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**LA THÈSE A ÉTÉ
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EXPRESSION, REGULATION AND PROCESSING OF LATE FUNCTIONS

INVOLVED IN THE

ASSEMBLY OF VACCINIA VIRIONS

by



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Department of Microbiology and Immunology

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

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ABSTRACT

Poxviruses are the most complex eukaryotic viral agents when considered in terms of size, morphology, and replicative strategy. Much of the life cycle of the prototypic member of this family, vaccinia virus, has been studied using metabolic inhibitors and, more recently, conditional-lethal temperature-sensitive (ts) mutants. In this study, biochemical, electron microscopic, and genetic analysis utilizing these tools has led to a greater understanding of the roles, first, of post-translational cleavage (PTC) and envelope assembly and, secondly, of host DNA-dependent RNA polymerase II (polymerase II) and ongoing host gene expression in vaccinia virus biogenesis.

Previously published investigations with inhibitors, such as rifampicin, and ts mutants revealed that PTC of virion core polypeptides is a necessary step for development of infectious, mature vaccinia virus. Present studies focused on the nature of the protease factor(s) required for vaccinia replication. To ascertain whether the proteolytic factor(s) can move freely through the cytoplasm, PTC occurring during complementation between cleavage defective and DNA(-) ts mutants was compared with that evident following induced syncytiogenesis, involving cells singly inoculated with wild-type (wt) and cleavage defective ts1085 virus. Since PTC can occur during coinfection but not after cell-cell fusion, the protease factor is presumed to be nondiffusible. Data from temperature shift experiments

with ts1085 indicate that the factor(s) for proteolysis is probably a short-lived activity. Affinity labeling suggests that the proteolytic factor may be a virus-induced, nonvirion polypeptide, p12.5 in molecular weight, possessing the specificity of chymotrypsin for protease inhibitors TPCK and ZPCK. Evidence indicating that the factor has a brief half-life implies that it must be synthesized on a continuous basis to effect viral maturation.

Five ts mutants of vaccinia virus which mimic the effects of rifampicin, being defective in assembly of the envelope and maturation, were characterized. Although the mutants showed a similar morphogenetic phenotype when examined by electron microscopy, analysis by isotopic pulse-chase experiments in which the viral polypeptides were separated by polyacrylamide slab gels, and complementation titrations between the mutants, indicate that each mutant produced a distinct defect. The data indicate that as many as four gene functions may be involved in the PTC process. A model of vaccinia self-assembly, which takes into account previous observations and current data, is proposed according to which induction of core enzymatic activities, internal differentiation, and acquisition of infectiousness are temporarily coordinated, closely coupled phenomena requiring PTC.

Concurrent with the study on PTC and assembly, the participation of host polymerase II in the vaccinia life cycle was examined by comparing efficiency of multiplication after treating the Ama^r sensitive and Ama. 102 drug resistant lines with amanitin. In the latter, resistance is due to a mutation in polymerase II. The toxin

reduces synthesis of virus-specified polypeptides and abolishes morphopoeisis in Ama⁺ but not in Ama 102 rat myoblasts without appreciably altering vaccinia DNA replication in either cell type. This implicates polymerase II in the expression of late virus functions.

Since the requirement for polymerase II in vaccinia virus replication may involve either viral or host cell gene transcription, the latter hypothesis was examined using intense γ -irradiation. Exposure of monkey BSC-1 or murine fibroblastic L-2 cells to 40 or 70 kilorad of γ -irradiation abolishes DNA and RNA synthesis almost entirely but reduces the formation of protein much less. A dose-response analysis of irradiation shows that synthesis of total RNA and the putative messenger component thereof are equally diminished. Induction of biological functions, namely the vaccinia virus-specified hemagglutinin activity and the interferon-mediated antiviral state were severely inhibited by γ -irradiation treatment. The combined data indicate that intense γ -irradiation is able to suppress host gene expression whether measured in terms of total and putative mRNA synthesis or biological function. However, vaccinia virus, as well as herpes simplex virus and vesicular stomatitis virus, are able to replicate fully in host cells made transcriptionally dysfunctional by γ -irradiation prior to inoculation. From this evidence, it is concluded that the requirement for polymerase II or subunit(s) thereof by a poxvirus is probably related to transcription from the viral, not the host cell, genome.

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LIST OF ABBREVIATIONS

APS	ammonium persulfate
BRdU	bromodeoxyuridine
CI	complementation index
Con A	concanavalin A
cpm	counts per minute
DEP	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA(-)	deoxyribonucleic acid synthesis defective
Do	dose of radiation that will kill 67% of the mammalian cells in culture
dpm	disintegrations per minute
EB	elementary body
EDTA	ethylenediamine-tetraacetic acid
FCS	fetal bovine serum
HA	hemagglutinin
HSV-1	herpes simplex virus type 1
INF	human leukocyte interferon
krad	kilorad
MEM	Eagle's minimal essential medium
MFM	methionine-free medium
m.o.i.	multiplicity of infection
MW	molecular weight

mu	millimicron
NM	nutrient medium
NP-40	Nonidet P-40
oligo(dT)	oligothymidylic acid
p	polypeptide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PFU or pfu	plaque-forming units
p.i.	post-infection
PMSF	phenylmethylsulfonyl flouride
poly(A)	polyadenylated
polymerase II	DNA-dependent RNA polymerase II
PPO	2,5-diphenyloxazole
PTC	post-translational cleavage
RBC's	red blood cells
SDS	sodium dodecyl sulfate
Str A	streptoviticin A
TCA	trichloroacetic acid
TEMED	N,N,N',N',-tetramethylethylenediamine
TLCK	N- α -p-tosyl-L-lysine chloromethyl ketone
TPCK	L-1-tosylamide-2-phenylethylchloromethyl ketone
TRIS	tris(hydroxymethyl)aminoethane
<u>ts</u>	temperature-sensitive

uCi	microcuries
ug	microgram
ul	microliter
UV	ultraviolet
VSV	vesicular stomatitis virus
<u>wt</u>	wild-type
w/v	weight to volume
w/w	weight to weight
ZPCK	N-carbobenzoxyl-L-phenylamine chloromethyl ketone
°	degrees centigrade

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In Dedication To
My Parents

Chapter 1

INTRODUCTION

Poxviruses are the most complex eukaryotic viral agents when considered in terms of size, morphology and replicative strategy (Luria et al., 1978). Elucidation of the scheme of replicative events has been undertaken by means of experiments employing metabolic inhibitors and electron microscopy (Dales and Pogo, 1981). Sambrook et al. (1966) were the first group of investigators to isolate conditional-lethal temperature-sensitive (ts) poxvirus mutants which permitted more detailed study of the replicative cycle of rabbit poxvirus. Other laboratories followed their lead (Padgett and Tompkins, 1968; Basilico and Joklik, 1968; Drillien et al., 1977; Chernos et al., 1978) and in 1978 Dales et al. reported the isolation of over 90 ts vaccinia virus mutants. Of the isolates, 78 were grouped on morphological observation into categories according to an ascending order of complexity reached in development. This broad spectrum of ts mutants has presented a means for incisive

investigations into the nature of vaccinia virus biogenesis.

Of particular interest were a group of mutants, group E, which were observed to mimic the effect of the antibiotic rifampicin on wild-type vaccinia morphogenesis, in that aberrant viral envelopes devoid of an external backing of spicules and having a supple and pleomorphic appearance were formed (Nagayama et al., 1970; Grimely et al., 1970). This antibiotic also inhibited the induction of certain core enzymes (Nagayama et al., 1970) and post-translational cleavage (PTC) of virus-specified precursor polypeptides to structural proteins found in the virion core (Kates and Moss, 1970a and 1970b). Stern et al. (1977) studied one of the group E mutants, ts1085, which under nonpermissive conditions mimicked the effects of rifampicin not only on envelope and virus formation but also the induction of core enzymes and PTC. While all of these defects were reversible upon "shift-down" to the permissive temperature, only assembly of immature virus envelopes could occur in the absence of continuing protein synthesis. Combined evidence from several lines of investigation indicates that PTC of core precursor polypeptides as well as the induction of core enzymes, virus maturation, and infectiousness must be temporally co-ordinated and somehow interconnected events. Data from this and previous studies suggest that proteins related to core enzymes and precursor polypeptides must be packaged within immature particles before PTC and differentiation into the core and lateral bodies, connected with virion maturation, can occur (reviewed in Dales and Pogo, 1981).

Towards the elucidation of the complex series of events leading to vaccinia virus assembly, efforts were turned towards determining what controls the maturation process and the nature of the protease involved in PTC. To this end, the five mutants belonging to group E were characterized to ascertain the extent of PTC in the mutant infections and the number of genes involved in the proteolytic process. Studies were conducted to determine the nature of the interaction of the proteolytic factor(s) with its substrate in vivo and the role of the envelope in this event. In addition, the protease itself was characterized in terms of its substrate specificity, size and location. This examination in considerable detail of the nature and role of the envelope and PTC in the assembly of this agent has culminated in a revised model of vaccinia virus biogenesis.

Concurrent with this investigation on virus assembly, the role of host activities in the expression of late Vaccinia virus functions was of considerable interest. While early virus functions and DNA replication are not dependent on host gene expression (Magee and Miller, 1962; Kajioaka et al., 1974) or even the presence of the nucleus (Prescott et al., 1971), the finding by Pennington and Follet (1974) that virus replication is inhibited in enucleated cells implied that a host nuclear function may be required for vaccinia assembly and maturation. In addressing this possibility, the initial experiments described in this discourse centered on the role of host transcriptional factor(s) in the life cycle of vaccinia virus, employing the toxin α -amanitin, a specific inhibitor of DNA-dependent RNA polymerase II (polymerase II) of animal cells (Roeder, 1976). The

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competence of cells exposed to intense γ -irradiation prior to infection to support virus replication was also examined, in anticipation that such treatment would render the cells transcriptionally dysfunctional. This assumption was critically tested and assessed in terms of the synthesis of total and putative mRNA as well as gene expression. Data from these studies and those conducted by other laboratories have been considered in the formation of a hypothesis concerning the role of polymerase II and ongoing transcription from the nucleus of the host in vaccinia virus biogenesis.

Chapter 2

HISTORICAL REVIEW

The pre-eminence of Poxviruses in the discipline of virology is undoubtedly due to the complexity associated with these agents. The elementary bodies (EB's), which are the extracellular infectious phase in the virus life cycle, are the largest in size and possess the highest degree of morphological intricacy of any eukaryotic virus (Luria et al., 1978). Vaccinia virus is the prototypic member of the genus Orthopoxvirus (ortho: from Greek orthos "correct"; pox: from Old English poc "pustule") consisting of antigenically similar viruses of mammals (Dales and Pogo, 1981). The other genera of the family Poxviridae are Avipoxvirus, Capripoxvirus, Leporipoxvirus, Parapoxvirus and Entomopoxvirus which constitute members of the fowl pox group, sheep pox group, myxoma group, orf group, and insect pox group respectively, and have been categorized by serological cross-reactivity (Matthews, 1979).

The chronology of the genus Orthopoxvirus can be dated back to the reign of Pharoah Ramses V (circa 1160 BC) who is believed to have been a victim of smallpox, caused by the species variola (Joklik, 1980). Indeed, the practice of variolation, a means of imparting immunity to this disease by inhalation of dried pustular material derived from infected individuals, was developed in ancient China. However, it was not until the publication in 1798 by Edward Jenner that a relatively safe and efficient means of prevention, the induction of a localized mild cowpox infection through the practice of vaccination, was described (Dales and Pogo, 1981).

Because of the large size of vaccinia virus, the EB could be observed by light microscopy and was first described by Buist in 1887 (Bland and Robinow, 1939). However, the modern era of poxvirus research did not commence until the technological advancements of the 1930's and 40's of the electron microscope for observation, virus propagation in chick embryos for large scale growth of the agents, ultracentrifugation for purification and analysis, and numerous biochemical and cytochemical techniques for dissection and determination of virus-associated phenomena (Dales and Pogo, 1981). The first definitive review on the biology and biochemistry of poxviruses was published at that time (Smadel and Hoagland, 1942) and has since been augmented by critical surveys by Joklik (1966, 1968), Fenner (1968), Woodson (1968), McAuslan (1969), Moss (1974, 1979) and most recently Dales and Pogo (1981).

2.1 The Virus Particle-Elementary Body

The advent of mass propagation techniques and ultracentrifugation made possible procedures for large scale purification of the EB's. Initially, virus inocula were administered to shaved areas of rabbit skin (Craigie, 1932) or chick embryos (Joklik, 1962). The availability of mammalian tissue culture systems made possible more controlled means of virus propagation from which progeny could be obtained in large quantities. Because the majority of progeny were trapped within the cell, cell disruption using repeated cycles of freezing and thawing (Green et al., 1942), homogenization (Dales and Mosbach, 1968) or ultrasonic oscillation (Stern and Dales, 1974) was an integral step in the purification protocols. After low speed centrifugation to remove nuclei and cellular debris, sedimentation into bands by ultracentrifugation through density gradients of cesium chloride (Planterose et al., 1962), sucrose (Smadel and Hoagland, 1942) or potassium tartrate (McCrea et al., 1961; Stern and Dales, 1974) would yield purified elementary bodies. However, a critical evaluation of purity, using electron microscopic and electrophoretic analysis, would favour the use of potassium tartrate gradients as the best medium for purification of EB's (Stern and Dales, 1974; Essani and Dales, 1979).

2.1.1 Morphology and Physical Composition

While poxviruses can be observed by light microscopy, and using this technique Ledingham (1931) was able to demonstrate agglutination of EB's by antisera, the introduction of the electron microscope made possible a thorough elucidation of the morphological structure of the virion. The rectangular or brick-shaped structure of the vaccinia EB's has a limiting outer membrane, a centric core or nucleoid and two lateral bodies (Green et al., 1942; Sharp et al., 1946; Dawson and McFarland, 1948; Stoekenius and Peters, 1955; Peters, 1956; Epstein, 1958; Dales and Siminovitch, 1961). The limiting outer membrane, or envelope, has been characterized by negative staining and freeze-etching techniques to be covered by surface ridges or tubular elements (Dales, 1962; Medzon and Bauer, 1970) wound around the virion in a left-handed sense (Nagington et al., 1964). The biconcave core is itself enclosed by a proteinaceous capsule (Noyes, 1962b) within which the viral DNA genome (Peters, 1956) appears as tangled fine threads or more concentrated bundles of filaments (Dales, 1963). The physical dimensions of the EB's of vaccinia virus are approximately 235-280 mu in length and 165-225 mu in diameter (Noyes, 1962a; Westwood et al., 1964) and have a mass of 5.5×10^{-15} g per EB (Joklik, 1966). Smadel et al. (1940) and Hoagland et al. (1940) are credited with first determining that the nucleoprotein of the virion contained DNA which has been established by electron microscopic measurements to be $120-130 \times 10^6$ daltons (Geshelin and Berns, 1974) and to comprise 5.6% of the virion by weight (Pfau and McCrea, 1962).

