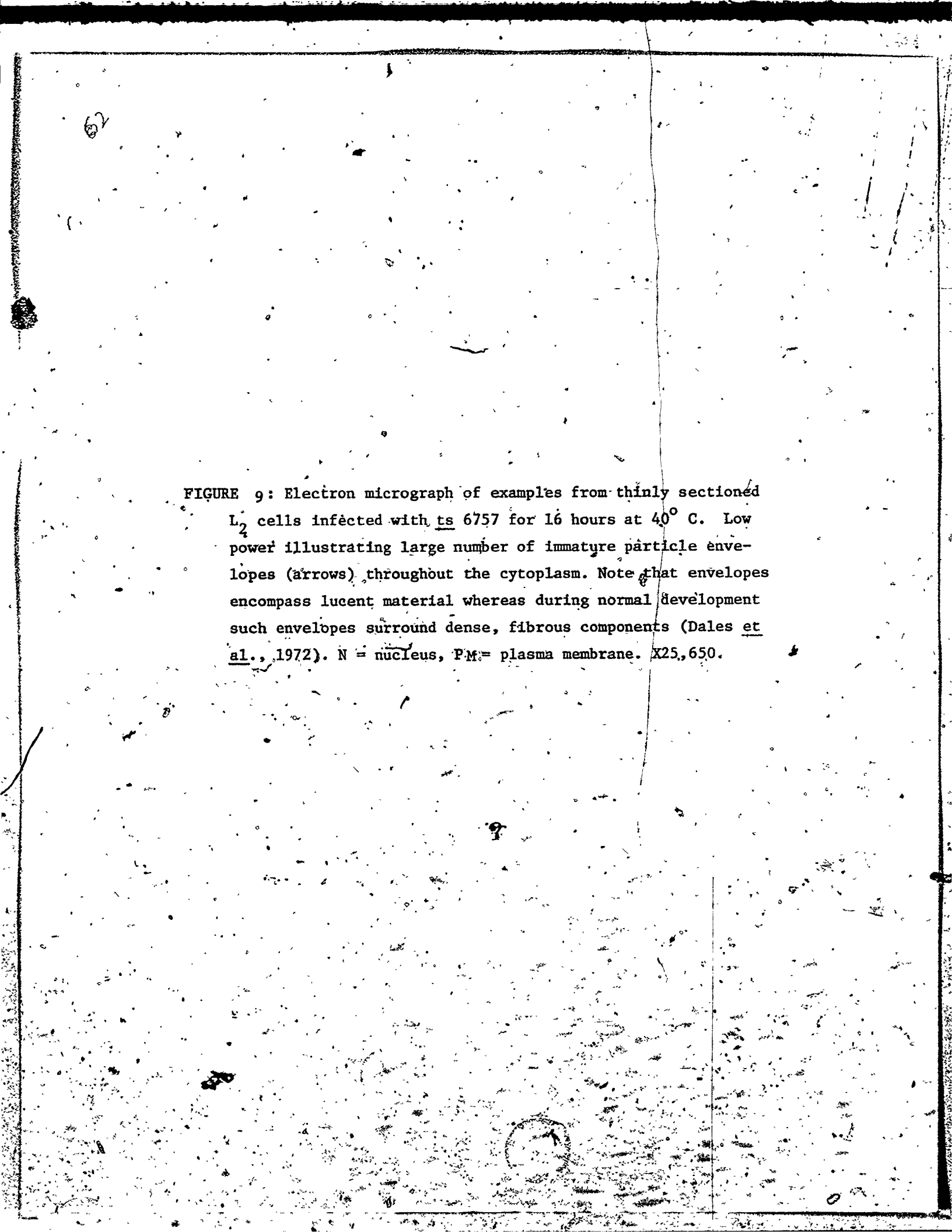


FIGURE 9: Electron micrograph of examples from thinly sectioned L_2 cells infected with ts 6757 for 16 hours at 40° C. Low power illustrating large number of immature particle envelopes (arrows) throughout the cytoplasm. Note that envelopes encompass lucent material whereas during normal development such envelopes surround dense, fibrous components (Dales et al., 1972). N = nucleus, PM = plasma membrane. X25,650.



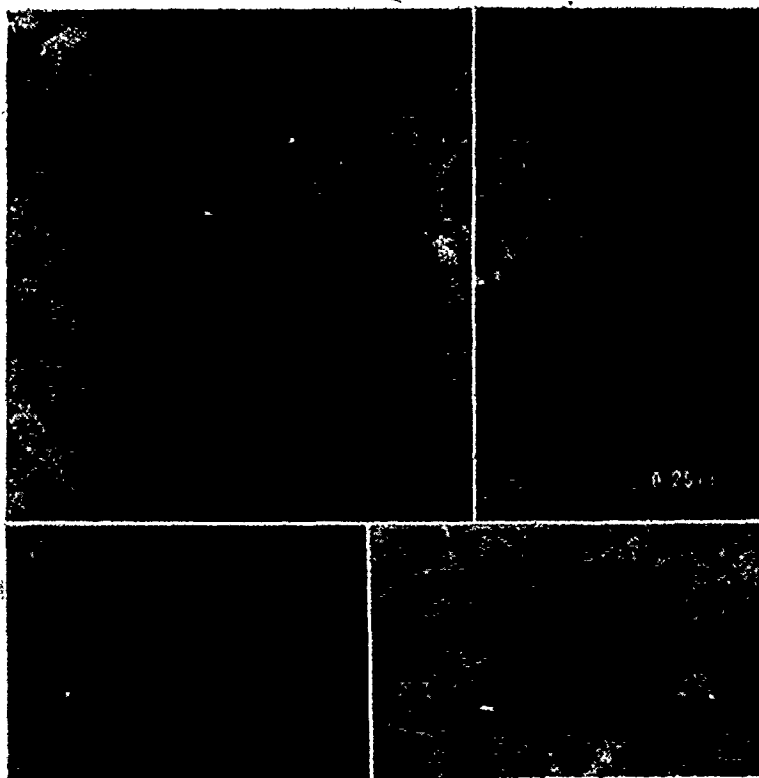
64

FIGURE 10: Selected area of cytoplasm from ts 6757 infected cells at higher resolution. The multilayered aberrant envelopes characterizing ts are clearly evident. Regular organization of spicules on envelopes is shown in side view (arrows) or tangential sections (double arrows). The spicule layer at 'S' is apparently detached from the adjacent membrane. x62,750



FIGURE 11: Selected areas of cytoplasm from ts 6757 infected cell at higher resolution. The detachment of spicule layer 'S' from membranes are even more evident. See Figure 10 for details.

A x 96,000; B, x 75,000; C x 112,000; D; x 112,000.



supports previous findings from this laboratory on the reversible addition of spicules to the bilayer in studies of defective assembly caused by rifampicin or elevation of temperature in the case of group E mutants like ts 1085 (Nagayama et al., 1970; Stern et al., 1977), which have phenotypic defects mimicking rifampicin. Thus aberrant vaccinia virus envelopes devoid of spicules are assembled under restrictive conditions but after return to permissive conditions the spicule layer becomes inserted onto the envelope (Stern et al., 1977). Additional involvement of the spicules in the process of morphogenesis became evident with another ts mutant 1911, which undergoes differentiation from immature into mature virions very slowly (Dales et al., 1978). During differentiation the external layer of spicules is peeled away at the same time that internal material is elaborated into core and lateral bodies, as evident from the examples in Figure 12. The electron microscopic images, therefore, indicated that spicules may exist in pools of a soluble material and can be added to or removed from immature viral envelopes depending on whether conditions prevailing for assembly are permissive or restrictive and according to the stage in the cycle of development.

Complete temperature related reversibility of ts 6757 assembly defects was ascertained by electron microscopy. However, resumption of normal development could occur within a limited period of 6-10 hr, beyond which any defective envelopes that had assembled remained unchanged. Electron microscopic analysis of samples taken during 24 hr also revealed that envelope formation with ts 6757 ceased at about 16 hrs. These findings provided a basis for designing experimental protocols related to biochemical studies described below.

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FIGURE 12A and B: Examples showing stages in assembly intermediate between immature and mature forms during infection with ts 1911. Note that the external spicule layer is in the process of detachment from the envelope (arrows). x136,200.



4.5 Polypeptides Induced During Infection by ts 6757 at the Restrictive Temperature:

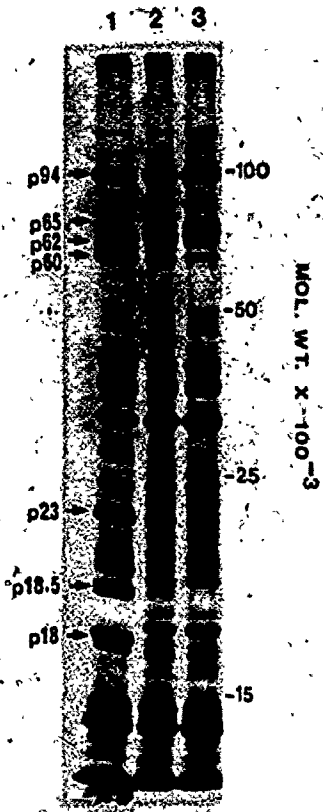
Analysis of 1-D SDS-PAGE of extracts isolated from pulse-labeled infected cultures and cultures kept in chase NM after labeling according to the protocols in Materials and Methods and the legend of Figure 13, showed that at the restrictive temperature most of the polypeptides synthesized under control of wild-type vaccinia virus were evident during infection with ts 6757. However, post-translational cleavage (PTC) of all precursors, including p94, 65, 25, and 18.3 was defective (Figure 13). The PTC defect was reversible upon shift-down to 33°C as evident from appearance of the products p65, 60, 23, 18.5, and 18 during interval of the chase (Figure 13, channel 2). Analysis by 2-D PAGE showed an electrophoretic pattern of spots that was identical with that described earlier (Essani and Dales, 1979) with cytoplasmic extracts from cells infected with the cleavage-defective ts 1085.

4.6 Isolation of Immature Viral Envelopes:

The overproduction and unusual assembly of immature virion envelopes (IE) as independent structures during ts 6757 infection at 40°C provided the source material of IE. A number of different approaches were initially employed in attempts to isolate the envelopes in a purified form. Variations in conditions of centrifugation, types of density gradients employed including use of potassium tartrate, sucrose and cesium chloride gradients, enabled concentration but not the purification of IE. The routine method finally adopted involved use of immunoagglutination. The approach was

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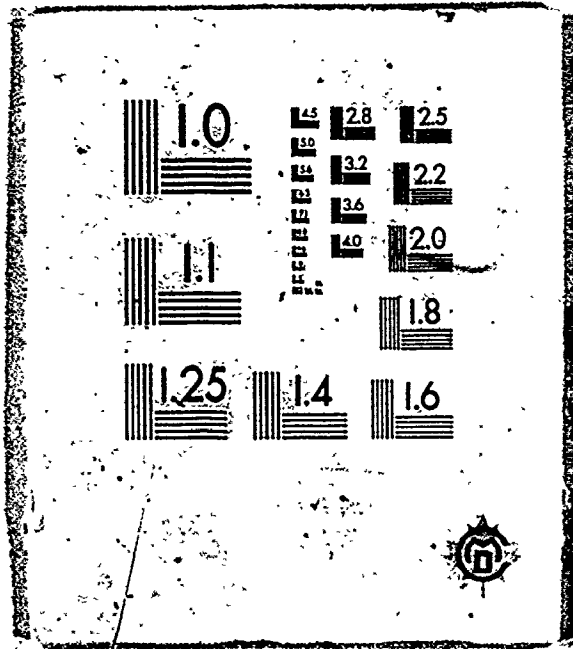
FIGURE 13: Analysis by 1-D PAGE of ts 6757. Autoradiogram shows [³⁵S]-methionine labeled virus-specified polypeptide bands. Cells infected for 9 hr were pulse-labeled with [³⁵S]-methionine for 1 hr and incubated for 8 hr in chase NM. Channel 1: pulsed at 32° and chased at 32°; 2: pulsed at 39° and chased at 32°; 3: pulsed at 39° and chased at 39°.



to obtain an antiserum directed against impure and concentrated IE prepared from infected HeLa cells, adsorb all the cell-directed antibodies from this serum with acetone powder of L₂ cells to enable specific agglutination of IE released from L₂ cells. Advantage was taken of effective inhibition by the vaccinia infection of host-related translation, ensuring that all labeled proteins synthesized during infection were virus specified. Immunoagglutinates obtained with anti-IE antisera analysed by 1- and 2-D PAGE contained several polypeptides identical to those present in soluble envelope material stripped from the surface of highly purified mature virions (Fig. 14, channels 3, 4, 5), indicating similarity in composition of both envelopes. However, the surface tubular element (STE) p58 polypeptide characterized by Stern and Dales, (1976a) from mature virus envelopes, evident in channel 3 of Figure 14 was absent from IE. This distinction was made evident more clearly when 7.5% 1-D acrylamide gels were used (data not shown). Further evidence for absence of STE from IE was obtained in neutralization tests whereby anti-IE serum failed to neutralize the infectivity of mature virus, while anti-p58 serum possessed neutralizing activity (Table II). On the other hand a 65K band, presumed to be p65E, absent from mature virus envelopes was evident in the IE preparations as shown in channel 4 of Figure 14. Two prominent core polypeptides p62 and p60 (Sarov and Joklik, 1972) evident in virions and purified cores (channels 1, 2, and 6) were absent from IE (channel 4 of Figure 14). The IE contained 9 major polypeptides, (p80, p65, p58.3, p42.6, p37, p23, p20, p13.7 and p11) at least three of which (p65, p20, and p11) were not detectable in envelope material from mature virions, while envelopes of mature virus

22

OF / DE



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FIGURE 14: Comparison between polypeptides present in mature virions and IE. Autoradiogram of SDS-PAGE, 1-D PAGE made from [³⁵S]-methionine labeled polypeptides after electrophoresis in 12.5% gels.

Channel 1: whole virions; 2: viral cores;
3: mature virion envelopes; 4: solubilized IE;
5: mature virion envelopes; 6: mature virions.

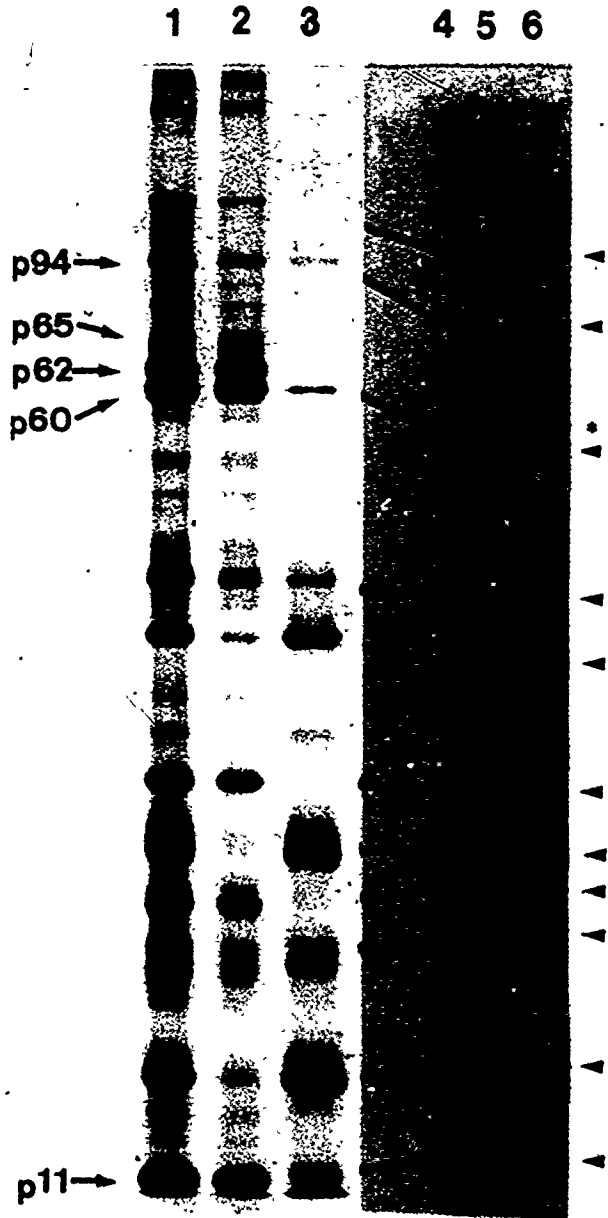


Table IINeutralizing activity of antisera^b

Antiserum	PFU/ml after incubation ^a
anti- IHD-W	2×10^4
anti- p58 (STE)	7×10^4
anti- p65E (2-D)	5×10^7
pre-immune	3.2×10^7

^aVirus suspension in 0.3 ml volume containing final concentration of 8.3×10^7 / ml was mixed with an equal volume of 1:5 diluted antiserum or pre-immune serum and incubated for 1 hr. at 37°C. The anti- IHD-W was raised by inoculating purified virus and anti-p58 serum after inoculating STE isolated from mature virions (Stern and Dales, 1976a) as described in Materials and Methods.

^bAdopted from Essani et al. (1982).

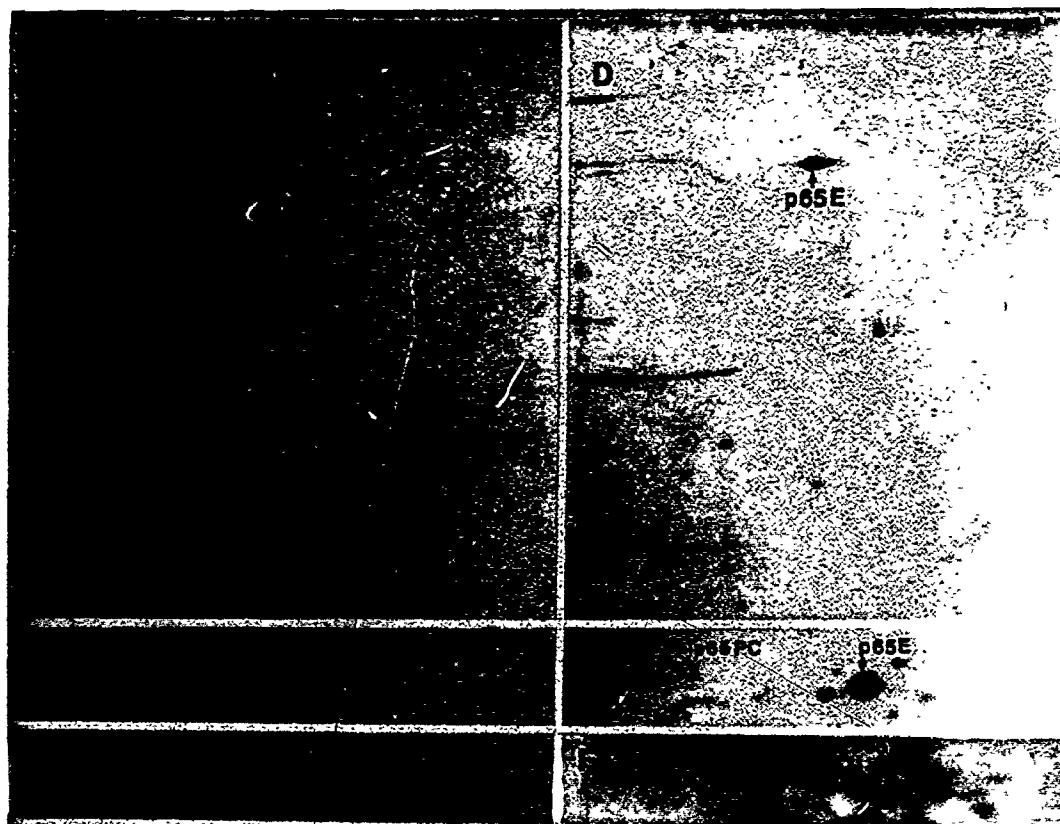
contained 12 major polypeptides, seven of which (p58, p23.6, p18.6, p18.1, p14.2, p12.8, and p10.2) were absent or undetectable in IE, indicating that mutual exclusiveness did not involve only p65E and the p58 STE.