2.1.2 Virion-Associated Proteins

The first analysis of vaccinia EB's by one-dimensional polyacrylamide gel electrophoresis (PAGE) was conducted by Holowczak and Joklik (1967) who reported 17 separable virion polypeptides. This laboratory was able to improve the resolving capability of the technique using longer disc gels and autoradiography and discerned 30 polypeptide bands (Sarov and Joklik, 1972) of which 5 were determined by lactoperoxidase iodination to be at or near the virion surface and 17 were associated with purified cores. Two of the core polypeptides, p62 and p60 accounted for over 50% of the weight of the EB and two proteins were glycosylated and determined to be absent from either the core or the envelope (Holowczak, 1970). Six phosphoproteins were also detected, one of which, p11, is a highly basic histone-like core polypeptide (Pogo et al., 1975). Other investigators have reported only one glycopeptide (Garon and Moss, 1971) and the absence of any phosphoproteins other than p11 (Rosemond and Moss, 1973; Pogo et al., 1975). Modifications to the PAGE system of Laemmli (1970) have resulted in the resolution of 56 bands by Stern and Dales (1976a) who also showed that the surface tubular element of vaccinia EB's consist of a single p58 polypeptide. More recently, the use of two-dimensional electrophoresis, consisting of isoelectric focusing in the first dimension followed by PAGE separation in the second (O'Farrell, 1975), has greatly enhanced the resolving power of electrophoretic analysis of polypeptides. In their original work, Essani and Dales (1979) were able to detect over 110 virion polypeptides ranging in MW from 10,000 to 100,000. Only one

glycoprotein, similar in MW to that reported by Garon and Moss (1971), and only one phosphoprotein, identical to the basic histone-like phosphoprotein reported by Rosemond and Moss (1973) were detected. The power of this analytical technique is underscored by a subsequent investigation in which it was determined that the temperature-sensitive (ts) lesion of a conditional-lethal vaccinia virus mutant manifested itself as a shift in isoelectric point of the p37 virion protein (McFadden et al., 1980).

2.1.3 Virion-Associated Enzymatic Activities

The degree of autonomy in the replicative cycle of a viral agent is determined, for the most part, by its ability to initiate expression of its own genetic information without host factors. Indeed, even if the virus coded for a multitude of replicative functions, it would still have to rely on host factors for their initial manifestation. Vaccinia virus carries within its EB at least 12 enzymatic activities, many of which are involved in transcription of the virus genome (Dales and Pogo, 1981). One of these activities is the virus-specified DNA-dependant RNA polymerase, found in the virion core and later in the cytoplasmic foci of virus replication, which is synthesized as a late function, i.e., after replication of the genome, in infected cells (Kates and McAuslan, 1967; Munyon et al., 1967; Kates et al., 1968; McRea and Szilagy, 1975). Nevins and Joklik (1977a) have purified this activity from infected cell cytoplasm and shown unequivocally that this α -amanitin resistant multimeric enzyme is virus-specified and distinct from the RNA

polymerases of the host, including the α -amanitin sensitive polymerase II involved in cellular mRNA synthesis (Roeder, 1976). Spencer et al. (1980) and Baroudy and Moss (1980) have purified the enzyme from the virion and have found it to be identical to the activity present in infected cells.

In addition to the polymerizing enzyme, other activities involved in the modification of the RNA transcripts are also found in the EB's. A terminal riboadenylate transferase, located in the core, is responsible for the addition of polyadenylic acid tracts to the 3' end of viral RNA following synthesis of the transcripts (Moss et al., 1973). The enzyme has been purified from infected cells and found to be unique when compared to nuclear or cytoplasmic enzymes of the host (Nevins and Joklik, 1977a). The addition of polyadenylic acid does not enhance translation of the viral RNA (Nevins and Joklik, 1975). Five enzymes, polynucleotide 5'-triphosphatase (Tutas and Paoletti, 1977), mRNA guanylyl transferase (Martin and Moss, 1975), mRNA (guanine-7)-methyl transferase (Martin and Moss, 1975), nucleoside-2'-O-methyl transferase (Wei and Moss, 1974) and 5'-phosphate-polyribonucleotide kinase (Spencer et al., 1978) are found in the core and are responsible of the formation of the 5' terminal methylated oligonucleotide or "cap" structure of the viral RNA. The presence of the cap structure on eukaryotic mRNA enhances initiation of translation (Shatkin, 1976) and vaccinia transcripts are no exception (Weber et al., 1977). There also exists, within the EB, an endoribonuclease activity which may be involved in processing high molecular weight precursor polycistronic RNA into monocistronic

transcripts (Paoletti and Lipinskas, 1978).

In addition to the activities involved in the autonomous synthesis of fully functional transcripts, there are, within the EB, three enzymes related to DNA and its functions. Two deoxyribonucleases, one with a low pH optimum and exonuclease activity and the other an endonuclease, were first reported by Pogo and Dales (1969a) and may be involved in removal of the virus genomic cross-links (Pogo, 1977) and/or inhibition of host DNA replication (Pogo and Dales, 1973). Bauer *et al.* (1977) discovered another core enzyme, DNA nicking-closing enzyme, which may have a transcriptional role in its ability to relax the viral superhelical DNA within the core.

The biochemical and electron microscopic cytochemical investigation by Gold and Dales (1968) revealed a nucleoside triphosphohydrolase (ATPase) activity associated with virion cores and a subsequent study by Paoletti and Moss (1974) disclosed the presence of two independent ATPase enzymes. The hydrolysis of ATP may be involved in a number of viral functions including extrusion of nascent transcripts from the core (Kates and Beeson, 1970a). A protein kinase, first discovered by Paoletti and Moss (1972), is also found in the core of the EB's and may be the phosphorylating activity in the production of phosphoprotein(s) mentioned above. Arzoglou *et al.* (1979) have reported a virion associated alkaline protease but the function of this activity is presently unknown.

2.2 Replication of Vaccinia Virus

In the Biology of Animal Viruses, Fenner et al. (1974) describe the major stages in the replicative cycle of lytic viruses. The first stage, penetration, involves the adsorption of the infecting agent to the cell, penetration of the virus into the cell, and uncoating or release of the infectious nucleic acid from the viral coat. During the next stage, termed the eclipse period, early viral transcription and translation precedes the replication of the viral genome. This is followed by late transcription and translation leading to the assembly and eventual release and dissemination of mature infectious progeny.

The attachment of an infecting vaccinia EB to a cell is initiated by a reversible electrostatic bond (Allison and Valentine, 1960) which is followed by an irreversible binding between virus ligands and host receptors, which may involve the surface tubular elements of the virion (Stern and Dales, 1976b). Entry of the particle into the cell usually involves viropexis, which is essentially the phagocytic engulfment of the intact virion (Dales and Siminovitch, 1961). Occasionally, fusion of the viral envelope and plasma membrane at the cell surface (Dales, 1973) or, under some circumstances, fusion of the plasma membrane with Golgi derived membrane enshrouding the EB (Payne and Norrby, 1978) occurs. The release of the core into the cytoplasmic milieu precedes the uncoating process which is directed, in part, by the virus itself (Cairns, 1960; Dales, 1963) during which time, the viral genome passes through breaks in the core coat (Dales, 1965). The viral contribution to this process involves immediate-early transcription of the genome within the core by the

forementioned virion DNA-dependent RNA polymerase (Kates and Beeson, 1970a and 1970b). Following uncoating of the viral DNA, early transcription, again mediated by the core RNA polymerase (Kates and Beeson, 1970a and 1970b), and early translation brings about the formation of viroplasmic foci, or "factories" (Cairns, 1960), in the cytoplasmic compartment. Envelopes are the first virion structures observed and are formed within the factories before the onset of DNA replication (Gaylord and Melnick, 1954; Dales, 1963; Kajioaka et al., 1964). The rigid spherical curvature of the envelopes depends on an external backing of spicules (Dales and Mosbach, 1968) and in the absence of this "exo-skeleton" from the bilayer as during morphogenesis in the presence of the antibiotic rifampicin, the aberrant envelopes appear supple and pleomorphic (Nagayama et al., 1970; Grimley et al., 1970). One of the many unique characteristics of poxviruses is that their envelopes are assembled de novo in contrast to the envelopes of "budding" viruses which are derived from intact host cell membranes, with minor modifications, during development (Dales and Mosbach, 1968; Dales and Pogo, 1981).

Among the 30 early polypeptides synthesized (Pennington, 1974) is the virus-specified DNA-dependent DNA polymerase or replicase (Magee and Miller, 1967), the exonuclease (Eron and McAuslan, 1966) and capping enzymes (Tutas and Paoletti, 1978; Bonne et al., 1977). The genome is replicated in a bidirectional process (Pogo and O'Shea, 1979) involving the formation of small DNA fragments linked by RNA primer sequences (Holowczak and Diamond, 1976) culminating in completely replicated genomes through elongation and ligation of the

DNA fragments (Pogo and O'Shea, 1978). Cross-links between the sister strands of the genome (Geshelin and Berns, 1974) are formed during the final stages of genome replication (Holowczak and Diamond, 1976). Arguments have been advanced that viral DNA replication is not limited to the cytoplasm but may require or occur in the nucleus (LaColla and Weissbach, 1975; Bolden et al., 1979). However, studies conducted by Magee and Miller (1962) and Kajioaka et al. (1974) regarding normal cytoplasmic DNA synthesis in cells pre-treated with the DNA cross-linking antibiotic mitomycin C and the competence of enucleated cells to support viral DNA synthesis (Prescott et al., 1971; Pennington and Follet, 1974; Hruby et al., 1979a) underscore the autonomous replication of the viral genome.

Following the period of maximum DNA synthesis, late transcription commences initiating the production of materials involved in virion assembly and maturation (Dales and Pogo, 1981). The shift from early to late transcription is somewhat loosely controlled as both classes may be found post-replication (Gda and Joklik, 1967; Dahl and Kates, 1970). Generally, however, there is good correlation between the appearance of early and late transcripts and corresponding protein classes (Estabén and Metz, 1973; Baglioni et al., 1978). Over 50 late proteins have been detected in the cytoplasmic factories (Pennington, 1974). Vaccinia-specified virion enzymes synthesized late include the two DNAases (Pogo and Dales, 1969b), the RNA polymerase (Kates et al., 1968) and the nucleotide phosphohydrolase (Pogo and Dales, 1969b).

While the evidence described above supporting the idea of autonomous early transcription is formidable, there has been a major controversy regarding the role of nuclear functions in late transcription (Pennington and Follet, 1974; Bolden et al., 1979; Hruby et al., 1979a and 1979b). The point of contention centers on the role of the host enzyme DNA-dependent RNA polymerase II, found by two laboratories to be obligatory for expression of late functions. (Silver et al., 1979; Hruby et al., 1979b). Whether this host function is involved in viral or host gene expression is one of the subjects of this dissertation.

The penultimate stage of vaccinia virus biogenesis, assembly and maturation, underscores the complexity of this agent. Dales and Pogo (1981) discuss five major events during the maturation process: envelopment of the virus particles, maturation of the envelope, post-translational cleavage (PTC) of virion structural polypeptides, induction of several core enzymes, and internal differentiation of the immature particle first into a nuclear body and finally into the core and lateral bodies of the mature EB.

Virus envelopes, which are backed by spicules and can be observed prior to the onset of DNA replication, are assembled surrounding a quantum of viral DNA plus early and late viral proteins required in the mature virion (Dales and Mosbach, 1968; Nagayama et al., 1970; Pogo and Dales, 1971; Stern and Dales, 1976a). The spherical immature particles, engulfed in a rigid spicule covered envelope, lose their spicules permitting the morphological change of the particle to the brick-shaped EB (Sarov and Joklik, 1973). The spicules are

replaced by surface tubular elements (Essani et al., 1982), one of the late components to be incorporated into mature virions (Stern and Dales, 1976a).

In the interval between formation of the immature particles and displacement of the spicules by the surface tubular elements, a complex series of events occurs internally culminating in the formation of core and lateral bodies. Certain core proteins are derived from higher molecular weight precursor polypeptides (Kates and Moss, 1970a and 1970b). Using tryptic peptide mapping techniques, Moss and Rosenblum (1973) were able to show a precursor-product relationship between polypeptides p94 and p62 and polypeptides p65 and p60 respectively. Stern et al. (1977) found, in addition, that virion polypeptides p23, p18.5 and p18 are also formed by PTC. Moreover, their study, which employed a temperature-sensitive vaccinia virus mutant defective in PTC, revealed that the precursor proteins were incorporated into immature particles in an uncleaved state, suggesting that processing may occur within the particle itself. The mutant used in this study mimicked the effect of the antibiotic rifampicin on wild-type vaccinia biogenesis in that envelope assembly was aberrant due to the lack of attachment of spicules to the envelope bilayer, PTC and maturation were blocked and induction of core-associated late enzymes was interrupted (Nagayama et al., 1970; Grimley et al., 1970; Kates and Moss, 1970a and 1970b). In both cases, viral DNA synthesis and formation of most early and late proteins, with the exception of late cleavage products, occurred normally. Perhaps most intriguing was the finding that reversal of the restrictive conditions, either by

removal of the drug or incubation at the permissive temperature, permitted spicule attachment resulting in normal membrane assembly and induction of core enzymes and PTC. However, the latter two processes were contingent on additional transcription and translation while normal membrane assembly was not. The implication was that normal membrane assembly is a prerequisite for PTC but by itself was insufficient to ensure cleavage. When taken together with the finding that inhibitors of translation alone will block PTC in wild-type infections (Stern and Dales, 1976a), there appears to be a tightly co-ordinated temporal control on the formation of the immature envelope, incorporation of the precursor polypeptides and subsequent cleavage into the virion core proteins culminating in the formation of the mature particle (Dales and Pogo, 1981).