4.7 Use of Specific Antisera for Partial Purification of IE:

Failure to purify IE by centrifugation methods alone made efforts turn towards the immunological approach. Anti-IE serum was prepared by injecting rabbits with pellets containing IE isolated from HeLa cells, as described in Materials and Methods. Antisera were thoroughly adsorbed with ruptured HeLa cells to remove cross reacting host antibodies and applied to IE preparations from L₂ cells. The material obtained by immunoagglutination was shown to contain IE, but analysis using light microscopy with fluorescent antibody staining revealed presence of considerable antibody reactivity to host cellular components. Another type of antiserum was raised in rabbit specifically directed against the major 65K polypeptide invariably present in immuno-agglutinates produced with the anti-IE serum. The polypeptide in question was isolated after 1-D SDS-PAGE like that in Figure 14, channel 4 and used as an immunogen. The antiserum raised in rabbits specifically precipitated a p65 polypeptide from solubilized material in the fraction enriched in IE and caused agglutination of intact IE isolated from infected cells. Unfortunately this anti-serum was also found to contain immunoglobulins not adsorbed by L₂ cell antigens, directed against host cell polypeptides which co-migrated in the 1-D PAGE with p65 of IE. The third and most satisfactory type of antiserum was obtained by injecting the 65K IE-associated polypeptide(s) isolated by

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FIGURE 15: Autoradiograms of labeled polypeptides in cytoplasmic extracts of ts 6757 infected cells. (A): Nine hours post infection at 32° cells were labeled with [³⁵S]-methionine for 30 minutes, cytoplasmic extract was prepared (Essani and Dales, 1979) and 50,000 cpm were loaded on 4% isoelectric focusing gel. Electrophoresis in the second dimension was through 11.5% SDS-polyacrylamide gel. (B): as (A) except that infection and labeling for 60 min was at 39°. (C): same preparation as in (B) sampled after 9 hr chase at 32°. (D): immunoagglutinates obtained with anti-p65E antisera were electrophoresed under identical conditions. (E): extracts from cells labeled as in (A) but treated also with 100 µg/ml of rifampicin. (F): extract from cells labeled as in (A) but treated with 20 µg/ml of Ara-C.



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immunoprecipitation and separated on 2-D gels like that shown in Figure 15D. When used for immuno-agglutination this antiserum, designated as anti-p65E, enabled routine isolation of concentrated IE preparations, as revealed by electron microscopy, in Figure 16. Additional evidence for the specificity of antibody-mediated agglutination was obtained by adding formaldehyde treated Staphylococcus aureus (Figure 16B). Therefore the p65 antigen must be a protein attached at the periphery of IE. To distinguish this polypeptide from the core-related 65K polypeptide (p65PC), the IE protein will be designated as p65E.

4.8 Relationship of p65E to IE:

Specificity of the anti-p65E serum was further evaluated by means of fluorescein antibody conjugates applied to ts 6757 infected cells. Observations from light microscopic images showed that the antiserum was specifically bound within cytoplasmic factories of infected cells where IE are assembled (Figure 17). This result strongly indicated absence of any contamination by host antigens from the material separated by 2-D PAGE used as an immunogen.

Concomitantly, when this antiserum was employed for immunoagglutination using ts 6757 infected cells as the source of IE, the entire IE were bound in the antibody complexes. Following 2-D PAGE two closely positioned spots of p65E became evident as shown in Figure 15D. Similarly, when IE material concentrated from cytoplasmic extracts by centrifugation was solubilized, immunoprecipitated, then separated by 1-D PAGE two closely contiguous bands with MW's

02

FIGURE 16 A and B: Selected area of thinly sectioned pellets containing concentrated ts 6757 immature particle envelopes. In (A) the envelopes were immunoagglutinated with antibody against p65E as described in Materials and Methods. The spicules (arrows) on the surface of viral envelopes are less distinctive than those illustrated in Figures 10 and 11, perhaps because they are obscured by immunoglobulin molecules. In (B) after agglutination with anti-p65E serum a suspension of Staphylococcus aureus, (protein A) was added to increase the size of the aggregates. Small segments of two bacterial cells (Bact) are evidently in contact with an aggregate of envelopes. Once again the spicule layer (arrows) has been obscured by fine, dense fibrils, presumably immunoglobulin molecules. x80,000.

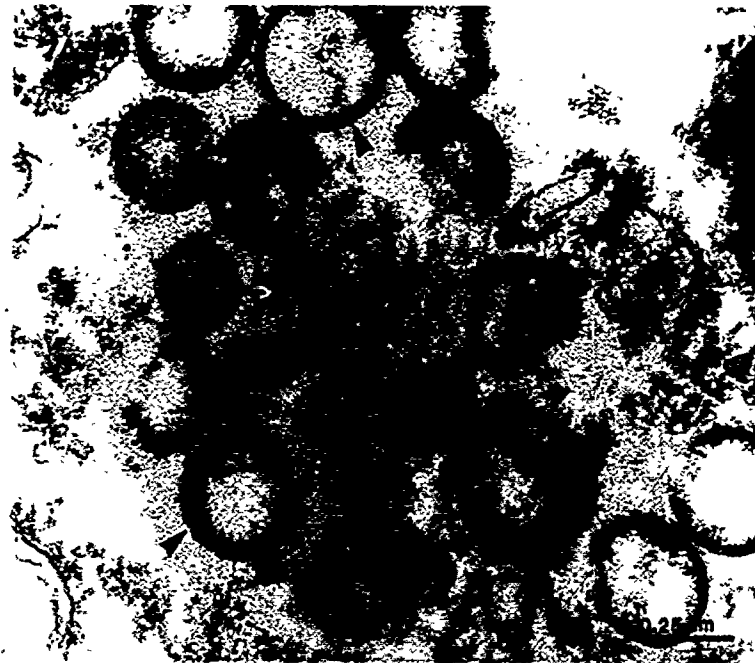
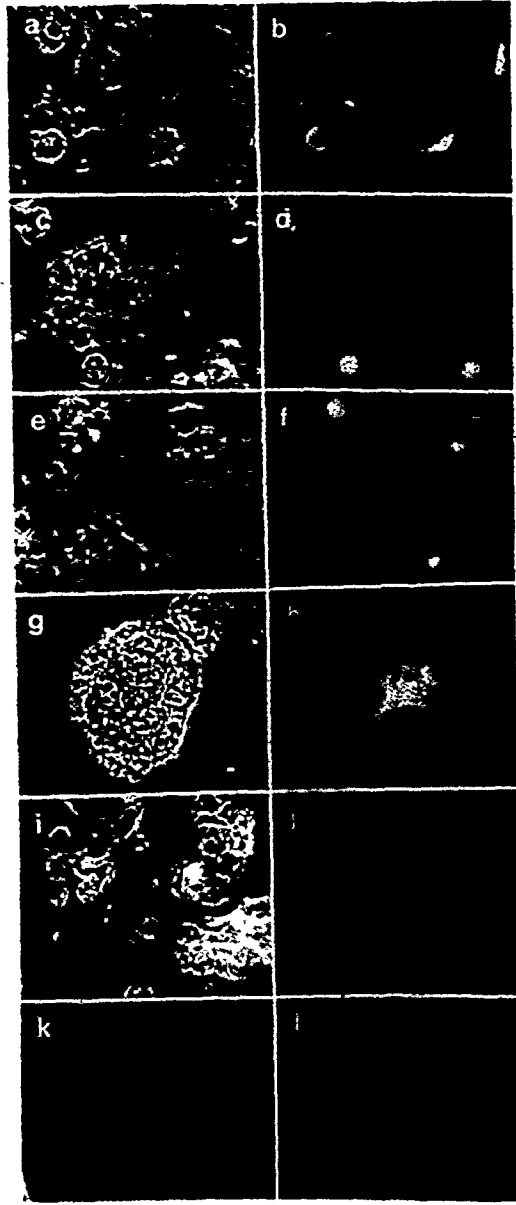


FIGURE 17: Fluorescent antibody staining applied to localize vaccinia induced antigens. L₂ cell monolayers were infected at a multiplicity of 10 pfu/cell, with either IHD-W strain of vaccinia (wt) or ts 6757 and incubated for 18 hrs at 32° or 40°. Micrographs a,c,e,g,i and k are phase-contrast images and b,d,f,h,j and l the corresponding images viewed under ultraviolet light. a and b ts 6757 infected at 40° reacted with anti-p65E (2-D) serum; c and d ts 6757 infected at 32° reacted with anti-p65E (2-D) serum; e and f wt infected at 40° reacted with anti-IHD-W serum; g and h wt infected at 32° reacted with anti-p65E (2-D) serum; i and j ts 6757 infected at 32° reacted with preimmune serum; k and l uninfected reacted with preimmune serum. x600.



corresponding to p65E appeared, as shown in channel 1 of Figure 18. These data strongly indicate that this antiserum contained monospecific antibodies directed against the p65E polypeptide(s).

4.9 Distinction Between p65E and p65PC:

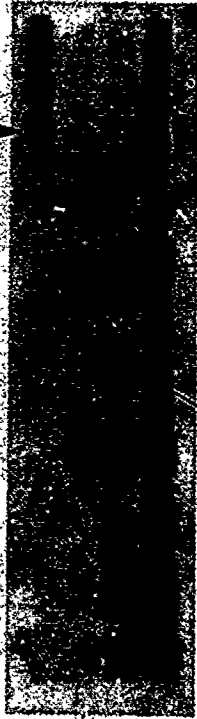
Immunological identification of p65E as a prominent polypeptide of IE most probably the spicule component, raised questions concerning the separate identity of this polypeptide from that of the core polypeptide precursor, p65PC, initially identified by Moss and Rosenblum (1973). These two proteins could be distinguished by 2-D PAGE analysis. It is evident from the autoradiograms, Figures 15A and B, that extracts from cells undergoing infection with ts 6757 under non-permissive conditions accumulated both polypeptides p65PC and a polypeptide at the position of p65E. Comparison between Figures 15B and C revealed that during chasing at the permissive temperature p65PC decreased while the product p60 increased in quantity. The 65K polypeptides, judging by their position in the electrofocusing dimension possessed different charges. Each of the p65PC and p65E components was resolved into 2 or 3 major spots, indicating minor charge heterogeneity. By contrast, immunoagglutinates formed with intact IE and anti-p65E serum contained only the more acidic 65K polypeptide(s) referred to as P65E (Figure 15D).

During treatment with the rifampicin, when aberrant IEs are formed lacking spicules (Nagayama et al., 1970), both p65PC and p65E were evident (Fig. 15E). This result is consistent with the observation that after removing the drug, normal IE with spicules are assembled even when protein synthesis is blocked, demonstrating that a

FIGURE 18: Autoradiogram showing the specificity of anti-p65E serum by 1-D and 2-D PAGE in 11.5% polyacrylamide gel. Immunoagglutination and immunoprecipitation conformed to the description in Materials and Methods. Channel 1: concentrated IE in pellets were solubilized, the insoluble material was removed by centrifugation, and the supernatant was reacted with anti-p65E serum and electrophoresed; 2: cell extracts containing intact IE reacted with anti-p65E serum; 3: solubilized envelopes from mature virions; 4: whole mature virions. Autoradiograms obtained after applying pre-immune serum under identical experimental conditions (not shown) did not reveal presence of any bands. Exposure was for 7 days.