Orthopoxviruses, as exemplified by vaccinia virus, are disseminated from the host cell by two major modes of release: extracellular dispersion or syncytial egress. Extracellular release often involves wrapping the mature EB's in Golgi membranes (Ichihashi et al., 1971; Morgan, 1976a). As Golgi vesicles surround the particle, fusion between the Golgi membranes occurs resulting in an EB wrapped in a double sac. After the wrapped EB has migrated to the cell surface, fusion between the outer Golgi derived membrane and the plasma membrane of the cell occurs, releasing the EB to the extracellular milieu. This mode of release is not particularly efficient since the majority of the progeny remain naked (i.e., unwrapped) and trapped within the cell but may be released by disruption of the host cell (Easterbrook, 1961; Appleyard et al.,

1971).

Both vaccinia and variola viruses give rise to variants with the ability to induce cell to cell fusion or syncytiogenesis (Cassel and Fater, 1958; Kaku and Kamohora, 1964; Ichihashi et al., 1971). This process requires migration of mature virions to the cell surface (Ichihashi et al., 1971) and is believed to be mediated by a host plasma membrane-associated virus-specified protein (Weintraub and Dales, 1974; Weintraub et al., 1977; Payne, 1979). Dales and Pogo (1981) postulate that variants capable of inducing cell-cell fusion may utilize this ability in cell to cell dissemination.

It is evident that vaccinia virus biogenesis displays a high degree of autonomy from host cell functions. From the very onset of the infectious process to the final dissemination of the mature progeny, the virus appears to direct, almost completely, its self-replication. Undoubtedly, the huge coding capacity for a myriad of enzymatic and structural proteins permits this highly autonomous virus to relocate the functional center of the cell from the nucleus to the cytoplasm, its site of replication (Luria et al., 1978).

2.3 Effects of Infection by Vaccinia Virus on

Host Cell Metabolism

Host cell metabolism is profoundly affected after infection by vaccinia virus. Cellular DNA, RNA, and protein synthesis may be suppressed or altered by virion components or virus-specified factors and are temporally dependent on the multiplicity of infection (Dales

and Pogo, 1981).

Host DNA synthesis is rapidly inhibited upon virus infection and may be due to degradation of nascent cellular ssDNA mediated by a virion core enzyme, the neutral pH nuclease (Pogo and Dales, 1973 and 1974). Preformed host dsDNA, however, is not hydrolyzed into reusable acid soluble products and remains essentially intact (Sheek and Magee, 1961; Kit and Dubbs, 1962b; Pogo and Dales, 1974). Parkhurst et al. (1973) have indicated that limited DNA breakdown does occur and may involve the migration of the core endonuclease to the nuclei (Pogo and Dales, 1973).

Kit and Dubbs (1962a) have shown that synthesis of cellular RNA is inhibited commencing 3 hr post-infection culminating in total suppression by 7 hr post-infection. This finding was supported by Becker and Joklik (1964) who also found that transport of cellular mRNA from the nucleus to the cytoplasm is blocked by 3 hr post-infection. Kit et al. (1964) have suggested that suppression of host RNA synthesis may be related to the decrease in uridine kinase activity following vaccinia virus infection.

One of the advantages of studying virus-specified protein synthesis in vaccinia virus infected cells is the rapid suppression of host protein synthesis. Within 20 minutes after inoculation, host protein synthesis is reduced and almost complete inhibition can be achieved within one hour post-infection using a sufficiently high input multiplicity of infection (Moss, 1968; Moss and Saltzman, 1968). A controversy surrounds the mechanism of inhibition of host

protein synthesis and evidence has been presented indicating that virus-specified transcription (Kit and Dubbs, 1962a; Bablanian et al., 1978), translation (Drillien et al., 1978) or indeed, the infecting virion itself (Moss, 1968; Ben-Hamida and Beaud, 1978) may be responsible.

2.4 Genetics

The genetic endowment of poxviruses has been studied using mutants or variants obtained spontaneously or after induction by physical or chemical means. The phenotypic expression of the genetic alterations include degree of virulence, thermosensitivity, pock or plaque type, serological relatedness, capacity for complementation and recombination, host restriction and defectiveness in virus-specific activities and assembly (Dales and Pogo, 1981). As an example, a mutant obtained by Dales et al. (1978), Ts9251, was chemically induced and then selected on the basis of thermosensitivity. After determining that at the restrictive temperature the mutant displayed an aberrant plaque type, electron microscopic analysis revealed a defect in virion assembly. A subsequent investigation revealed that the mutation involved a change in the isoelectric point of a single protein and further that the mutation could be pinpointed to a single base alteration in the genome of the mutant (Essani and Dales, 1979; McFadden et al., 1980). Thus intensive and sensitive analysis of poxvirus mutants is presently possible permitting a thorough elucidation and dissection of the multi-faceted process of virus biogenesis.

2.4.1 Reactivation

Reactivation is the non-genetic process whereby the viable genome of the reactivable virus contained originally in an EB which has been made dysfunctional by heat or high salt protein denaturation can be expressed culminating in the production of its normal progeny only when inoculated into a suitable host in conjunction with a rescuing virus (Dales and Pogo, 1981). The original study on non-genetic reactivation, or the Berry-Dedrick phenomenon, involved the rescue of heat-inactivated myxoma virus by co-infection with rabbit fibroma virus in rabbits which would then develop myxomatosis (Berry and Dedrick, 1936). Indeed, rescue of the reactivable virus could occur even if the genome of the rescuing virus had been inactivated by physical or chemical mutagens (Joklik et al., 1960a and 1960b; Fenner and Woodroffe, 1960). This underscores the non-genetic nature of the reactivation phenomenon because no productive exchange of genetic material or nascent expression can occur. It is postulated that the rescuing virus contributes factors required for uncoating and initial expression of the reactivable virus genome (Dales and Pogo, 1981).

2.4.2 Spontaneous Mutants

Spontaneous mutations can phenotypically manifest themselves as alterations in virulence (Baxby, 1975), host range (Sambrook et al., 1965), thermosensitivity (Shelukhina et al., 1979) and the virus-specified hemagglutinin activity (Weintraub and Dales, 1974). Sharp and McGuire (1970) revealed that virus progeny derived from plaque-purified isolates displayed variability in structure and

infectiousness. This may be explained by the discovery by Wittek et al. (1977 and 1978) and McFadden and Dales (1979) that vaccinia virus stocks derived from cloned isolates contain heterogeneous genomes of varying lengths due to spontaneous non-lethal terminal sequence deletions. As a case in point, a spontaneous mutation of monkeypox, phenotypically expressed as non-hemorrhagic "white" pocks as compared to normal hemorrhagic red pocks (Sambrook et al., 1969) results in a major deletion of the terminal portion of the genome (Archard and Mackett, 1979). While the terminal region of the genome displays significant plasticity, the internal tract is highly conserved not only within a given stock of vaccinia virus but, indeed, among members of the Orthopoxviruses in general (Muller et al., 1977; Esposito et al., 1978).

2.4.3 Chemically-Induced Mutants

Chemical mutagens are capable of producing deletion, insertion, frame-shift and point, both missense and nonsense, mutants (Goodenough and Levine, 1974). Perhaps the most useful are conditional-lethal, temperature-sensitive (ts) mutants which possess a unique defect in each isolate and only single as opposed to double or multiple mutations (Sambrook et al., 1966) because the mutation may be uniquely defined and often phenotypically reversable. Sambrook et al. (1966), employing the chemical mutagen bromodeoxyuridine (BRdU), were the first group to produce poxvirus ts mutants and twenty-six isolates derived from a parental rabbitpox strain were characterized. Other laboratories followed in producing chemically-induced ts mutants.

defective in assembly and maturation (Drillien et al., 1977; Dales et al., 1978; Chernos et al., 1978; Condit and Motyczka, 1981) while other mutants which are drug resistant or dependent (Appleyard and Way, 1966; Subak-Sharpe et al., 1969a and 1969b; Nagayama et al., 1970; Moss et al., 1971; Katz et al., 1973a and 1973b) have been isolated.

A most ambitious project of generating and characterizing a broad spectrum of ts mutants was undertaken by the group of investigators headed by S. Dales. Mutagenesis of purified stock IHD-W vaccinia virus was conducted using N,-N-dimethylnitrosoguanidine and about 90 ts mutants were isolated. The mutants were categorized into 17 classes based on a scheme of ascending progression of development after electron microscopic observation of thinly sectioned infected cells grown at the restrictive temperature (Dales et al., 1978).

Recombination analysis of a group of morphologically similar mutants, group E, indicated that each mutation is located in a separate and distinct locus (Lake et al., 1979; K. Essani, doctoral dissertation, 1982). Further evidence suggesting that each of the 90 mutants may carry a single point mutation comes from the studies of the mutant designated ts9251, in which a specific base alteration occurs at one unique site (McFadden et al., 1980; Schumperli et al., 1980).

Recombination analysis of the aforementioned group E mutants and a subsequent study of a group of mutants defective in DNA synthesis (McFadden and Dales, 1980) has also indicated that genes involved in related functions such as assembly and DNA synthesis respectively, are most probably not located in close proximity on the genetic map. No

doubt further studies employing conditional-lethal temperature-sensitive mutants will be prominent in the elucidation of poxvirus genetics and biogenesis.

Chapter 3

MATERIALS AND METHODS

3.1 Chemicals and Isotopes

L- ^{35}S methionine (sp.act. 100-1010 Ci/mmol; 1Ci=37GBq), U- ^{14}C uridine (350 mCi/mmol), L- ^3H amino acid mixture (NET-250), 5- ^3H uridine (25 mCi/mmol), methyl- ^3H thymidine (77Ci/mmol) were purchased from New England Nuclear Corporation. G- ^3H ZPCK (330 mCi/mmol) was prepared by Amersham Corporation employing random labeling of ZPCK by means of the catalytic exchange method in tritiated aqueous medium. Polyethylene glycol 6000 (PEG) was obtained from BDH Chemical Co., oligo(dT)-cellulose type 3 from Collaborative Research Inc., diethylpyrocarbonate (DEP) from Sigma Chemical Co. and Nonidet P40 (NP-40) from Shell Oil Company. Acrylamide and bisacrylamide, electrophoretic grade, were purchased from Eastman, sodium dodecylsulfate (SDS) and N,N,N',N'-tetramethylethylenediamine (TEMED) from Bio-Rad Laboratories. Human leukocyte interferon (INF) was kindly provided and standardized by Dr. C. Tan, University of

Calgary. The Conavalia ensiformis lectin (concanavalin A type IV) (Con A) and protease inhibitors phenylmethylsulfonylfluoride (PMSF), L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK), N- α -p-tosyl-L-lysine chloromethyl ketone (TLCK), and N-carbobenzoxyl-L-phenylamine chloromethyl ketone (ZPCK), were purchased from Sigma Chemical Co. Streptomycin A was a gift from Upjohn, rifampicin and actinomycin D were obtained from Sigma and the Amanita phalloides toxin α -amanitin was purchased from Boehringer Mannheim. All tissue culture media, antibiotics, and sera were obtained from Flow Laboratories, GIBCO, and Microbiological Associates. Unless otherwise indicated, all remaining chemicals and materials were reagent grade and were purchased from Fisher Scientific Limited.

3.2 Cells and Culture Methods

The majority of experiments employed monolayer cultures of the L-2 substrain of mouse L-929 fibroblasts (Rothfels et al., 1959). A clone of a rat myoblast line, L6H9, designated Ama⁺ and an α -amanitin-resistant mutant, Ama 102 derived therefrom, both kindly provided by M.E. Pearson (University of Toronto) and the monkey BSC-1 cell line, obtained from Connaught Laboratories, Toronto, were also used. All cultures were maintained in nutrient medium (NM) consisting of Eagle's (1959) minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FCS), 100 units/ml penicillin and 100 ug/ml streptomycin. Monolayer cultures, on plastic culture dishes (Falcon Plastics) were incubated at 37° in Hotpack incubators gassed with a

humidified mixture of air and 5-10% carbon dioxide. Suspension cultures of L-2 cells, between 2×10^5 to 1×10^6 cells/ml, were maintained as described above with gentle agitation in NM supplemented with 0.1% (w/v) methylcellulose (c.p.s. 15; Dow Chemical).

3.3 Viruses: Prototype and Mutant Virus Isolates

The IHD-W subtype, isolated initially from the IHD-J parental strain by Hanafusa et al. (1959) was the prototypic vaccinia strain used in this study. This subtype is distinguished from its hemagglutinin-inducing parental strain by its inability to express at the cell surface an active hemagglutinin and by its ability to elicit polykaryocytosis in mammalian and avian cells (Ichihashi and Dales, 1971; Weintraub and Dales, 1974). Dales et al. (1978) have derived numerous diverse temperature-sensitive (ts) mutants from the IHD-W subtype using the mutagen N,N'-dimethylnitrosoguanidine, which lend themselves to a more thorough elucidation of vaccinia biogenesis. Ninety mutants have been grouped into 17 categories according to the complexity in morphological development reached at the restrictive temperature of $39-40^\circ$. The mutants used during this study consist of the five members of the E category, ts1085, ts7743, ts9203, ts9251 and ts9383 as well as the single member of the C category, ts6389. A brief phenotypic description of these mutants has been presented in Table I. Virus development of members of the E group is blocked at about the same stage; numerous viroplasmic foci are formed, each surrounded by flexible envelope segments but which are devoid of spicules, and DNA paracrystals accumulate. The C category mutant,

TABLE I

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

Category	Description	No.	Relative ^c PFU/ml 33°/40°
C	Rudimentary virus membranes with spicules in foci of viroplasm and defective in DNA synthesis.	6389 ^d	2,100:1
E	Foci of viroplasm, aberrant membranes without spicules and accumulation of DNA paracrystals.	1085 ^e 7743 ^d 9203 ^d 9251 ^d 9383 ^d	100:1 100,000:1 1,000:1 81,000:1 100,000:1

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

b, adopted from Dales et al., 1978.

c, based on two or more independent titrations.

d, mutants selected after mutagenesis with nitrosoguanidine and replicated in the presence of bromodeoxyuridine.

e, mutants selected after mutagenesis with nitrosoguanidine.

ts6389, exhibited foci of viroplasm containing curved segments of envelope backed externally by a layer of spicules and was found to be defective in viral DNA synthesis (Dales et.al., 1978). In this study, the syncytogenic IHD-W subtype of vaccinia virus is designated as wild-type (wt).

The Indiana strain of vesicular stomatitis virus (VSV) originally obtained from P.W. Choppin (Rockefeller University, New York) and the herpes simplex virus, type 1 (HSV-1), originally a clinical isolate from herpes keratitis obtained through L. Hatch (Victoria Hospital, London, Ontario) were also used in this study.