1 2 3 4

P65E



pool of spicule material^c may exist. Treatment with cytosine arabinoside (ara-C), which blocks expression of late proteins during vaccinia virus infection but not assembly of normal IE, did not prevent the synthesis of p65E but inhibited p65PC production, as demonstrated by 2-D PAGE in Figure 15F.

Another approach to investigate the separate identity of the 65K polypeptides was peptide mapping employing limited proteolysis as initially described by Cleveland et al., (1977). Autoradiograms (Figs. 19A and B) revealed clearly that p65PC and p65E were distinctive proteins. Incidentally the electrophoretic patterns in Figure 19A obtained after limited proteolysis with trypsin and in Figure 19B by Staphylococcus aureus V8 protease provided convincing evidence confirming the origin of the p60 core product polypeptide as a derivative from p65PC.

4.10 ts 9251 Possesses an Additional EcoRI Restriction Site:

Five ts mutants, being defective in envelope self-assembly at a specific stage in virus development under restrictive conditions, have been included in group E (Dales et al., 1978). This specific stage in virus development (Figure 1) has been characterized by appearance of numerous viroplasmic foci surrounded by flexible envelope segments which are devoid of spicules, thus morphologically mimicking the defects induced by the antibiotic rifampicin (Nagayama et al., 1970). An earlier study (Stern et al., 1977) with one member (ts 1085) of the group has revealed that biochemically the defect was related, as with rifampicin (Moss and Rosenblum, 1973), to a block in post-translational cleavage required for virus maturation. Subsequent

FIGURE 19: Peptide mapping by limited proteolysis. The two vaccinia-induced 65K proteins and the p65PC product, polypeptide 60K were excised from slab gels after separation of [35 S] methionine labeled polypeptides solubilized from highly purified virions, or IE immunoagglutinates. p65E and p65PC were isolated from 2-D gels. Each well of the 18% polyacrylamide gels was loaded with 2-3 pieces excised from dried gels, as evident by vertical streaks at the upper end of each channel. Constant 25 mA current was applied till the dye marker ran out at the bottom. After fluorography by the method of Laskey and Mills, (1975), exposure to produce latent images on x-ray films was for 12 days. (A) represents the cleavage patterns obtained by trypsin and (B) shows the peptides obtained by digestions with Staphylococcus aureus V8 protease. In (A) Channel 1: p65E obtained after immunoagglutination of solubilized IE; 2: p65E isolated from gels after 2-D PAGE; 3: p65PC obtained as in 2; 4: core protein p60 product derived from p65PC.



investigation (Lake et al., 1979) has established that four out of five ts mutants are defective in post-translational cleavage of one or more precursor polypeptides.

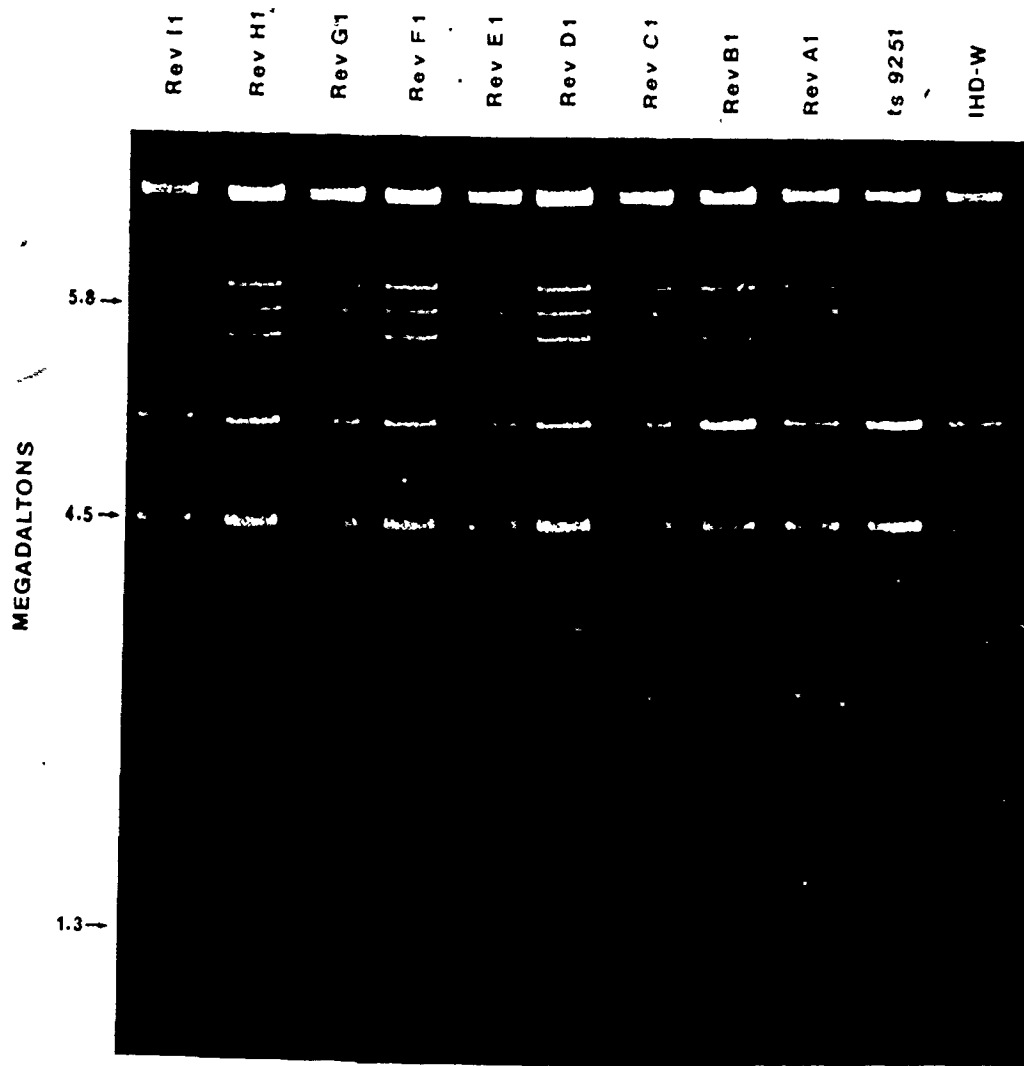
The only group E isolate evincing no processing defect of precursor polypeptide is ts d9251, which is very thermosensitive at the restrictive temperature and reverts infrequently, $<1:10^5$, to the wild-type phenotype. To characterize this mutant, biochemically, the DNA from this mutant was screened for any alterations in the primary structure using restriction endonucleases. A unique alteration has been identified by the restriction enzyme EcoRI (Fig. 20), but not by other enzymes employed including HindIII and BamHI. Comparing the pattern of bands generated by EcoRI digestion of parental IHD-W strain of vaccinia with that of ts 9251 it appeared that fragment D of 5.8×10^6 daltons is replaced by two new fragments with a collective molecular weight equal to that of the missing fragment (Fig. 20). It was later proved by southern blot hybridization that two new fragments in ts 9251 were derived from fragment D of parental wild-type vaccinia (Schumperli et al., 1980).

4.11 EcoRI Restriction Site Resides at the Temperature

Sensitive Locus in ts 9251:

Of particular interest here is the relationship of this novel EcoRI site to the phenotype of ts 9251. If this altered base sequence is the authentic site of the ts mutation, as opposed to that of some "silent" mutation, it might be expected that ts⁺ revertants could be found in which back-mutation had occurred whereby the mutated site would now again be recognized as an EcoRI site. Therefore,

Figure 20: Agarose gel electrophoresis of EcoRI-digested DNA from wild type vaccinia, ts 9251, and nine spontaneous ts^+ revertants of ts 9251. Virus stocks were prepared, viral DNA extracted on lysis gradients, and the purified DNA digested with EcoRI as described in Materials and Methods. Electrophoresis was performed for 16 hrs. at 50 V in 0.7% agarose and the DNA visualized with 1 ug/ml ethidium bromide. Molecular weight markers were the products of λ plac5 digested with EcoRI or HindIII. The sequences present in the 5.8 megadalton fragment are present in the 4.5 and 1.3 megadalton fragments shown by the arrows.



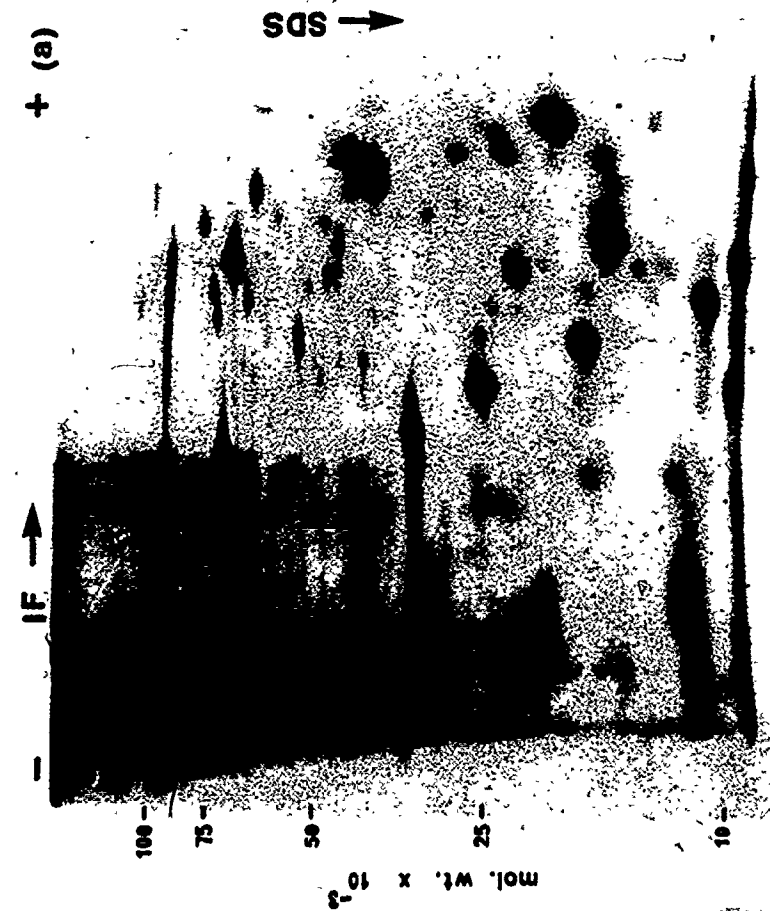
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independent ts⁺ revertants of ts 9251 were selected, cloned twice, and subjected to restriction enzyme analysis as before. Comparing the band pattern of several revertants with that of the parental ts 9251 DNA (Fig. 20)., it is evident that eight out of nine which became biologically changed from ts to ts⁺ also now exhibited the profile associated with wild-type vaccinia. However, one of the revertants to the ts phenotype, ts 9251 REVBI, retained the EcoRI digestion pattern of the parent ts 9251. These findings imply that when ts 9251 reverted to ts, mutation occurred at either the original mutant locus or at a second, independent and alternate site.