3.4 Propagation of Viruses

Monolayers of L-2 cells were used for IHD-J, IHD-W, ts mutants or VSV propagation. In the case of vaccinia virus and its mutants, the cells were infected with one plaque-forming unit (pfu) per cell; i.e., the multiplicity of infection (m.o.i.) was 1. Following an adsorption period of 1 hr at 4⁰ (Pogo and Dales, 1971), the unadsorbed inoculum was removed with repeated washings with MEM, fresh NM was added and the cultures incubated at the appropriate temperature for 24-36 hr. The infected cell monolayer was scraped with a rubber policeman and the cells were concentrated by centrifugation at 160g for 10 min. The cells were resuspended in a small volume of MEM diluted 1:4 using sterile-deionized water and were kept on ice for 20 min. to allow for cell swelling. The swollen cells were homogenized in a tightly-fitting Dounce homogenizer using approximately 25 strokes. The homogenate was subjected to 500g for 5 min. to pellet

nuclei and large cellular debris, from which the supernatant fraction was pooled with the NM recovered from the infected cells. This pooled virus suspension was centrifuged at 2000g for 20 min. in a Sorval high-speed centrifuge using a SS-34 rotor. The virus in the supernate was concentrated by high-speed centrifugation at 50,000g for 60 min. and resuspended in a small volume of NM. This crude virus preparation, for use as a virus stock for further infections, was divided into aliquots and stored frozen at -70° . Prior to titration of this virus stock, an aliquot was thawed at 37° and then sonicated on ice for two-30 second pulses in a sonicator (type Sonicup SE101, Sorensen, Zurich) to disperse virus particles which had aggregated into clumps during storage at -70° .

L-2 cells, used for propagation of VSV, were inoculated at an m.o.i. of 0.1 pfu/cell. After the inoculum had been allowed to adsorb for 1 hr at 37° , the unadsorbed virus was removed, fresh NM was added and the infected cells incubated at 37° for 18-24 hr. The medium was removed and spun at 2000g for 20 min. in a Sorval high-speed centrifuge using a GSA rotor to remove intact cells and cellular debris. The supernatant fraction was dispensed into 3 ml aliquots and stored at -70° . Prior to titration and experimentation, clumps of virus particles were dispersed using a single 30 second sonic pulse.

BSC-1 cells were used for propagation of HSV-1, using an inoculum containing 0.1 pfu/cell. After a 1 hr adsorption period at 37° , the unadsorbed virus was washed away from the monolayer, fresh NM was added and the virus was allowed to replicate at 37° for 18-24 hr. The medium was then centrifuged at 2000g for 20 min. in a GSA rotor. The

supernatant fraction was divided into 3 ml aliquots and stored at -70° . These aliquots were thawed at 37° and sonicated on ice for a 30 second period prior to titration.

3.5 Titration of Viruses

Titration of vaccinia virus and VSV were conducted using L-2 cells while HSV-1 was assayed using BSC-1 cells. Monolayer cultures were grown in 6-well dishes (Falcon Plastics or Costar) to confluence, approximately 1×10^6 cells per well, and were inoculated in duplicate with 0.2 ml of the appropriate ten-fold serial dilution of thawed and sonicated stock virus, using MEM as the diluent. After a 1 hr adsorption period at 4° , during which the dishes were gently rocked to evenly disperse the inoculum and to prevent cell drying, 3 ml of overlay medium, consisting of NM supplemented with 0.5% methylcellulose (4000 c.p.s.), was added. Cells infected with vaccinia virus were incubated at either 33, 37, or $39-40^{\circ}$ as indicated for 48 hr while HSV-1 or VSV inoculated monolayers were incubated at 37° for 36 hr. The overlay medium was then carefully removed, the monolayers fixed with 2 ml of neutral buffered formalin for 10 min. and then stained with 1 ml of 0.1% crystal violet. After removing the staining solution, the monolayers were gently washed in tap water and dried. Using an illuminated magnifying glass for enumeration, the plaques appeared as clear areas of cell lysis on a purple stained monolayer.

3.6 Isotopic Labeling and Vaccinia Virus Purification

To prepare isotopically labeled vaccinia virus, monolayer cultures of L-2 cells, grown in 100-mm plastic dishes (Falcon), were inoculated with an m.o.i. of 10-20 pfu/cell. After allowing for virus adsorption at 40° for 1 hr with gentle rocking, the inoculum was removed and the monolayers washed twice with MEM. The cultures were then incubated for 24 hr at 37° in the presence of 5 μ Ci/ml [³⁵S]methionine in MEM containing 5% FCS and one-third the normal concentration of methionine. The cells were harvested by scraping, mixed with 2 X 10⁷ unlabeled infected cells, which acted as carrier material, and crude virus was prepared as described earlier. The virions were then purified as described by Stern and Dales (1974) with some minor modifications. Briefly, the crude virus preparation was layered onto 2 ml of 1.46 M sucrose in 10 mM Tris-HCl (pH 8.1) and centrifuged at 50,000g for 90 min. in a Beckman ultracentrifuge using a SW-56 rotor. The resulting pellet was resuspended by sonication in a small volume of 0.42 M potassium tartrate in Tris-HCl (pH 8.1) and subjected to gradient centrifugation at 50,000g for 60 min. through a 0.85-2.13 M potassium tartrate solution prepared in 10 mM Tris-HCl (pH 8.1). The virus band was removed, diluted in the Tris-HCl solution and sedimented by centrifugation for 30 min. at 50,000g. The presence of cellular debris in the final purified virus preparation was checked by electron microscopy.

CYTOLOGICAL METHODS

3.7 Cell-Cell Fusion

Fusion between infected cells was initiated by a modified method of Mercer and Schlegel (1979). Briefly, semiconfluent (1.5×10^6 cells/plate) 60-mm monolayer cultures of L-2 cells were inoculated with ts1085 at a m.o.i. of 10. Following 10 hr incubation at the nonpermissive temperature, $39-40^\circ$, the monolayers were washed with MEM and placed for 1 hr at 39° in NM containing 10 ug/ml of Con A to enhance cell-cell contact with the 3×10^6 cells/dish added from suspension. The latter had, prior to plating, been infected with either wt or ts1085 at a m.o.i. of 10 and incubated at 39° for 9 hr. The mixed adherent and added cells were incubated for 1 hr at 39° to establish more stable contact. The Con A-containing medium was then removed and replaced for 1 min. by carefully adding 1 ml of MEM containing 44% polyethylene glycol (PEG) and 10% dimethyl sulfoxide (DMSO), prepared as described by Davidson and Gerald (1976). Upon removal of the PEG-containing medium, by washing three times with prewarmed MEM + 10% DMSO, each plate received 5 ml of NM and incubation continued at 33 or 39° for 8 hr prior to harvesting cells for electron microscopy, analysis using polyacryamide gel electrophoresis (PAGE), or enumeration of polykaryocytes.

Efficiency of fusion was ascertained after fixation in situ with 2% glutaraldehyde and staining with Giemsa. For enumerating nuclei following fusion, cells were released from monolayers by trypsinization, disrupted in lysis buffer (1% NP-40, 10 mM Tris-HCl,

pH 7.2, and 1 mM magnesium chloride) and the free nuclei centrifuged into pellets at 800g for 5 min. The pellets were washed twice with phosphate buffered saline (PBS) (Dulbecco and Vogt, 1954), resuspended in 10 ml PBS and the nuclei enumerated using a hemocytometer. Cultures prepared as described above with the omission of PEG were used as controls.

3.8 Assays for Vaccinia Hemagglutinin Titer

Hemagglutinin (HA) was assayed as described by Ichihashi and Dales (1971) with minor modifications. Monolayer cultures of L6H9 rat myoblasts, either Ama⁺ or Ama 102, (10 cells/plate) were infected with IHD-J at a m.o.i. of 5 pfu/cell as described earlier. The infected cultures were incubated in the presence or absence of metabolic inhibitors to 20 hr p.i. The cells were washed with PBS lacking calcium and magnesium salts (PBS-A), scraped and spun into pellets at 1000g for 10 min. The cell pellet was resuspended in 0.25 ml of PBS-A supplemented with 1% FCS. Cell rupture, using a 60 second sonic pulse, was monitored by phase-contrast microscopy and 50 ul of two-fold serial dilutions of the cell lysate was dispensed into a 96 conical-well microtitration plate (Linbro). To each well was added 50 ul of White Leghorn chicken red blood cells (RBC's) which were prepared as follows: the RBC's were washed three times and resuspended in 200 times their packed volume using PBS-A plus 1% FCS. After gentle agitation the plates were incubated at 37° for 1 hr before the HA end point agglutination titer was determined.

3.9 Electron Microscopy

Methods for collecting and preparing cell samples, thin sectioning and examination by transmission electron microscopy were the same as those described by Dales and Mosbach (1968). 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) or were removed from the plastic using cold (0-4°) 0.12% trypsin solution in PBS. The trypsin was inactivated by adding NM and gentle aspiration using a Pasteur pipet released the cells. The cells were spun into pellets at 500g for 5 min. and fixed with 2% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4. Samples fixed in situ or after release and centrifugation were post-fixed with 1% osmium tetroxide prepared in 0.05 M phosphate buffer (pH 7.4). Dehydration through an ethanol series (50-95%) was preceded by a single PBS wash and followed by final dehydration in two washes of absolute ethanol in the case of cells fixed in situ or two washes of propylene oxide when the cells were pelleted. The samples were then embedded in epoxy resin (Epon) prior to sectioning (60-100 nm) with diamond knives using a Porter-Blum MT2-B ultramicrotome. The thin sections were post-stained with alcoholic uranyl acetate followed by Reynold's (1963) lead citrate solution and examined using a Phillips EM 300 operated at 80 kV.

3.10 Inhibitors of Virus Replication

Synthesis of RNA was suppressed by adding NM containing 4 ug of actinomycin D per ml to infected cells at the times indicated (Shatkin, 1965). Similarly, protein synthesis was inhibited with streptoviticin A (Str A) (Ennis and Lubin, 1964) at a concentration of 10 ug/ml of NM unless otherwise stated (Dales and Mosbach, 1968). Rifampicin, an antibiotic which simultaneously blocks vaccinia virus morphopoesis and PTC in a reversable manner (Nagayama et al., 1970) was used at a final concentration of 100 ug/ml. The toxin α -amanitin was used at concentrations which specifically inhibit DNA-dependent RNA polymerase II (polymerase II) (Roeder, 1976). Cells were exposed to 2-10 ug/ml of α -amanitin from 10 to 24 hr prior to and during virus infection, as indicated, according to Somers et al. (1975a). Human leukocyte interferon (INF), kindly provided and standardized by Dr. C. Tan, University of Calgary, was used to induce an antiviral state in BSC-1 cells. Monolayer cultures, each containing 1.2×10^6 cells, were treated with 100 units/ml of INF for the indicated duration. After removal of the INF by repeated washings with MEM, the monolayers were inoculated with VSV at a m.o.i. of 0.1 pfu/cell. Following incubation for 10 hr p.i., the infectious virus formed was assayed as plaque-forming units on monolayers of L-2 cells. Cell monolayers untreated with INF but otherwise incubated under identical conditions were used as the controls for replication of VSV. The protease inhibitors PMSF, TLCK, TPCK, ZPCK, and ^3H ZPCK were dissolved in DMSO and used in MEM containing 1% DMSO. Cell monolayers were washed twice with MEM containing 1% DMSO prior to treatment with the protease

inhibitors.

3.11 γ -Irradiation of Cells

Confluent or nearly confluent monolayers grown for 3 days after subculture were placed on ice and irradiated using a ^{60}Co source (Gamma cell 220, Atomic Energy Canada, Ltd., calibrated to deliver 110 rad/second; 1 rad = 1×10^{-2} J/kg). The NM was then replaced and cultures were returned at incubators and kept at 37° for at least 1 hr prior to experimentation.

3.12 Determination of Cellular Macromolecular Synthesis using Isotopic Labeling

Rates of host cell DNA, RNA, and protein synthesis were measured as follows: The NM was removed, the monolayers were washed with PBS and incubated at 37° for 1 hr in MEM containing |methyl- ^3H | thymidine (1 uCi/ml), |5- ^3H | uridine (1 uCi/ml), or | ^{35}S | methionine (1 uCi/ml). The monolayers were placed on ice and isotopes were removed by washing twice with cold PBS. Cells were harvested by scrapping into 2 ml of PBS and following addition of SDS at a final concentration of 1%, the cell suspension was agitated on a vortex mixer to effect cell rupture. Macromolecules were precipitated by addition of TCA at a final concentration of 10%. Before precipitating cell material labeled for DNA and RNA with TCA at 0° , calf thymus DNA was added as carrier (final concentration 40 ug/ml). The precipitates were trapped on glass fiber filters (Reeve-Angel 934AH), washed sequentially with 10% TCA and 95% ethanol, and placed in vials containing toluene-based

scintillation fluid. For determining counts per minute (cpm) a Beckman LS-350 scintillation counter, with an efficiency for ^3H of 46%, was employed. In some instances, cell material was kept frozen at -20° , then thawed prior to precipitation with TCA.

3.13 RNA Extraction and Oligo(dT)-Cellulose Chromatography

To facilitate mRNA selection on oligo(dT)-cellulose columns, the nascent RNA was labeled with $|5\text{-}^3\text{H}|$ uridine or $|U\text{-}^{14}\text{C}|$ uridine (2.5 $\mu\text{Ci/ml}$) at 37°A for 2 hr as described above. Cell cultures were washed once with ice-cold PBS before RNA extraction. To facilitate comparisons between different samples, aliquots of $|U\text{-}^{14}\text{C}|$ uridine-labeled material, derived from unirradiated L-2 cells, were mixed with samples of the $|5\text{-}^3\text{H}|$ uridine-labeled BSC-1 cells prior to RNA extraction.

RNA was extracted using the guanidine-HCl technique of Strohman et al. (1977). Four 100-mm cell monolayers were scraped on ice directly into 2 ml/plate of ice-cold solution A, consisting of a 19:1 mixture of 8 M guanidine-HCl:2 M potassium acetate (pH 5.0). The plates were rinsed with 1 ml of solution A carried through the four plates. The suspended cells were homogenized on ice in a loose-fitting Dounce homogenizer using 10 strokes, precipitated with ethanol (final concentration 60%) and placed at -20° overnight. The mixture was then centrifuged at 2500g for 10 min. in a Sorval high-speed centrifuge using a SS-34 rotor. The precipitate was carefully drained and resuspended in 5 ml of solution B, consisting of a 19:1 mixture of 8 M guanidine-HCl:0.5 M EDTA (pH 7.0). After

resuspending the pellet using a vortex mixer, 2 M potassium acetate (pH 5.0) was added to a final concentration of 0.1 M and ethanol, one-half the volume of the mixture, was added for precipitation at -20° overnight. The precipitate was pelleted by centrifugation at 10,000g for 10 min. using a HB-4 rotor, resuspended in 1 ml of 20 mM EDTA (pH 7.0) and extracted with 3 ml of chloroform:butanol (4:1). After gentle mixing and brief centrifugation to separate the phases, the aqueous phase was removed and the organic phase re-extracted twice with 1 ml EDTA. The pooled aqueous phases were precipitated with sodium acetate (pH 6.0) (final concentration, 3.0 M) and placed at -20° overnight. The suspension was spun at 12,000g for 1 hr in a HB-4 rotor to pellet the precipitate, which was then washed with 95% ethanol. After air drying, the precipitate was dissolved in 1 ml of distilled water and made 0.2 M in potassium acetate. Ethanol was added to a final concentration of 67% and the suspension stored at -20° overnight.