4.12 Polypeptide Alterations Associated with True and Pseudoreversion Phenomenon in ts 9251:

A polypeptide alteration associated with ts 9251 could also be demonstrated, employing our modification of the O'Farrell two-dimensional polyacrylamide gel analyses. Among virus-specified polypeptides of this mutant, identifiable in cytoplasmic extracts of infected cells, is a 37K protein (pI=7.6) with an apparently altered charge (compare Figs. 21a and f). To ascertain whether revertants of ts 9251 had been altered in the same gene as the ts protein induced by ts 9251 or at another locus, a comparison was undertaken employing 2D PAGE. The fluorograms revealed that all labeled proteins in extracts from cells infected with ts 9251 were the same as those after infection with the ts⁺ revertants with the exception of the singled-out 37K protein which in revertants ts 9251 A1, C1, and D1 now migrated consistently to a slightly more basic isoelectric point in the gel (Figs. 21b, d, e). Therefore the ts site-specific revertants

FIGURE 21: Two-dimensional gel electrophoresis of vaccinia-specified polypeptides in extracts of infected cells. (a) IHD-W wild type; (b) ts 9251 Rev A1; (c) ts 9251 Rev B1; (d) ts 9251 Rev C1; (e) ts 9251 Rev D1; (f) ts 9251. Cultures of L₂ cells were inoculated independently with one of the above types of vaccinia at a multiplicity of 10 PFU/cell and incubated throughout at 40° C. Eight hours after infection cultures were placed in methionine free medium, then pulse-labeled with [³⁵S]-methionine at 20 μCi/ml for 60 min as described in Materials and Methods. The sample preparation and the electrophoretic procedures were also as described earlier. Isoelectric focusing gels, used in the first dimension, contained ampholytes of pH 2/11 (Brinkman) and 10% SDS-polyacrylamide gels were employed in the second dimension. Samples loaded onto each gel contained 150,000 cpm in 10-20 μl volumes. Dried gels were exposed at -70° C for 10 days. Arrows point to 37 K polypeptide. The gel in panel f was slightly underexposed and several of the viral polypeptides are not apparent.



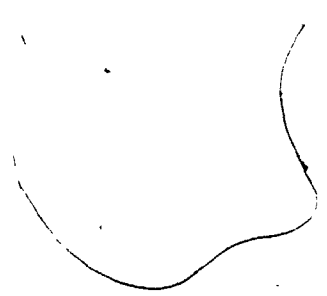
(A1, C1, and D1) induce a 37K protein with an isoelectric point like that encoded in the parental IHD-W virus. It is highly noteworthy that in ts 9251 REV B1 which had retained the novel EcoRI site and is therefore a "suppressed" revertant the 37K protein appeared to possess an isoelectric point intermediate between ts 9251 and wild-type vaccinia (Fig. 21c). The alterations in pI were checked further by subjecting to 2D-PAGE mixed extracts from infected cells inoculated with wild-type and ts 9251 or revertants of vaccinia. Whenever the 37K polypeptide with pI of the mutant virus was induced it could be readily distinguished from that of either IHD-W or the different revertants (Fig. 22).

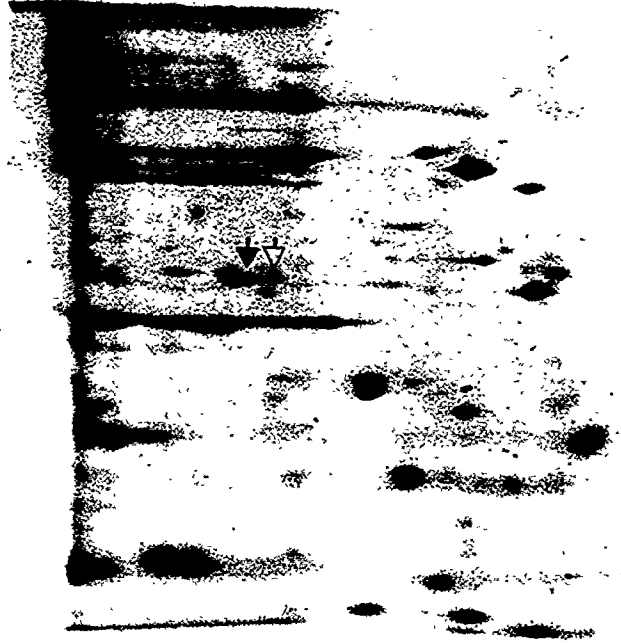
4.13 Recombination Analyses of Group E Mutants:

Once the biochemical basis of the ts defect was established in each of the group E mutants, it was possible to focus attention to the genetic aspects of these mutations investigating the relative position of genes involved in the expression of group E phenotype, their order on the genetic map and their relationship with the rifampicin resistance which mimics the group E phenotype, by recombination analyses involving two and three-factor crosses. Although poxviruses have been subjected to such analyses in numerous studies (Padgett and Tompkins, 1968; Chernos et al., 1978; Lake et al., 1979; Condit and Motyczka, 1981), little or no attention has ever been paid to define optimum conditions required for recombination analyses. Attempts were therefore made to determine the influence of multiplicity of infection

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Figure 22: Two-dimensional electrophoresis of a mixture of IHD-W and ts 9251 specified polypeptides in extracts of infected cells. Samples shown in Figure 21(a) and (f) were mixed in equal volumes and 150,000 cpm were loaded onto the isoelectric focusing gel. The open arrow points to 37 K polypeptide with an altered isoelectric point specified by ts 9251 and the closed arrow indicates equivalent wild type induced 37 K polypeptide. The sample preparation and electrophoretic conditions were as described in Figure 21.





and incubation period on recombination frequency (%RF), prior to detailed recombination analyses involving two and three-factor cross strategies.

4.14 Effect of Multiplicity of Infection on Recombination Frequency:

To evaluate the multiplicity effect two ts mutants (ts 9251 and ts 6389) were selected and used in two factor cross experiments. Each experiment was repeated three times independently, under an identical set of conditions. Table III summarizes the results of such experiments. When cells were infected at the moi of 1 or less, lower RF values were obtained reflecting asynchrony in the infection cycle. The RF optimum was obtained at an moi of more than 5 and remained constant even when pfu of 20/cell was used. Two way analysis of variance was performed on the data set presented in Table III, demonstrating no statistically significant variability in data among experiments (Table IV). The significant differences between the means of treatment (moi) as revealed by the least significant difference at an arbitrary probability level of 0.05 ($LSD_{0.05}$) are indicated underneath Table IV. The means of treatment that do not differ by LDS are underlined. Other mutant pairs in similar experiments exhibited an identical behavior (data not shown) showing consistency of the idea that results obtained with ts 9251 x ts 6389 pertain to all vaccinia mutants employed in this investigation. The relatively high recombination frequency values at low moi may be due to non-infectious particles which might participate in recombinational events.

Table III

Relationship between the multiplicity of infection and the recombination frequency as determined by two-factor crosses between ts 6389 and ts 9251.

Multiplicity of Infection	%RF ^a Mean±SE
0.1	2.7±0.4
0.2	7.3±0.8
0.5	8.3±0.3
1.0	8.9±0.6
5.0	11.7±0.6
10.0	11.2±1.0
20.0	13.1±0.3

a = per cent (%)RF was calculated per formula in Materials and Methods and the mean was computed from three independent experiments.

SE = Standard error.

Table IV

Analysis of variance summary table for the influence of moi on recombination frequency.

Source of variation	SS	DF	MS	F
Treatments	273.304	6	45.550 ³³	31.88 ^{***}
Blocks	2.5925	2	1.29625	0.9074 ns
Residuals	17.1415	12	1.4284	-
Total	293.038	20	-	-

*** = $p < 0.001$

SS, sum of squares;

DF, degrees of freedom;

MS, mean square

Standard error of differences of means ($s_{\bar{d}}$) = $\sqrt{\frac{2s^2}{r}} = 0.97584$
 where s = residual mean square; r = number of replicates.

$LSD_{0.05} = s_{\bar{d}} \times t_{0.05}$ (with residual DF) = 2.1263

Mean of %RF	2.766	7.366	8.300	8.933	11.700	11.200	13.166
moi	0.1	0.2	0.5	1.0	5.0	10.0	20.0

4.15 Relationship Between the Duration of Infection and Recombination Frequency:

Another parameter checked was the influence of the duration of infection on %RF. Crosses between ts 6389 and ts 9251 revealed that incubation beyond 24 hrs caused a marked increase in the recombination frequency, but the %RF values were the same if cells were incubated for 8-24 hrs. (Table V): Increase in RF values observed at 36 hrs. post infection suggests that recombinants emerging earlier might infected the small fraction of cells in culture not initially involved in the replicaton cycle. It is therefore important to select a time of sampling whereby only the cell population undergoing infection by the initial inoculum is involved in measuring the efficiency of recombination.

An identical statistical analysis of raw data shown in Table V again confirmed that %RF values obtained during each independent experiment were not significantly different. A comparison of %RF values at various time intervals (hours post infection) clearly demonstrated that the only significantly different %RF was obtained at 36 hours post-infection (Table VI), as apparent also from raw data and indicated earlier.

4.16 Recombination Analyses by Two Factor Crosses:

All possible crosses between six ts mutants were analysed by two factor cross strategy. When samples for titration were taken at 18 hpi, the %RF values ranged between 0.4-13.8. The additive %RF values between various crosses were satisfactory, as expected from such studies. The resulting map with the order of genes and %RF between

Table V

Influence of the duration of infection on the recombination frequency between ts 6389 and ts 9251.

Hours Post-Infection	%RF ^a Mean±SE
8	13.7±0.2
16	13.9±1.0
24	13.6±0.6
36	40.1±1.0

a, the %RF was computed as described in Materials & Methods.
Mean was calculated from three independent experiments.

Table VI

Analysis of variance summary table for the influence of duration of infection (hpi) on the recombination frequency.

Source of variation	SS	DF	MS	F
Treatments	694.419	3	231.473	191.298***
Blocks	4.227	2	2.113	1.746 ns
Residuals	7.264	6	1.210	-
Total	705.91	11	-	-

*** = $p < 0.001$
 ns = non-significant.

$$s_{\bar{d}} = 0.8981$$

$$LSD_{0.05} = 2.1976$$

Mean of %RF	13.733	13.933	13.600	40.166
hpi	8	16	24	36

various crosses are shown in Fig. 24A. However, when the incubation time was extended to 36 hpi, the %RF remarkably increased ranging from 5.8-56. The additivity of %RF values was satisfactory with the exception of the ts 1085 x ts 9203 cross, which on repeated assays gave 10 fold lower values than the expected (Fig. 23). It may further be noted that the gene order on the map was completely altered, generating a genetic map (shown in Fig. 23) which was identical to the one published earlier (Lake et al., 1979) but with unusually higher recombination frequency values. The duration of infection, therefore, seems to influence even the order of the genes on the map, and care should be taken to avoid the second cycle of replication which tends to increase the number of recombinants in such assay systems.

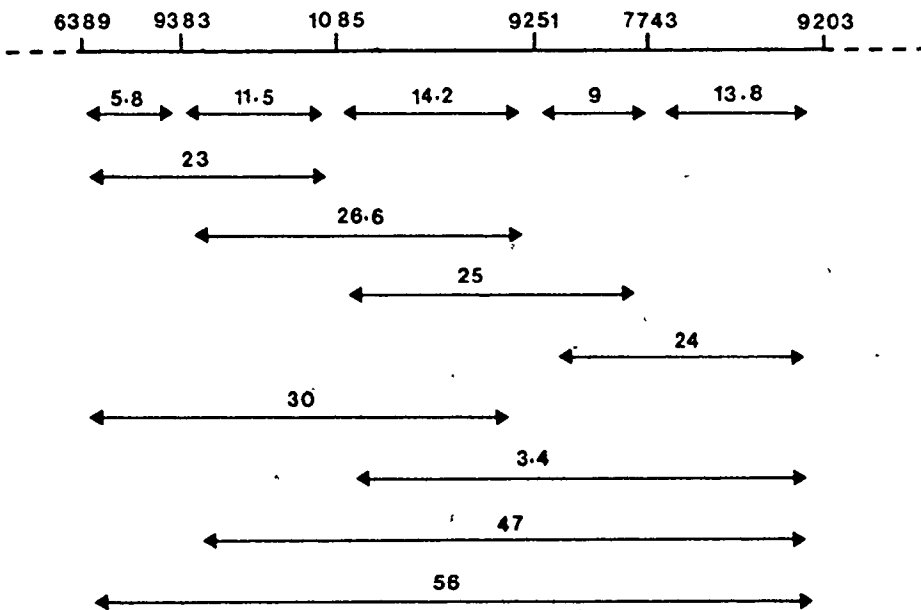
4.17 Recombination Analyses by Three Factor Crosses and the Influence of Acquisition of Rifampicin Resistance on ts 7743:

In all the ts double mutants (tsR) the rifampicin resistance marker was genetically identical since it was introduced by recombination from the same wt R isolate. The results obtained by three factor cross experiments provided further evidence that the rifampicin-resistance always mapped at the same locus in all the crosses tested. Furthermore the maps generated by two and three factor crosses were virtually identical (Fig. 24). However, one of the double mutant isolates (ts 7743RI) repeatedly mapped at a different position which was confirmed by employing several crosses (Fig. 11C). It is worthy mentioning that ts 7743 upon acquiring rifampicin resistance displayed a highly unexpected and complex behaviour.

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FIGURE 23: Recombination map of six ts mutants of vaccinia virus. ts 6389 is a DNA-minus mutant while remaining five ts mutants are defective in envelope self-assembly mimicking the effect of rifampicin (Dales et al., 1978). Numbers between arrows represent %RF indicating the percentage recombinants which have ts⁺ character. The %RF was calculated per formula in Materials and Methods. Samples for titration were obtained 36 hours post-infection.

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Unlike all the remaining mutants described in this report, the recombinants (tsR) obtained by crossing ts 7743 and wtR fell into three different categories: (i) two isolates (ts 7743RI and ts 7743RIII) retained total temperature sensitivity and acquired resistance for rifampicin, like all other recombinant isolates obtained by crossing ts x wtR; (ii) two isolates (ts 7743RII and ts 7743RV) completely lost the ts trait while retaining small plaque size characteristic of ts parent unlike the rest of the recombinants; and (iii) one isolate (ts 7743RIV) became cold sensitive (Table VII).

These unexpected observations added extra complexity to the earlier experience of so called marker movement (Figure 24C). Though we do not have enough conclusive data at present to provide any explanation for this preliminary observation, it appears that the ts mutation and the mutation for rifampicin resistance are interacting in some way thereby generating perhaps some sequences on the genome which may directly influence the recombination frequency. Such so called "special sites" have been reported in bacteriophage lambda (Stahl, 1979). Such sites are not present in the wild-type genome, but may be induced as mutations. It has further been suggested that such "special sites" in genetic recombination may be more widely spread than originally envisaged (Rosamond, 1980). Clearly more work is required to support and understand this preliminary observation. It is, however, clear by segregation analyses that the ts lesion in ts 7743 and rifampicin resistance do not involve the same gene. Although these two mutations are located very closely (see Fig. 24), it was possible to isolate spontaneous revertants from ts 7743 R III which lost the temperature sensitivity but retained the rifampicin

FIGURE 24 : Recombination map based on two and three factor crosses among ts mutants and rifampicin-resistant recombinants (tsR⁺). In two factor crosses (A) %RF represents percentage recombinants which have ts⁺ character. Arrows with bar ($\leftarrow\text{---}\rightarrow$) in three factor crosses (B) indicate the frequency of ts⁺R recombinants from which the locus for rifampicin resistance was calculated. Arrows without bar ($\leftarrow\text{---}\rightarrow$) between two markers represent total recombination frequency which was calculated without regard to rifampicin selection and includes ts⁺R⁺ and ts⁺R⁻ recombinants. Samples for titration were obtained 18 hpi and the recombination frequency (%RF) was computed as described in Materials and Methods. In (B) ts 7743R represents ts 7743RIII isolate. In (C) two different recombinants (tsR⁺) derived from ts 7743 were mapped and arrows represent total %RF measuring the distance between two ts markers.

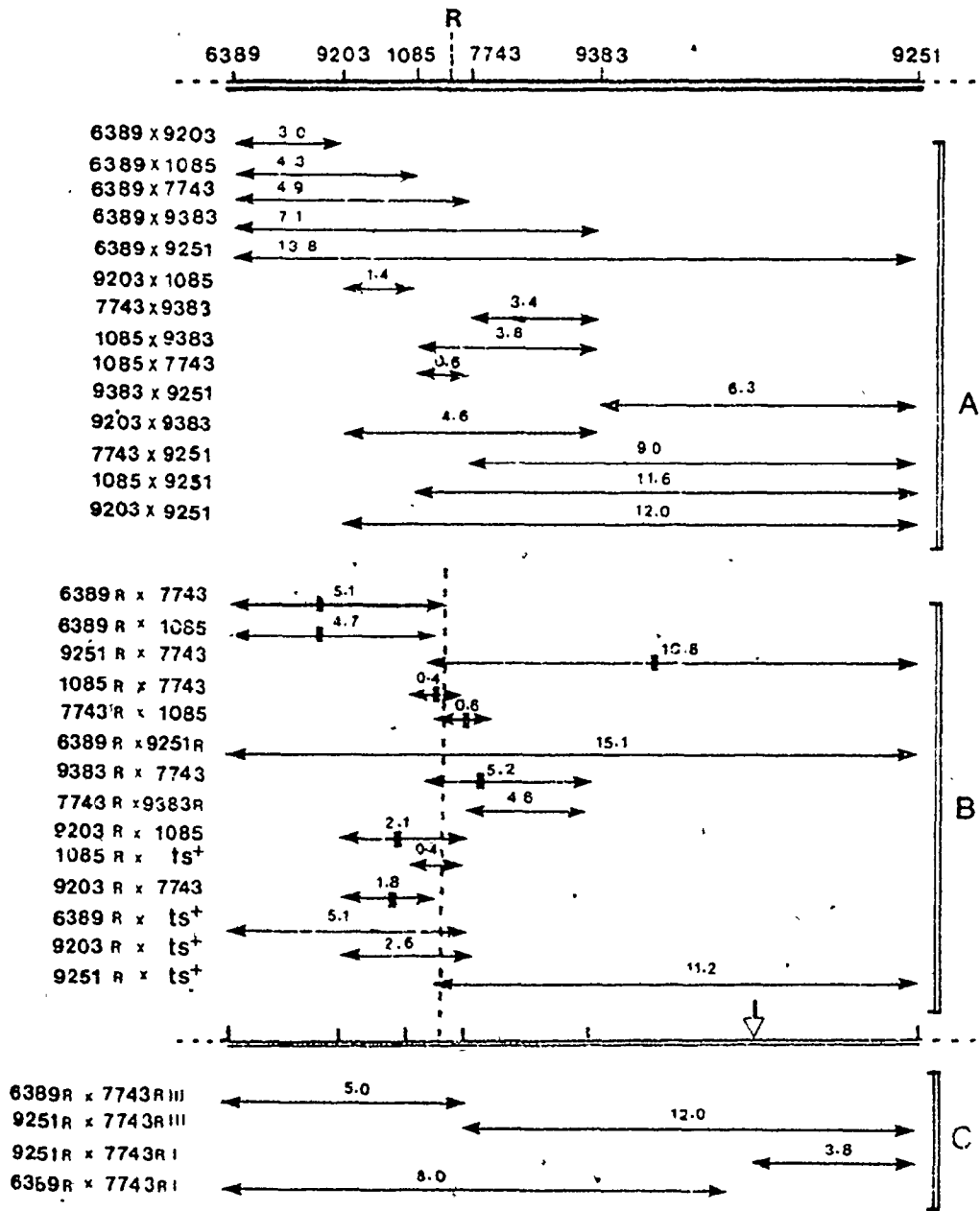


Table VII

Influence of acquisition of the rifampicin-resistance marker on temperature sensitivity of group E mutants.