The purified RNA was washed twice in 95% ethanol and applied to oligo(dT)-cellulose columns for chromatography by a modified method of Avim and Leder (1972). Approximately 0.3 mg of RNA, dissolved in binding buffer containing 10 mM Tris-HCl (pH 7.5) and 0.5 M KCl, was applied to 0.1 g of oligo(dT)-cellulose packed into columns, previously washed with binding buffer. Elution of the unabsorbed material by continuous washing with binding buffer was followed by elution of bound material using 10 mM Tris-HCl (pH 7.5). Calf thymus DNA was added as carrier to eluted fractions and the samples were precipitated with TCA as previously described. All reagents used in

the RNA extraction and oligo(dT)-cellulose chromatography were previously treated with DEP to minimize activity of RNase.

3.14 Determination of Cytoplasmic DNA Synthesis in Vaccinia Virus-Infected Cells

Cytoplasmic DNA synthesis in IHD-W-infected cells was measured by continuous labeling at 37° for 4 hr after inoculation with |methyl-³H| thymidine (1 uCi/ml) as described by Pogo and Dales (1971). Monolayer cultures of 3 X 10⁶ cells/plate were inoculated at a m.o.i. of 10 as described previously. After exposure to the isotope, the cells were washed three times with ice-cold MEM, scraped and resuspended in a solution containing 0.25 M sucrose and 10mM KCl. The labeled cells were allowed to swell, then were disrupted in a Dounce homogenizer. The cell homogenate was separated into a supernatant cytoplasmic fraction and a crude nuclear fraction by centrifugation at 800g for 5 min. TCA-precipitation of the cytoplasmic fraction was conducted as described above and its radioactivity measured in a scintillation counter.

3.15 Determination of Nuclear and Cytoplasmic RNA Synthesis in Vaccinia Virus-Infected Cells

To ascertain rates of |5-³H|uridine (10 uCi/ml) incorporation into RNA of pre-irradiated (70 kilorad), vaccinia virus-infected cells, 60-mm monolayers of L-2 cells were pulse-labeled for 15 min. at 37° commencing 8 hr p.i. The labeled cultures were immediately chilled to 0°, washed twice with ice-cold PBS, and lysed in lysis

buffer containing 1% NP-40, 10 mM Tris-HCl (pH 7.4) and 1 mM magnesium chloride by agitation on a vortex mixer. Nuclei freed by the procedure were centrifuged into pellets at 800g for 5 min. The pellets were washed with PBS and resuspended in lysis buffer. SDS was added at a final concentration of 1% to both the nuclear and supernatant cytoplasmic fractions. The TCA-precipitable material was prepared as described previously and uridine incorporation into macromolecular material measured in a scintillation counter.

3.16 Determination of Protein Synthesis in Vaccinia

Virus-Infected Cells

The effect of protease inhibitors on rates of protein synthesis in vaccinia-infected cells was determined as follows: Suspensions of 2×10^7 L-2 cells in 1 ml NM were inoculated for 1 hr at 4° with wt vaccinia virus at a m.o.i. of 10, then were washed twice with MEM to remove unadsorbed virus, resuspended in 50 ml of NM containing 0.1% methylcellulose, and incubated at 37° for 9.5 hr with agitation. The cells were collected into pellets by centrifugation at 800g for 10 min., washed once with MEM, and resuspended in 50 ml MEM containing final concentrations of 1% DMSO and 0.1% methylcellulose. Str A or one of the protease inhibitors was added to duplicate 1 ml aliquots and the samples incubated with agitation at 37° for 1 hr in a water bath. ^{35}S methionine was added at a final concentration of 0.25 $\mu\text{Ci/ml}$, incubation continued at 37° for 1 hr, whereupon the samples were cooled and kept on ice during addition, with simultaneous mixing on a vortex mixer, of SDS at a final concentration of 1% and

bovine serum albumin at a final concentration of 0.1 mg/ml. Protein was precipitated with 10% final concentration of TCA as described above and the incorporation of isotopically labeled methionine into macromolecular material was measured in a scintillation counter.

3.17 Preparation of Isotopically Labeled Cytoplasmic Extracts for Polypeptide Analysis

Labeled cytoplasmic extracts were prepared by infecting monolayer cultures with vaccinia virus, 10 pfu/cell, as previously described and incubating the cultures prior to, during, and following labeling at 33, 37 or 39-40° as indicated. Virus polypeptides were labeled with ^{35}S methionine (10-25 uCi/ml) or ^3H amino acids (10 uCi/ml) from 9 to 10 hr p.i. unless otherwise stated. In the case of methionine labeling, infected cells were incubated for 30 min. in methionine-free MEM medium (MFM) prior to exposure of the cells to the label which was also dissolved in MFM. The pulse was terminated by washing the monolayers with prewarmed NM and the chase medium used after methionine labeling contained five times the normal concentration of methionine. In experiments involving cell-cell fusion, the adherent ts1085-infected cultures were labeled prior to initiation of the fusion process.

The labeled cells were gently scraped away from the plastic, washed in PBS and disrupted by agitation in 100 ul of lysis buffer as described above. The cell lysate was either treated with 50 ug of pancreatic deoxyribonuclease I per ml (Worthington) for 15 min. at 4° to hydrolyze the DNA or was fractionated into crude nuclear and

supernatant cytoplasmic fractions by centrifugation at 800g for 5 min. No protein degradation was observed during either of these procedures. TCA-precipitable material contained in the samples were assayed for radioactivity by previously described methods.

3.18 In Vitro Post-Translational Cleavage Assay

Monolayers of L-2 cells, 1×10^7 cells/plate, were infected with wt or ts1085 vaccinia virus and labeled with ^{35}S methionine as described in the previous section. Following exposure to the radioisotope, the monolayers were washed twice with cold PBS and gently scraped away from the plastic, on ice, into cold PBS. The cells were spun into pellets and resuspended in 0.5 ml of lysis buffer lacking NP-40. The labeled cells were allowed to swell, then were disrupted in a Dounce homogenizer. The supernatant cytoplasmic fraction was obtained by centrifugation at 800g for 5 min. which was then divided into four 0.1 ml aliquots. An equal volume of identically prepared unlabeled cytoplasmic extract derived from wt-infected cells was added to each sample containing labeled material. Dissociation buffer (see section 3.20) was added to stop any polypeptide hydrolysis immediately or following incubation of the samples for 4 hr at 33, 37 or 39°. NP-40 (final concentration 0-5%), deoxycholate (final concentration 0-5%), calcium chloride (final concentration 0-50mM), 2-mercaptoethanol (final concentration 0-25mM) and/or Tris-HCl (pH 6 or pH 9) (final concentration 25mM) were/was added to the mixture of labeled and unlabeled material at the start of the in vitro incubation period. After the addition of the

dissociation buffer, the samples were boiled for 2 min. and stored at -20° to await SDS-PAGE analysis.

3.19 Labeling of Polypeptides with ^3H ZPCK

Four 100-mm monolayer cultures were infected with wt or ts1085 vaccinia virus at a m.o.i. of 25. At 10 hr p.i., the NM was removed, the monolayers washed once with MEM containing 1% DMSO and ^3H ZPCK at a final concentration of 10^{-4} M was added to the cells. After 3 hr incubation at 33, 37, or 39° , cells were harvested by scraping, washed twice with MEM + 1% DMSO, and disrupted in lysis buffer as described above. The cytoplasmic supernatant fraction was layered on a 1% deoxycholate, 10 mM Tris-HCl (pH 7.4) cushion and centrifuged at 10,000g for 20 min. to concentrate into pellets the virus "factories" (Pogo and Dales, 1969) which were then used for SDS-PAGE analysis.

The ability of the radiolabeled ZPCK compound to inhibit PTC had been confirmed prior to its use in labeling experiments.

3.20 SDS-Polyacrylamide Gel Electrophoresis

Cell extracts and purified virus were prepared for polyacrylamide gel electrophoresis (PAGE) by mixing 1 volume with an equal volume of dissociating buffer (70 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.001% bromophenol blue). The samples were boiled for 2 min. The dissociated proteins were separated by electrophoresis on 15-cm 11% polyacrylamide slab gels of 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. The ratio of acrylamide to

bisacrylamide was 30/0.8. The polymerization of the gels was initiated by adding 100 ul of 10% ammonium persulfate (APS) and 20 ul of N,N,N',N'-tetramethylethylenediamine (TEMED) per gel. The stacking gel consisted of 4% acrylamide in 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS, and the electrophoresis buffer was 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. Samples were adjusted to give comparable amounts of radioactive label and loaded into 8 mm-wide wells. The gels were run at a constant current of 25 mA until the tracking dye reached the bottom of the gel (Studier, 1973). In the case of ^3H ZPCK labeling experiments, 10-20% continuous gradient gels were employed and initiation of polymerization was achieved using 50 ul of 10% APS and 10 ul of TEMED per gel.

Following electrophoresis, the gels were fixed and stained for 2 hr in a 50% methanol, 7% acetic acid, and 0.025% coomassie brilliant blue R solution and destained in a 50% methanol and 7% acetic acid solution.

3.21 Fluorography and Autoradiography

Following the destaining process, SDS-PAGE gels were prepared for autoradiography or fluorography. Autoradiograms were produced by re-hydrating the gels in water for 1 hr, drying on Whatman 3MM filter paper, employing a slab gel dryer (Model 224, Bio-Rad Laboratories), then exposing the gel to Kodak NS-T2 film. The fluorographic technique of Bonner and Laskey (1974), used to amplify latent images in autoradiograms, involved the dehydration of the destained gels in DMSO. The gels were then impregnated in a 20% (w/w)

2,5-diphenyloxazole (PPO) DMSO solution for 3 hr, re-hydrated in water for 1 hr, dried and exposed to Kodak X-Omat (PR/R) film at -70° .

GENETIC ANALYSIS

3.22 Checkerboard Series of Complementation Among Group E and DNA(-) Mutants

Complementation assays were done in monolayer cultures of L-2 cells grown in flat-bottomed glass tubes (3×10^5 cells). The five assembly-defective ts mutants (ts 1085, ts7743, ts9203, ts9251, and ts9383) and the ts mutant defective in viral DNA synthesis (ts6389) were diluted to deliver 40 pfu/cell. The mutants were mixed in every pairwise combination before inoculation. The mixed infections received 20 pfu/cell of each mutant (total of 40 pfu/cell), and the single infections 20 pfu/cell. Following an adsorption period of 1 hr at 4° , the unadsorbed inoculum was removed with repeated washings with prewarmed MEM (40°) and the cultures incubated at $39-40^{\circ}$ for 24 hr. The virus yield from each infection was titrated at 33° and the complementation index (CI) for each cross calculated as follows:

$$CI = (A \times B) / A + B ,$$

where (A X B) is the yield of the mixed infection titrated at 33° , and A and B are the yields of the single infections titrated at 33° . A complementation index greater than 3 was considered a positive indication of complementing gene functions.

3.23 Detailed Complementation Analysis Between ts1085 and ts6389

Complementation between ts1085 and ts6389 was tested using 35 mm monolayer cultures (1×10^6 cells/plate) singly or doubly infected with each mutant at a m.o.i. of 5 pfu/cell to measure rescue in terms of infectivity and at a m.o.i. of 10 for SDS-PAGE analysis of virus polypeptides. After adsorption at 40° for 1 hr, the unadsorbed inoculum was removed by repeated washings of the monolayer with MEM. After a 1 hr incubation period at $39-40^\circ$, uneclipsed virus was neutralized with NM containing antivaccinia serum during a further 1 hr incubation at 39° . The antiserum was removed by repeated washings with prewarmed MEM (39°) and cultures either harvested immediately or after a further incubation for 22 hr at 33 or 39° for determination of progeny yield by plaque assay on L-2 cells at 33° . Cultures used for virus polypeptide analysis were maintained throughout at $39-40^\circ$ and were pulse-labeled with ^{35}S methionine ($10 \mu\text{Ci/ml}$) from 9 to 10 hr p.i. as described previously. Samples were either harvested immediately or 8 hr later after incubation in chase medium. Particle counting by quantitative electron microscopy (Dales, 1963) was made on singly or doubly infected 60 mm monolayer cultures (3×10^6 cells/plate) inoculated with individual mutants at a m.o.i. of 2. Data was obtained by examining 100 thinly sectioned cell profiles per sample.

3.24 Isolation and Identification of Progeny Derived after
Complementation

The phenotype of progeny derived after complementation from the mixed infections with ts1085 and ts6389 at 39-40° was checked by isolation of individual progeny and SDS-PAGE analysis. At 24 hr p.i., cultures inoculated with both mutants and incubated at 39° were harvested and the mixed infection lysate was prepared using the protocol for crude virus preparation. The lysate was then diluted and plated on 100 mm monolayers of L-2 cells. After a 1 hr adsorption period at 4°, unadsorbed virus was removed by repeated washings with MEM and the infected monolayers were incubated at 33° under overlay medium until some areas of limited cell fusion was observed. Individual, well separated plaques were removed by suction into a Pasteur pipet and the material removed was placed in a tube containing 1 ml of NM. The plaques were grown into virus stocks and subjected to SDS-PAGE analysis as described previously.

Chapter 4

RESULTS

4.1 Requirement for Post-Translational Cleavage During Vaccinia Virus Maturation

Previously published investigations with inhibitors (Nagayama et al., 1970) and a temperature-sensitive (ts) mutant (Stern et al., 1977) revealed that post-translational cleavage (PTC) of virion core polypeptides is a necessary step for development of infectious, mature vaccinia virus. A chance selection of a group of conditionally lethal ts mutants of vaccinia, group E, (Dales et al., 1978) which morphologically mimic the effects of the antibiotic rifampicin on wild-type (wt) virus morphogenesis has allowed an examination in considerable detail of the nature and the role of the envelope and PTC in virus assembly culminating in a revised model of vaccinia biogenesis.