Mutant (tsR ⁺)	Relative ^a PFU/ml 33/40	Plaque ^b Type 33/40
6389RI-IV ^c	700-1025:1	S/VS
1085RI-IV	675-1330:1	S/S
9203RI-VIII	340-540:1	S/S
9251RI-V	75-1300:1	S/VS
9383RI-IV	90-1000:1	S/VS
7743RI ^d	1200:1	S/VS
7743RII	1:1	S/S
7743RIII	1015:1	S/VS
7743RIV	1:26	S/S
7743RV	1:1	S/S
wtR	1:1	L/L

a, based on three or more independent titrations.

b, diameter observed at 48 hpi under overlay medium.

c, rifampicin-resistant isolate number.

d, because of wide variability, titre of individual isolates is presented.

L, large (1.5mm).

M, medium (0.5-1.0 mm).

S, small (0.2-0.4 mm).

VS, very small (<0.1 mm).

resistance. A total of 24 spontaneous revertants were isolated by incubating ts 7741RIII at 40°C as described in Materials and Methods. Twenty two of these isolates lost temperature sensitivity while retaining the rifampicin-resistance (results not shown).

CHAPTER 5

DISCUSSION AND CONCLUSIONS

The conditional lethal mutants described in this thesis have been isolated by Dales et al. ., (1978) and are defective in envelope self-assembly. Five of these mutants have been categorized in group "E" and morphologically mimic the effect of the antibiotic rifampicin (Dales et al.., 1978). Previous studies employing a series of pulse-chase experiments followed by SDS-PAGE analyses have clearly demonstrated that all, but one, group "E" mutants are defective in the proteolytic cleavage of one or more precursor polypeptides essential for virus maturation (Stern et al.., 1977; Lake et al.., 1979). The only isolate (ts 9251) exhibiting no defect in proteolytic cleavage of precursor polypeptides encouraged the search for a more sensitive technique for resolving minor alterations in the polypeptides. A two-dimensional electrophoresis technique (O'Farrell, 1975) was modified and successfully applied in the analyses of vaccinia virus polypeptides, as described later. The study was then continued into the genetic aspects of mutations involving envelope self-assembly and a recombination map indexing group E mutations and a locus for rifampicin resistance, was constructed.

5.1 Analytical Technique for the Separation of Vaccinia Virus

Polypeptides:

The genome of vaccinia virus comprises double stranded DNA of molecular weight greater than 120×10^6 daltons (Muller et al.., 1977;

McCarron et al., 1978; Esposito et al., 1978; McFadden and Dales, 1979), approximating that of the T-even bacteriophages with a potential coding ability for 200-300 proteins (Luria, 1978). Only O'Farrell's two dimensional electrophoretic system (O'Farrell, 1975; O'Farrell et al., 1977) is capable of resolving such a large number of polypeptides. In contrast, the one dimensional system of Laemmli (1970) can not resolve more than 56 structural polypeptides from vaccinia virus (Stern and Dales, 1976a). We, therefore, undertook to apply O'Farrell's technique to vaccinia in order to resolve a wider spectrum of structural polypeptides and to detect minor alterations therein (Essani and Dales, 1979; McFadden et al., 1980; Essani et al.), as explained later.

The results obtained during the early phase of these studies revealed that poxviruses cannot be satisfactorily solubilized using the lysis buffer described by O'Farrell (1975). Ames and Nikaido (1976) working with membrane proteins from Salmonella typhimurium, E. coli and animal cells arrived at the same conclusion. Addition of SDS has long been known to optimize the solubility of such proteins. It could, however, be argued that SDS would bind to proteins, thus causing alterations in their isoelectric points, and so the use of SDS should be avoided. Several earlier reports (Weber and Kuter, 1971; Tunzynski et al., 1978; Dottin et al., 1979) had convincingly demonstrated that SDS is effectively removed from proteins in higher urea concentrations, in the presence of NP-40 during isoelectric focusing (IF). O'Farrell (1975) has further observed that in such systems SDS combines with NP-40 so as to form micelles which, upon electrophoresis migrated towards the acidic end of the IF gel. Our

earlier observations have suggested that SDS does alter the shape of the pH gradient in IF gels, but that the isoelectric point of the polypeptide seems not to be altered (Essani and Dales, 1979). In addition to the vaccinia proteins, the dissociation solution formulated during this study has also been applied to identify dihydrofolate reductase (DHFR) isolated from Chinese hamster ovary cells, and SDS was found not to influence the isoelectric point of DHFR polypeptide resolved by two-dimensional gels (Flintoff and Essani, 1980; Essani and Flintoff, unpublished observations). Similar results have also been reported with bovine serum albumin and catalase (Danno, 1977). It may, therefore, be concluded that since SDS is stripped away during isoelectric focusing, so as not to influence the isoelectric point, its use in dissociation solution to facilitate the complete separation of polypeptides which otherwise can not be resolved, is essential. Therefore, the modification adopted with the vaccinia system has general applicability for separation of proteins in organized biological structures of higher order. Confirmatory results have recently been reported Kilpatrick and Rouhandeh (1981) who analyzed the polypeptides of monkey poxvirus.

5.2 Structural Polypeptides of the Virion:

The attempt made to enumerate structural polypeptides was based entirely on gel analyses. All necessary precautions were taken to assure the purity of each virus preparation. The virus purification procedure employed in this study (Essani and Dales, 1979) was more extensive than reported in any previous investigation. A number of viral structural polypeptides like p58, Gp34, p25, p18, and p11 have

been resolved as single spots on two-dimensional gels. Taking all above observations together one can assume with some degree of assurance that all or most of the 111 spots resolved by 2-D gel analysis represent individual authentic virion polypeptides. More recent studies on the monkey poxvirus which has been shown to contain about 115 polypeptides (Kilpatrick and Rouhandeh, 1981), further confirms the results reported here. The strongest biochemical evidence supporting the existence of a large number of polypeptides in the virion has been documented by Isle et al., (1981) who have selected early and late mRNAs by hybridization to cloned HindIII DNA fragments of vaccinia virus DNA derived from the left half of the genome. The translation of these mRNAs in a cell-free system resulted in 115 polypeptides. These data also endorse our previous assumption that vaccinia DNA, like that of T-even bacteriophages has a coding capacity for about 200 proteins.

Despite the available evidence from 2-D gel analysis for existence of 111 polypeptides, none of the polypeptides resolved was above 96K and none below 8K. Yet it is known from SDS-PAGE analysis that other proteins of higher molecular weight exist in the virion, as evident also in Figure 6. Therefore the present count of structural proteins must be an underestimate and one can anticipate that with further improvement in the technology additional virion polypeptides will be demonstrated.

5.3 Isolation of Immature Viral Envelopes:

The significance of this study must be examined in the context of previous investigations (Dales and Mosbach, 1968; Nagayama et al., 1970; Stern and Dales, 1976a,b; Stern et al., 1977) emphasizing the fundamental role of the poxvirus envelope in virion morphogenesis. The observations reported here were made possible by the availability of a conditional lethal mutant, ts 6757 which overproduces IE that are assembled separately from virions (Dales et al.; 1978) and a reproducible 2-D PAGE system (Essani and Dales, 1979) modified for analysis of poxvirus polypeptides from the method of O'Farrell (1975).

Isolation of IE, made possible by the ts 6757 phenotype led in turn to the preparation of anti-IE serum. The application of anti-IE serum to enrich IE from cells isotopically labelled during the infection, when host protein synthesis is effectively blocked, furnished an assay system and initial criteria for claiming that several polypeptides including p65E are authentic IE components encoded by the virus genome. Preparation of p65E, from 2-D gels, enabled the production of anti-p65E serum. Immunoprecipitation and SDS-PAGE analysis indicated, however that two distinctive, albeit very closely related, polypeptides may be formed during infection, probably implying inherent heterogeneity in MW and/or charge of the p65E component of IE. Future analyses of the primary gene products such as those synthesized during in vitro translation will resolve this issue.

Peptide mapping by limited proteolysis and 2-D PAGE provided data for concluding unequivocally that a late virus 65K polypeptide which is also evident in cell extracts must be the precursor p65PC of the 60K core polypeptide described by Moss and Rosenblum (1973) and is unrelated to p65E.

Formation of immunoaggregates between intact isolated IE, specific anti-p65E antiserum and protein A of Staphylococcus aureus cells clearly demonstrated that p65E is available for antibody binding at the surface of the IE, reinforcing the notion that p65E and spicules are one and the same material. This view is supported by a previous analysis of vaccinia virus development in which electron microscopic and 1-D PAGE observations led to the conclusion that an intermediate particle in virus assembly, evidently surrounded by spicules contains a prominent 65K polypeptide (Sarov and Jiklik, 1973).

The immunological approach was fruitful not only in providing evidence for identity of p65E as the likely spicule protein but also information about the transitory association of p65E with the envelope. Combined biochemical, PAGE and electron microscopic findings revealed that the spicules attach to the lipoprotein bilayer during assembly of IE and thereby provide a rigid "exoskeleton" determining the diameter and spherical form of envelopes surrounding immature virions.

That assembly of the envelope bilayer need not be coordinated with spicule attachment is clear from experiments with inhibitors like rifampicine or ts mutants with a rifampicin-like phenotype which produce readily reversible defects in IE assembly (Grimley et al.,

1970; Nagayama et al., 1970; Stern et al., 1977). When infected cells are returned to permissive conditions the spicules become spontaneously attached to the bilayer even in the absence of ongoing translation, indicating that intracellular pools of spicules must accumulate. This interpretation is entirely consistent with the presence of p65E in extracts of cells undergoing infection by ts 1085 or any other group E mutant under non-permissive conditions.

During vaccinia virus maturation the surface of the virion that was presumed to undergo alteration as a consequence of replacement of spicules by an externally positioned p58, identified as the polypeptide of surface tubules or STE characterized by Stern and Dales (1976a). This notion was confirmed by present data based on experiments with a slowly maturing ts mutant 1911 and analysis of IE and mature virions with anti-p65E and anti-p58 antisera. It should be mentioned that surface antigenic dissimilarity of immature and mature vaccinia virus was suggested by the immunoelectron-microscopic in the analysis of Morgan et al., (1962). Previous and current results are consistent with the notion that spicules may provide the developing vaccinia particles with a scaffolding, somewhat analogous to that described in the assembly of the head of tailed bacteriophages (King et al., 1973; Showe and Black, 1973; Fuller and King, 1981). Unlike the polypeptide involved in the bacteriophage scaffold, the spicule component p65E evidently is turned over, as suggested by pulse-chase experiments and 2-D electrophoresis. One may speculate that spicules are dismantled after detachment from IE during maturation of the virus.