4.1.1. Phenotypic Characterization of E Group Mutants

The five isolates employed in this study belong to the E category in the spectrum of assembly mutants selected previously by Dales et al. (1978). The mutant designated ts1085 was produced after mutagenesis with N,N'-dimethylnitrosoguanidine (N-G) while the other mutants, ts7743, ts9203, ts9251 and ts9383 were selected after N-G treatment of a virus inoculum followed by replication of this inoculum in the presence of bromodeoxyuridine (BUdR).

When replicating at 40° this group is characterized by the aberrant formation of virus envelopes (Figures 1-4). Usually these envelopes consist of a bilayer, unit membrane backed externally by a layer of spicules which endow the envelope with rigidity and a curvature so as to form spheres enclosing immature particles (Dales and Mosbach, 1968). When the ts defect was expressed in the extreme, only flexible sheets of unit membrane, like those identified by arrows in Figures 2 (inset) and 3 were evident while the rigid, curved segments were almost entirely missing. More usually, however, the envelopes of normal structure, containing spicules, were contiguous with sheets of flexible membranes as in ts1085 described previously (Stern et al., 1977) and shown in Figure 4 and in ts9203, illustrated in Figure 1. The accumulation of DNA paracrystals like those shown in Figure 3 was another common finding with group E mutants. The aberrations with ts1085 and ts9203 are very similar to those that develop in the presence of the drug rifampicin (Nagayama et al., 1970; Moss, 1974).

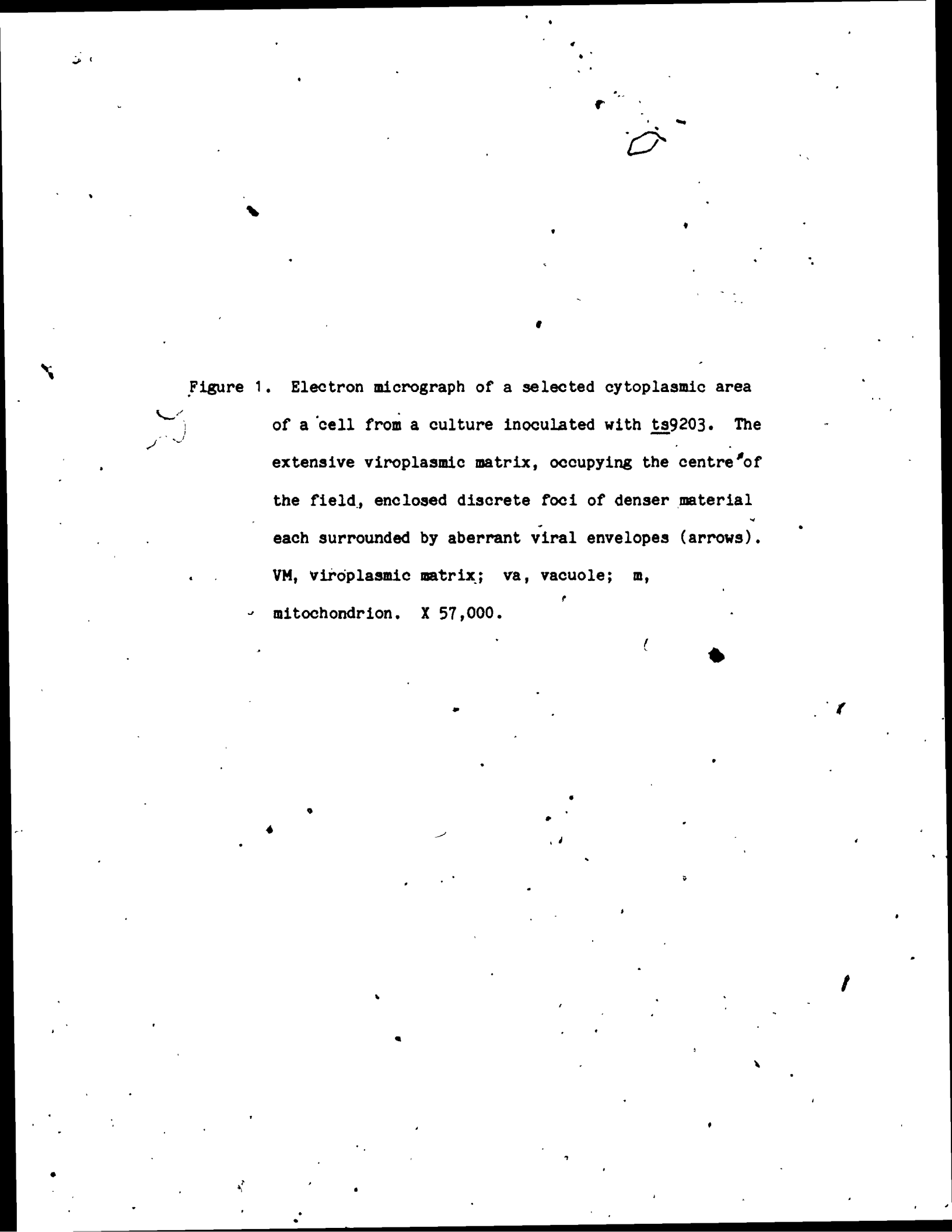


Figure 1. Electron micrograph of a selected cytoplasmic area of a cell from a culture inoculated with ts9203. The extensive viroplasmic matrix, occupying the centre of the field, enclosed discrete foci of denser material each surrounded by aberrant viral envelopes (arrows). VM, viroplasmic matrix; va, vacuole; m, mitochondrion. X 57,000.



10µm

30

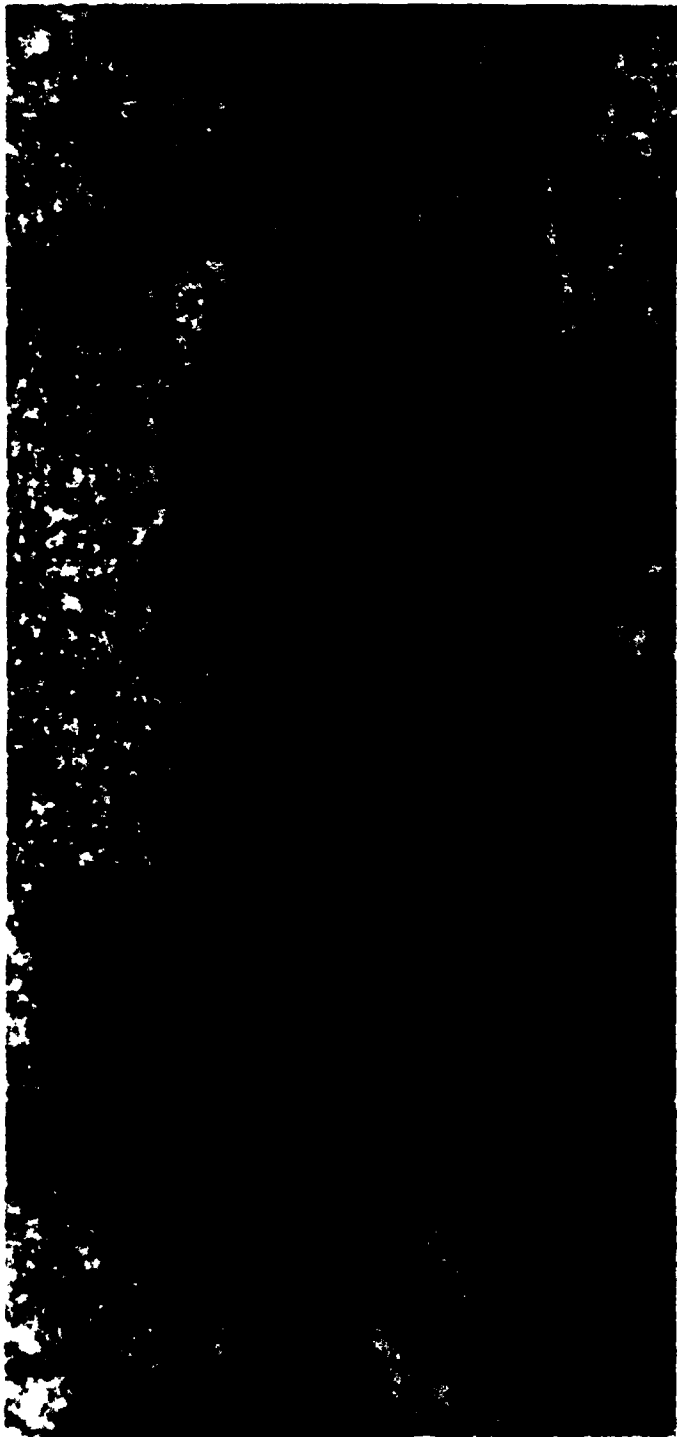
3

Figure 2. Other examples showing selected areas of cytoplasm from cells infected with ts9251. In the inset, the viroplasmic matrix is enclosed by aberrant envelopes indicated by arrows. The extensive viroplasmic matrix occupying the centre area contains aberrant as well as normal (I) immature forms of vaccinia virus. The mature particles, identified by arrows, illustrate that occasionally there was a breakthrough whereby morphogenesis progressed all the way to maturation. VM, viroplasmic matrix; m, mitochondrion. X 31,500. Inset X 54,000.

Figure 3. Similar example of Figure 2. illustrating the appearance of virus structures following infection with ts7743. Development generally stopped prior to the assembly of complete, rigid, spherical envelopes, as evident from the presence of flexible membranes (arrows) and DNA paracrystals. However, a few immature particles of normal appearance (I) were visible. VM, viroplasmic matrix; va, vacuole. X 52,000.



Figure 4. Example of a selected cytoplasmic area of a cell from a culture inoculated with ts1085. The viroplasmic matrix or factories (F) contain aberrant virus envelopes (arrows) which appear as flexible "unit" membranes devoid of spicules. Occasionally, normal immature envelope structures (arrowheads) can be observed. X 72,000.



With one of the mutants under study here, ts7743, envelope formation conformed more to the normal process, as evident by the presence of numerous partially complete envelopes and even some spherical particles (Fig. 3). Mutants ts9251 and ts9383 behaved in an anomalous manner at 40°, because both abnormal envelope malformations and development of mature virions were observed (Fig. 2). Therefore, from the point of view of morphogenesis these mutants must be termed leaky. However in terms of infectivity, as measured by PFU, ts9251 and ts9383 are tight mutants (Table I) (Dales et al., 1978).

Previous studies with ts1085 (Stern et al., 1977) indicated that the defects, as with rifampicin (Moss and Rosenblum, 1973) were related to a block in PTC. Since the five ts mutants studied appeared to be arrested at about the same stage of development (Dales et al., 1978), it was necessary to determine whether the late proteins were processed normally at the restrictive temperature.

The polypeptide patterns for the five ts mutants along with an identically treated DNA defective mutant, ts6389, and the wt-infected cells are shown in Figures 5 and 6. Incidentally, polypeptides mentioned hereafter will be identified by their molecular weight, so that p100 is a polypeptide migrating in the described system with an approximate molecular weight of 100,000 daltons. The polypeptide patterns produced by all the ts mutants at 33° were essentially similar to the profile obtained with wt virus at 40°. More specific differences were identified in the cytoplasmic extracts prepared from ts mutant infections at 40°. The processing of the major core polypeptides, p62 and p60, from their higher molecular weight

Figure 5. Autoradiogram of a slab gel prepared from infected cytoplasmic extracts of L-2 cells and purified virus. Cultures were inoculated with wt, ts1085, ts7743, or ts9251 vaccinia virus and incubated at the designated temperatures. Each culture was pulse-labeled 10 hr p.i. with 25 uCi/ml ^{35}S methionine and further incubated for 8 hr in chase medium. The influence of temperature shifts on the processing of individual polypeptides is evident. In each case, the temperature during labeling is the upper number of the ratio and the temperature during the chase is the lower number. The vertical scale showing molecular weight $\times 10^{-3}$ was calculated from polypeptides used as MW standards and is also drawn on the basis of known molecular weights of several major virion polypeptides (Stern et al., 1977). The arrows denote the positions of the seven polypeptides considered in this analysis: p94, p65, p62, p60, p23, p18.5, and p18. Channel marked V = purified vaccinia virus.

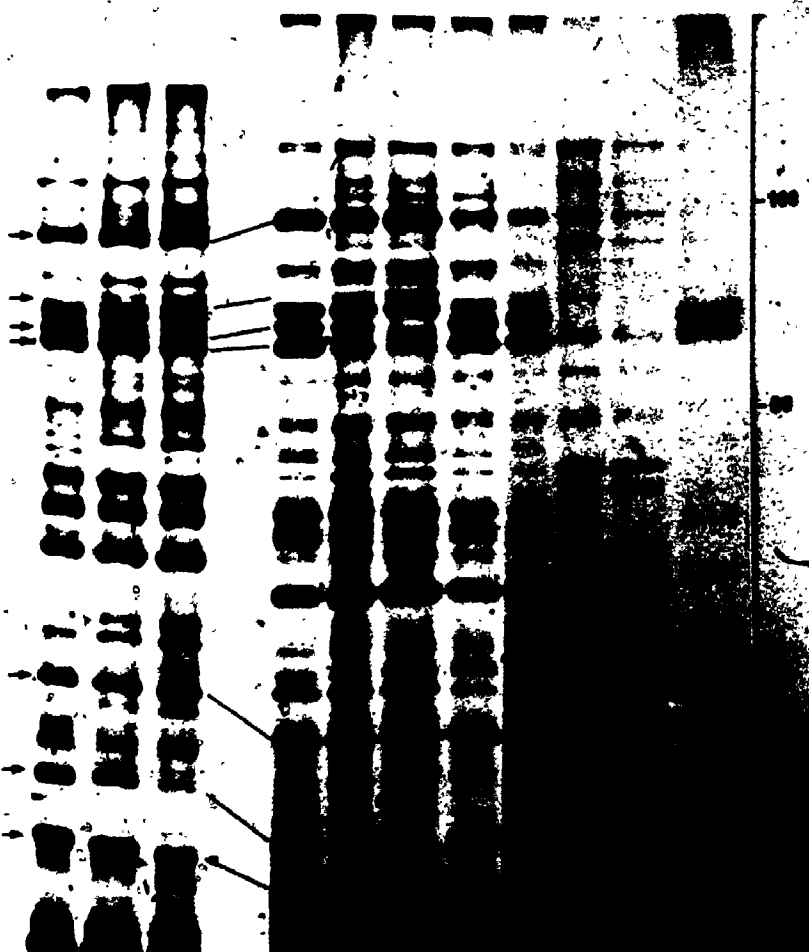
Figure 6. Autoradiogram of a slab gel similar to that shown in Figure 5. showing labeled polypeptides occurring in the cytoplasmic extracts of cells infected with ts9383, ts9203, and ts6389. Infected cultures were labeled for 1 hr with ^{35}S methionine at 10 hr p.i. followed by an eight hr chase period. The temperature during labeling is the upper number of the ratio and the temperature during the chase is the lower number. The arrows denote the positions of p94, p65, p62, p60, p23, p18.5, and p18, respectively. Channel V: purified vaccinia virus.

18 9383
33 40 40
33 33 40

18 9203 WI
33 40 40 40
33 33 40 40

18 6389
33 40 40
33 33 40

V

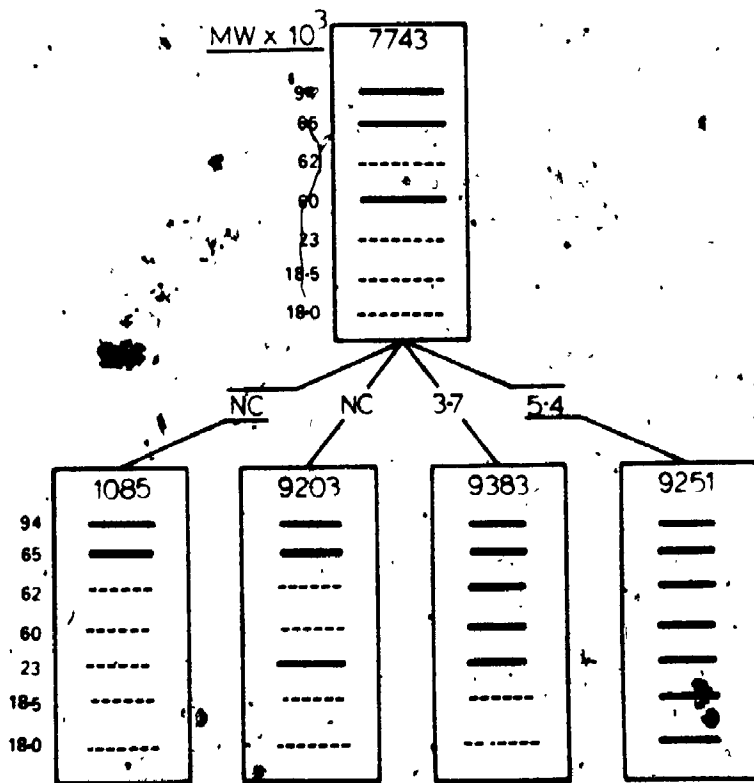


mol. wt. x 10⁻³

precursors, p94 and p65, respectively, has been shown previously to be defective for ts1085 (Stern et al., 1977). In the current experiments, the precursor p94 was present in all the mutant infections at 40°. Processing of this precursor to the core polypeptide p62 was affected in infections with ts1085, ts7743, and ts9203, but not with ts9383. Accumulation of the precursor p65 was also evident at 40° in all the cytoplasmic extracts from infections with the 5 ts mutants, but processing to p60 was blocked or reduced in quantity in the case of ts1085 and ts9203. In the case of three other polypeptides, p23 was reduced or absent from ts1085 and ts7743, while p18.5 and p18 were missing or present in small quantities in all but ts9251. These results indicate that the defect in PTC was different in the case of each mutant, diagrammatically presented in Figure 7. The polypeptide pattern for infections with ts9251 appeared to be an anomaly to the proposed grouping of the ts mutants on the basis of their morphological appearance under the EM, since some morphologically mature progeny were found and both precursors were apparently processed with equal efficiency at 33 and 40°. However, it is not known whether one or both products are fully functional, because little infectious progeny appeared at 40° despite the presence of mature-like particles (Fig. 2). The variability in processing indicates that either precursor can be cleaved in the absence of the other, so that the enzymology of the reaction is not as tightly coupled as the assembly process.

If the infected cells were labeled at 40°, but incubated for a further 14 hr at 33°, all the precursors were processed normally.

Figure 7. Schematic representation of the processing of virion polypeptide precursors p94 and p65 during infection with individual ts mutants. ———, Normal or accumulated amount of polypeptide; -----, polypeptide absent or present in reduced amount. The rates of complementation between ts7743 and the four other mutants ranged from 5.4 to no complementation (N.C.).



PRECURSOR - PRODUCT
RELATIONSHIP

indicating that the cleavage defects were related to a temperature sensitivity.

The pattern of polypeptide bands produced by ts6389 was typical of that expressed in a mutant defective in DNA synthesis (Pennington, 1974). Only the early, prereplicative polypeptides were evident.

4.1.2 Complementation Between Group E and ts6389 Mutants

To determine whether the ts mutations of group E were localized in different or identical genes, a checkerboard series of complementation experiments was undertaken in collaboration with J.L. Lake. Although large variability was recorded from experiment to experiment involving specific crosses, complementation was repeatedly observed with most of the mutants. A complementation index greater than 3 was taken as positive evidence that genetic interaction had occurred. The mutants ts1085, ts9203, ts9251, and ts9383 readily complemented each other, and all gave high values when crossed with the DNA(-) ts6389. Since ts9251 was apparently not defective in proteolytic cleavage, the crosses with this mutant were discounted as concerning determination of the number of genes involved in group E defects. Most dual infections yielded good complementation, ranging from 8.8 to 143 (Table II). The strikingly high value obtained in the cross ts9251 X ts9383 may in some way be related to the leakiness of the phenotype, described above.

In mixed infections of the group E mutants involving ts7743, complementation was either very poor or nonexistent. On the other

TABLE II

Complementation Between the Mutants

Mutant	<u>ts1085</u>	<u>ts7743</u>	<u>ts9203</u>	<u>ts9383</u>	<u>ts9251</u>	<u>ts6389</u>
<u>ts1085</u>	—	N.C.	8.8 ^a	62	3.2	33
<u>ts7743</u>		—	N.C.	3.7	5.4	52
<u>ts9203</u>			—	49	8.4	50
<u>ts9383</u>				—	143	53
<u>ts9251</u>					—	38
<u>ts6389</u>						—

a, The complementation indices (average of two replicates) were calculated by dividing the mixed infection yield with the sum of the corresponding single infections.

N.C., No complementation detected.

hand, a cross between ts7743 and ts6389 outside this group gave high complementation values. Since ts7743 was able to both complement outside group E defectives and did not interfere with replication of wt vaccinia virus, there was no evidence to indicate that it might be a "trans-acting dominant mutant" (Carstens and Weber, 1977). Our observations could be explained if one assumes that ts7743 mutation was being expressed in a polar fashion with respect to the functions necessary for post-translational cleavage.

The high degree of complementation between members of the group E mutants, with the possible singular exception of ts7743, indicated that each mutation occurred in a distinct locus. In an attempt to more fully elucidate the role of the envelope and PTC in vaccinia virus assembly, the complementation between one member of the group E mutants, ts1085, and the DNA(-) mutant, ts6389, was studied in detail. In agreement with the findings presented earlier in Table II, the data on the yield of progeny, expressed as formation of PFU, indicated that these two mutants can complement, the complementation index, as shown in Table III, being 19.5. While this value was lower than that given in Table II, the experimental protocol used in these separate experiments was somewhat different; specifically, the input multiplicity of infection here was one-fourth of that used in generating the data presented in Table II. Any possibility that the increase in PFU after dual infection resulted from appearance of revertants with wt phenotype was eliminated by analysis of plaque-purified progeny, as described below. Complementation data on PFU were corroborated by particle counting in the electron microscope.

TABLE III

Stages in virus assembly during single and dual infection with ts mutants

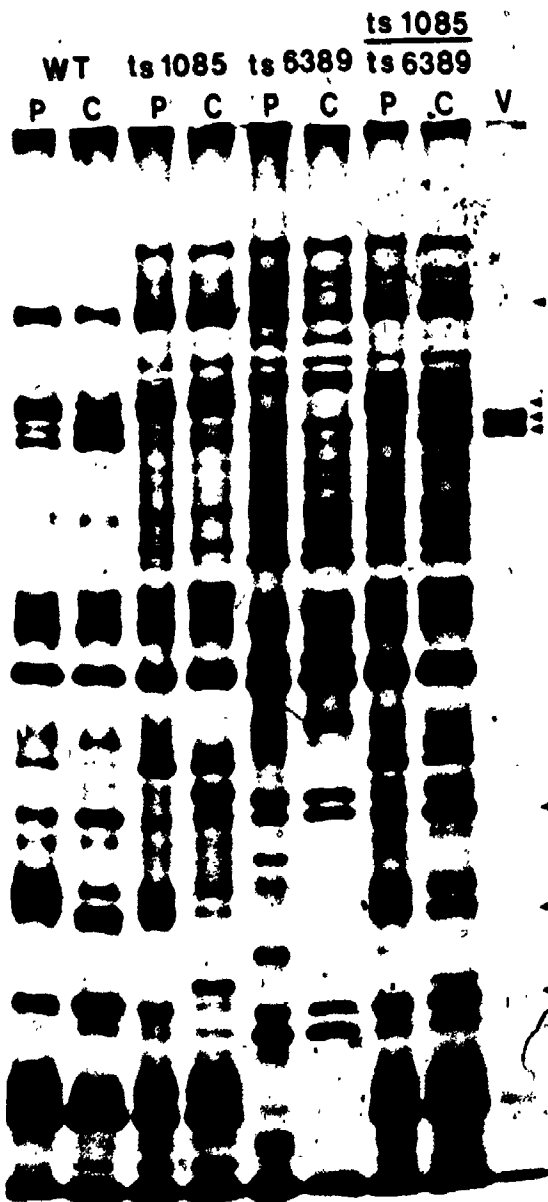
temp.	Virus titer ^a		Electron Microscopy ^b			
	pfu/ml x 10 ⁻⁵		Foci of Vioplasm (Factories)		Immature particles	Mature particles
			early	late		
33°	650		0	65	1091	2667
39°	1.3		8	51	360	42
33°	120		20	38	516	1154
39°	0.4		59	7	159	48
39°	33		14	35	211	477

Confluent 60-mm culture plates were infected at an m.o.i. of 2 with ts1085 or ts6389 or 2 of each of the two mutants, incubated at 33° or 39° for 24 hr and sampled for virus titer and electron microscopy.

- a. Virus titers were determined by plaque assay on L-2 cells at 33°.
- b. The electron microscopy data were obtained by examining 100 thinly sectioned cell profiles per sample.
- c. Early factories were those from which recognizable virion structures were absent.
- d. In this category were included complete spherical particles and immature forms with incomplete membranes.

Various stages of virus development, evident in thinly sectioned cells, were enumerated according to the procedure of Dales (1963). It is clear from the data in Table III that dual infection with ts1085 and ts6389, under nonpermissive conditions, produced about five times more mature virions than did the sum of infections with individual mutants. By contrast there was no increase, even perhaps a reduction, in the number of incomplete and complete immature particles following dual infection (Table III). Defectiveness of ts6389 in DNA synthesis was reflected in the number of early factories, i.e., those which did not contain any recognizable virion structures. Rescue of one ts mutant by the other became also evident from enhanced PTC of all precursor polypeptides, as demonstrated by means of SDS-PAGE and autoradiography. Gels such as that shown in Figure 8 revealed, first of all, that in cells infected with wt vaccinia virus and harvested immediately after the pulse-labeling with ^{35}S methionine, the cleavage products p62, 60, 23, 18.5, and 18 were present in low amounts but increased in quantity after an 8 hr chase at 39-40°. With ts1085, also evident in Figure 8, PTC was blocked effectively during the chase at restrictive temperature. In the case of ts6389, the DNA mutant, the precursor polypeptides being late functions (Stern *et al.*, 1977) did not appear during the pulse-labeling and therefore failed to generate cleavage products during the chase at 39°. Following dual infection with ts1085 and ts6389, p94 and p65 precursors were synthesized, then underwent normal processing as evident from the presence in Figure 8 of the five cleavage product bands following the 8 hr chase under nonpermissive conditions.

Figure 8. Rescue of the defect in post-translational cleavage by complementation during dual infection. Autoradiograph was prepared from cytoplasmic extracts following single or double infections with ts1085 and ts6389. Cultures maintained throughout at 39 were pulse-labeled with ^{35}S methionine 9 to 10 hr p.i. and either harvested immediately (P) or 8 hr later (C), after incubation in chase medium. Arrowheads indicate positions of p94, p65, p62, p60, p23, p18.5, and p18, respectively. Channel V: purified vaccinia virus.



To establish that enhanced PTC and mature virus production were, indeed, the consequence of complementation, not reversion of mutated virus to the wt phenotype, progeny from dual ts1085 + ts6389 infection at 39° were inoculated after suitable dilution onto L-2 cell monolayers and allowed to form plaques under methylcellulose. Twenty individual plaques were picked, most were grown into virus stocks, and subjected to SDS-PAGE analysis, reproduced in Figure 9. Among progeny from randomly isolated plaques, six displayed a protein banding profile identical with that of ts6389 and four with that of ts1085 confirming the evidence for bonafide complementation.

Taken together, the data from the complementation experiments provide evidence for close linkage between development of mature, infectious virions, and normal PTC.