5.4 Thermo-labile Lesion in ts 9251:

A survey of ts mutants in our collection (Dales et al., 1978), for any alteration in the primary DNA structure employing a number of restriction endonucleases by Dr. Grant McFadden revealed a novel restriction site that was identified by the enzyme EcoRI. Of particular interest was the relationship of this novel restriction site to the phenotype of ts 9251. Spontaneous revertants were selected, cloned twice and subjected to EcoRI analysis. The notion behind the selection of spontaneous revertants was that if altered base sequence is the authentic ts mutation site, then back mutation would restore the sequence generating a wt EcoRI profile, as opposed to that of a 'silent' mutation. All revertants, with the exception of Rev B1 regained the EcoRI digestion pattern of parental wild-type, implying that when ts 9251 reverted to ts, back mutation occurred at either the original ts locus or at a second independent and alternate site. Polypeptide analysis employing two-dimensional electrophoresis revealed a 37K polypeptide with an altered isoelectric point, associated with ts 9251. The alteration in isoelectric point was checked further by subjecting to 2D PAGE mixed extracts from infected cells inoculated with wild-type and ts 9251 or revertants of vaccinia. Whenever 37K polypeptide with an isoelectric point of the mutant virus was induced it could be readily distinguished from that of either wild-type or different revertants.

One may interpret these findings as showing that in revertants A1, C1, and D1 the DNA codes for the same amino acid sequence as that of the equivalent protein of wild-type virus, but in the case of revertant B1, the mutation, and hence amino acid sequence at the ts locus, was retained while a second alteration elsewhere in the cistron appeared which restored the wild-type phenotype linked to the ts ts reversion. Invoking another, less likely, explanation one may suppose that the 37K protein serves as a defective substrate for some thermolabile function induced by ts 9251. The defect in 37K would then be related to modification in the charge associated with infection by ts 9251 but not by any of the revertants. There are 3 distinct genetic pathways by which temperature sensitive mutants of viruses or cells can lose the ts phenotype: (i) true reversion by a mutational event resulting in a restoration of the original wild-type nucleotide sequence; (ii) pseudo-reversion or intragenic suppression by mutation within the same cistron but at a different locus than the original ts-inducing lesion; (iii) extragenic suppression via a second mutation outside the mutated gene. The later two classes are similar in that both retain the original base sequence of the ts site but are phenotypically suppressed by a second mutation. The existence of extragenic suppression in a eukaryotic system has now been well documented in reovirus (Raming et al., 1977; Raming and Fields, 1979). It is shown here that both true and pseudoreversion within the same gene can be demonstrated in ts 9251 of vaccinia which has been mutated into a novel EcoRI site (GAATTC) at the ts locus.

It is the first demonstration of a novel restriction endonuclease site in a mutant of any eukaryotic virus which can be directly correlated with the mutant phenotype by reversion analysis. Previous investigations in other virus systems in which genomes from ts mutants were found to have novel restriction profiles have failed to demonstrate any such correlation with reversion (Vogel et al., 1979). Analysis of the different revertants of ts 9251 documents compelling evidence supporting the notion that the ts lesion resides at a single base pair alteration whereby the altered nucleotide sequences in any one particular revertant may or may not re-establish the sequence present in the wild-type virus.

5.5 Recombination in Group E Mutants:

The identification of phenotypic defects in all 5 ts mutants categorized in group "E" (Stern et al., 1977; Dales et al., 1978; Lake et al., 1979; Essani and Dales, 1979; McFadden et al., 1980), made it feasible to probe the genetic aspects of envelope self-assembly. The relative positions of genes on a recombination map were determined and their relationship with the rifampicin resistance was evaluated. It was particularly intriguing that all these mutants phenotypically mimic the effect of rifampicin (Moss et al., 1969; Nagayama et al., 1971; Pogo, 1971; Stern et al., 1977; Dales et al., 1978; Lake et al., 1979). Since the precise mode of action of this antibiotic in eukaryotic system is not well understood, the selection of rifampicin-resistance as a marker for genetic studies, like those described herein, may be debatable. Our choice of rifampicin-resistance was based on previous observation that incorporation of the

marker for rifampicin-resistance reduced the tightness of ts trait so that the mutants became leaky, and plaque morphology was altered, indicating interaction between two mutations. Nevertheless a tentative map for group E mutations, based on recombination analyses employing three-factor cross strategy, was established (Lake et al., 1979).

The principle underlying genetic mapping by recombination analyses presumes that exchanges occur with uniform frequencies at all points along a chromosome's length (Goodenough and Levine, 1974). Accepting this assumption as "universal truth", one can presume that the further apart two genes are on the chromosome, the more likely it is that they will be separated by a genetic exchange, and therefore the frequency of recombination (expressed as %RF) would be higher, suggesting that two markers are far apart. This fundamental principle has not always been fulfilled in genetic studies of recombination. This implies the occurrence of isolated hot spots on the genome which influence the frequency of recombination in that region (Rosamond, 1980). Such sites, termed Chi, have been detected in bacteriophage lambda. Chi sites are not present in the wild-type bacterial virus lambda, but are found as mutations that arise in response to selective pressure on a specific part of lambda life cycle and are of fundamental importance in recombination (Faulds et al., 1979; McMillin et al., 1974; Malone et al., 1978; Sprague et al., 1978; Stahl, 1979; Stahl and Stahl, 1977; Stahl et al., 1975). It was further suggested that such sites in genetic recombination may be more widely spread than originally thought (Rosamond, 1980). However, to date there is no

direct evidence that such sites exist in vaccinia virus genome, but his phenomenon may explain a preliminary observation of so-called "marker movement", as described later.

In the early phase of genetic studies, attempts were made to standardize the system and evaluate the influence of multiplicity of infection and the duration of incubation on resulting recombination frequency, employing two ts mutants in two-factor cross experiments. It was evident that at lower multiplicities asynchrony in the infection cycle reduced recombination events. Higher recombination frequencies at 36 hours post-infection do not seem to suggest that recombination may occur late during the replication cycle or there may be more than one cycles of a recombination event. Unfortunately, to date no such report describing optimum conditions for recombination in poxvirus mutants is available for comparison.

The recombination frequency values documented in this report seem to be precise and dependable: (a) over all additivity of the RF was satisfactory; (b) all possible crosses in two-factor cross and a wide number of crosses in three-factor cross strategy were examined and each was independently repeated at least 3 times; (c) both ts and rifampicin-resistance (R) markers were highly stable with low reversion frequency; (d) the marker for R was genetically identical in all isolates used; (e) the optimum conditions for recombination were relatively well defined; and (f) the genetic maps generated by two and three-factor cross experiments were virtually identical. The %RF obtained between various crosses varies from 0.4-15.1, and are

analogous to those reported in comparable studies with two factor crosses of poxvirus ts mutants (Padgett and Tompkins, 1968; Ghenden, 1972; Chernos et al., 1978; Condit and Motyczka, 1981).

Although phenotypically all ts mutants classified in group E are identical, the temperature sensitive lesions in the genome seem to be widely spread. The morphogenetic basis of the aberration therefore appears to be even more complex than it was thought before. The position of the marker for rifampicin-resistance, between ts 1085 and ts 7743, which in turn are closely located, deserves more attention. Despite the differences in the genetic maps generated earlier (Lake et al., 1979) and the one outlined in this report, complementation analysis revealed that ts 1085 was capable of complementing all group E mutants, while no complementation was detected between ts 7743 and ts 1085.

The interaction between ts 7743 and R marker was significantly evident during generation of double mutants (ts R). Although, we have been successful in obtaining two isolates which retained the ts trait, one isolate lost the temperature sensitivity and the other acquired cold-sensitivity. However, the suppression of ts trait by introducing a new mutation is not confined to poxviruses. Similar phenomenon has been reported with poliovirus ts mutants (Cooper et al., 1970) and E. coli mutants (Ward and Gottesman, 1981). At present, we are unable to evaluate the significance or mode of interaction between ts 7743 and R mutations, but segregation of two markers in spontaneous revertants obtained from one of the recombinants suggested the presence of ts 7743 lesion and the marker for rifampicin-resistance on two different genes. Apart from three categories of recombinants

(ts 7743R), so-called "marker movement" of ts marker (see Figure 24C) presented a unique problem. Since no conclusive data is available to furnish an explanation, it should only be regarded as a preliminary observation. It may be, however, postulated that a minor error in the recombination event perhaps generates a small set of sequences which can influence the recombination frequency. Such "special sites" have been reported in bacteriophage lambda (Stahl, 1979). The two-dimensional analyses of polypeptides induced by mutants and recombinants failed to reveal any alterations which could be attributed to the rifampicin-resistance.

5.6 Conclusions:

Taken together the results described in this thesis clearly indicate that poxviruses are even more complex than had been anticipated. A number of new viral polypeptides have been revealed and in case of p55E polypeptide a structure-function relationship has been established. In one of the group E mutants (ts 9251) which possesses a novel EcoRI restriction endonuclease site in the fragment D of the parental genome, the ts polypeptide with an altered isoelectric point was identified. Two-dimensional gel analyses with an altered isoelectric point was identified. Two-dimensional gel analyses of ts⁺ revertants derived from ts 9251 documents first demonstration of a novel restriction endonuclease site in a mutant of any eukaryotic virus which can be directly correlated with the mutant phenotype.

Genetic analyses of the group E mutants which are defective in envelope self-assembly at restrictive temperature, employing two and three-factor crosses revealed that the genes participating in envelope assembly are scattered rather than clumped together; suggesting a complex morphogenetic bases of envelope assembly. The locus for rifampicin-resistance which mimics the group E mutations morphologically, and ts 7743 locus was found to be located closely, but on different genes. A variety of interactions between two loci was evident. Though the precise nature of these interactions remains unexplained, the so-called 'movement of markers' perhaps suggest the existence of 'special sites' analogous to chi sites in bacteriophage lambda. The 'special sites' could be generated as a result of mutations. The future attempts into the physical mapping of group E mutations may help identify such sites and their sequences may provide further insights into the biochemical basis of genetic recombination.

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