4.1.3 Attempt to Transfer Capacity for PTC by Cell-Cell Fusion

The above results showing that different ts mutants can act in a complementary manner to facilitate PTC raised the questions concerning the mobility and intracellular site where factor(s) required for processing may be active. Experiments designed to answer these questions involved coalescence of cytoplasmic compartments from cells singly infected with cleavage defective ts1085 and wt vaccinia virus. Cultures inoculated with ts1085, and maintained at the restrictive temperature were pulse-labeled with [³⁵S] methionine for 1 hr commencing at 9 hr p.i., while companion cultures, infected independently with wt vaccinia virus were incubated for 10 hr without protein labeling. Then cells, in the process of infection by the wt


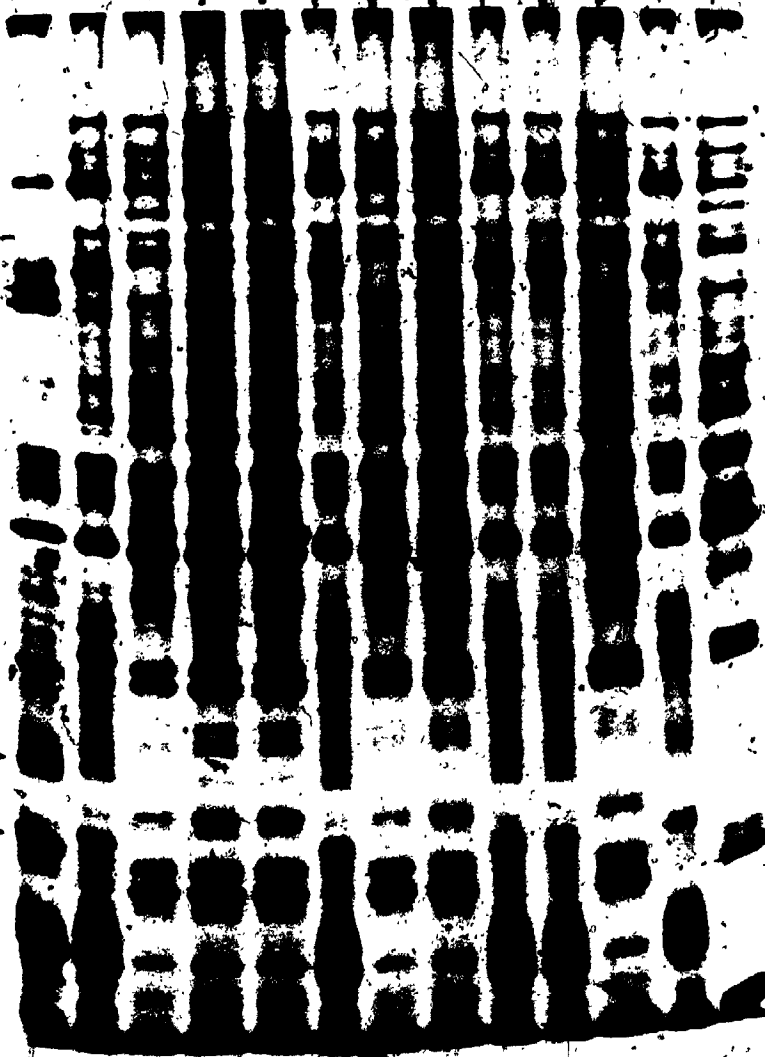


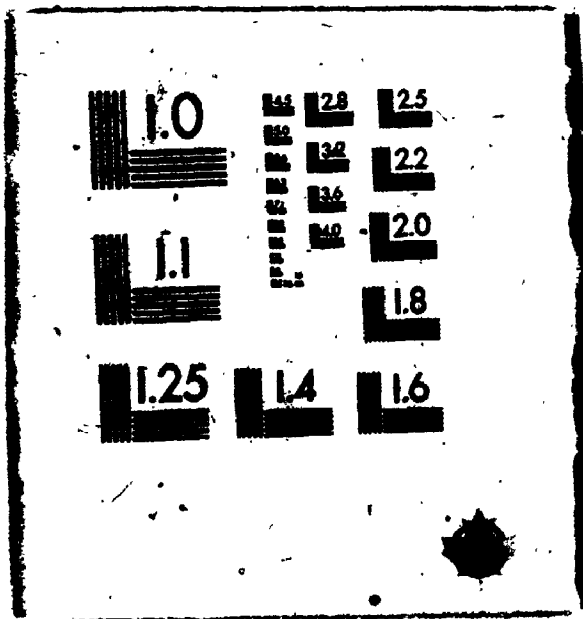
Figure 9. Protein profile phenotypes of progeny derived from co-infection with ts1085 and ts6389. Autoradiograph was prepared from cytoplasmic extracts following infection with individual stocks of plaque-purified progeny obtained originally from the dual infection incubated at the restrictive temperature. Cultures maintained throughout at 39° were pulse-labeled with ^{35}S methionine 9 to 10 hr p.i. and further incubated for 8 hr in chase medium. Arrowheads indicate positions of p94, p65, p62, p60, p23, p18.5, and p18, respectively.

WT 1085 6388

1085/6388 PROCEED



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and mutant viruses, were induced to fuse under the influence of PEG, as described under Materials and Methods, and incubation resumed at 33 or 39-40°.

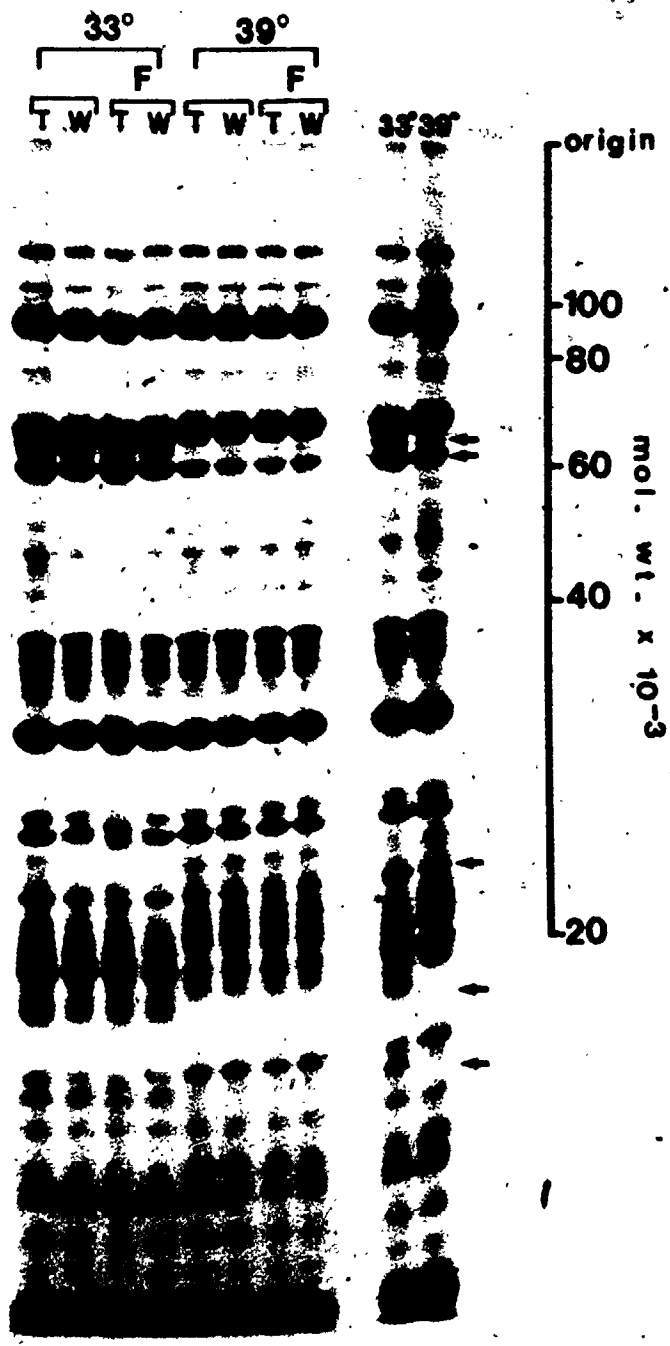
An electron microscopic image of a typical "hybrid" polykaryocyte from a culture maintained at the nonpermissive temperature is shown in Figure 10. The cytoplasm of such fusion-induced polykaryocytes contained viroplasmic foci characteristic of both wt and ts1085 infections and numerous mature particles. Approximately one-third of all cells present on the monolayer were bi- or multinucleated. Fusion also produced a 48% increase in the total number of nuclei in the monolayer culture as compared to monolayers not subjected to cell-cell fusion but otherwise treated similarly. The morphological observations indicated that extensive fusion between cells, independently infected with ts1085 or wt vaccinia virus, must have occurred.

To ascertain whether it is possible to transfer a factor(s) from wt-infected cells which elicits PTC of ts1085 proteins at 39° by means of cell-cell fusion, extracts from labeled cells were analyzed by PAGE. It is necessary to point out that the bulk of virus polypeptides resides within the isolable virus factories (Stern et al., 1977). It was evident from the band pattern in Figure 11 that, regardless of induced fusion, polypeptides synthesized after infection with ts1085 underwent PTC at 33° but failed to do so at 39°. This result indicated that any factor(s) involved in proteolysis of vaccinia core polypeptides which were active during infection with the wt could not effect PTC of ts1085 precursor proteins concentrated in

Figure 10. Electron micrograph of a selected area of a thinly sectioned polykaryocyte. Cell-cell fusion mediated by PEG involved cultures independently infected at 39° with ts1085 or wt vaccinia virus. Following coalescence of cytoplasm from the two infections, both aberrant factories (F), typical of ts1085 development, and normal immature (I) and mature (M) virions, associated with wt infection, were evident. N, nucleus; m, mitochondrion. X 14,250.



Figure 11. Failure to rescue the ts1085 defect in PTC by cell-cell fusion induced with PEG. Autoradiograph was prepared from extracts of cells inoculated with ts1085, which were inoculated, pulse-labeled with ^{35}S methionine, and then fused (F) with either wt-infected (W), unlabeled cells or with ts1085 infected (T), unlabeled cells at 39° , as described in Materials and Methods. After formation of the polykaryocytes, incubation was continued at 33 or 39° . The two channels at the extreme right represent material from cells infected with ts1085 which had not been subjected to fusion with PEG. Arrows are at positions p62, p60, p23, p18.5, and p18, respectively.

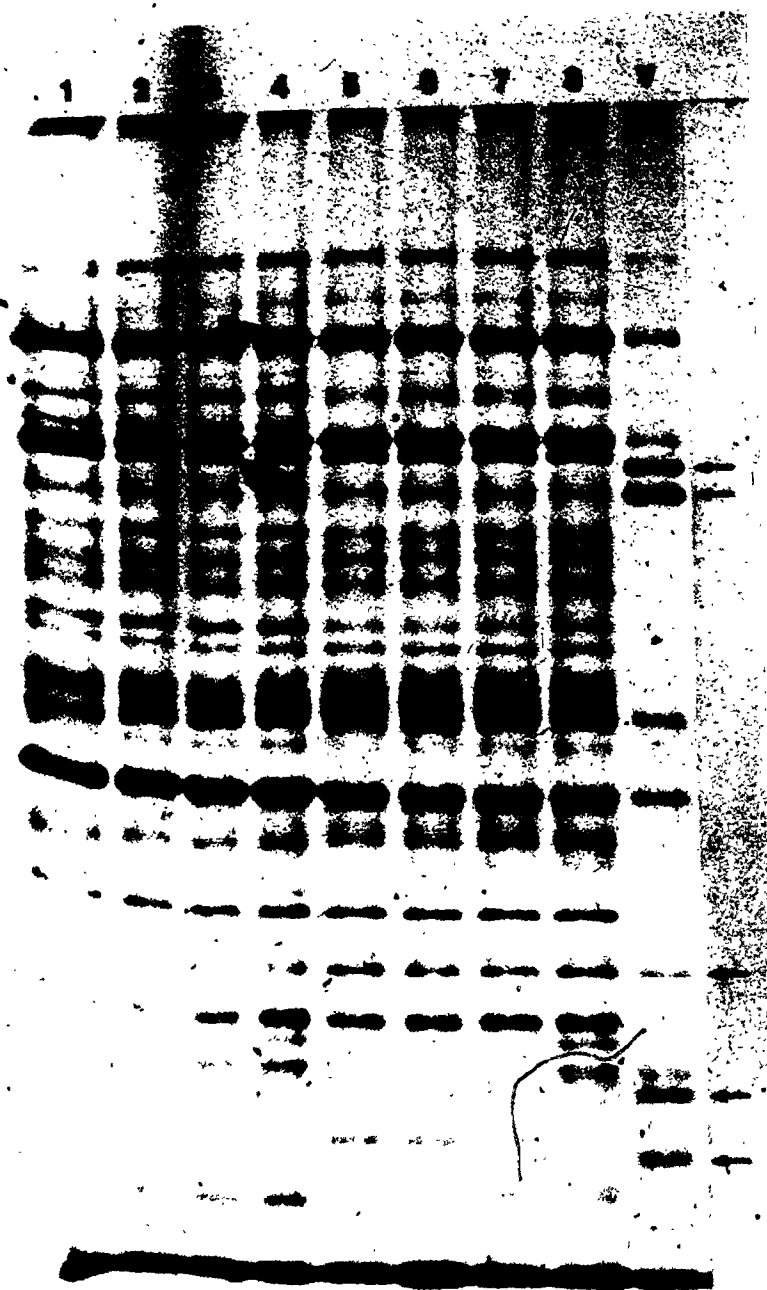


viroplasmic foci, following cytoplasmic mixing.

Another approach towards characterization of the factor(s) functioning in PTC was to isolate cytoplasmic components containing the factories for investigation as follows: The entire cytoplasmic material or concentrated factories from unlabeled cells infected for 10 hr with wt vaccinia virus were mixed with comparable material from cells infected with ts1085 for 10 hr at 39° and labeled with [³⁵S] methionine 9-10 hr p.i. Incubation of this mixture under a variety of conditions, such as different temperature or pH, in the presence of cations (calcium or magnesium), detergents (NP-40 or deoxycholate) or reducing agents (2-mercaptoethanol), failed to bring about cleavage of preformed isotopically labeled precursor polypeptides as exemplified in Figure 12 where formation of products p62 and p60 was not enhanced. Thus in line with data revealed by PAGE analysis following cell-cell fusion experiments, the results indicated that cytoplasmic components isolated from wt vaccinia-infected cells did not induce fusion of core protein precursors specified by ts1085.

From these combined in vivo and in vitro experiments it may be deduced that any late protein factor(s) required for PTC of core polypeptides was not a readily diffusible cytoplasmic component which could be moved between cytoplasmic factories. Conversely, such factor(s) may be able to elicit proteolysis only when present in close proximity to the site of synthesis or interaction with the core precursor polypeptides.

Figure 12 ✓ Autoradiogram showing failure to obtain PTC products in vitro. Cytoplasmic extracts were prepared from cells infected with wt (channels 1-4) or ts1085 (channels 5-8) pulse-labeled from 9 to 10 hr p.i. and harvested immediately. Following cell rupture in lysis buffer lacking NP-40, the cytoplasmic fraction was obtained by differential centrifugation. Samples were divided into four aliquots into which dissociation buffer was added immediately (channels 1 and 5) or following 4 hr incubation at 33° in the presence of an equal volume of unlabeled cytoplasmic extract obtained similarly from wt-infected cells (channels 2-4 and 6-8). In some samples, NP-40 (final concentration 1%) (channels 3, 4, 7, and 8) and 2-mercaptoethanol (final concentration 10mM) (channels 4 and 8) were added at the start of the in vitro incubation period. Arrows denote the positions of p62, p60, p23, p18.5, and p18, respectively. Channel V: purified vaccinia virus.

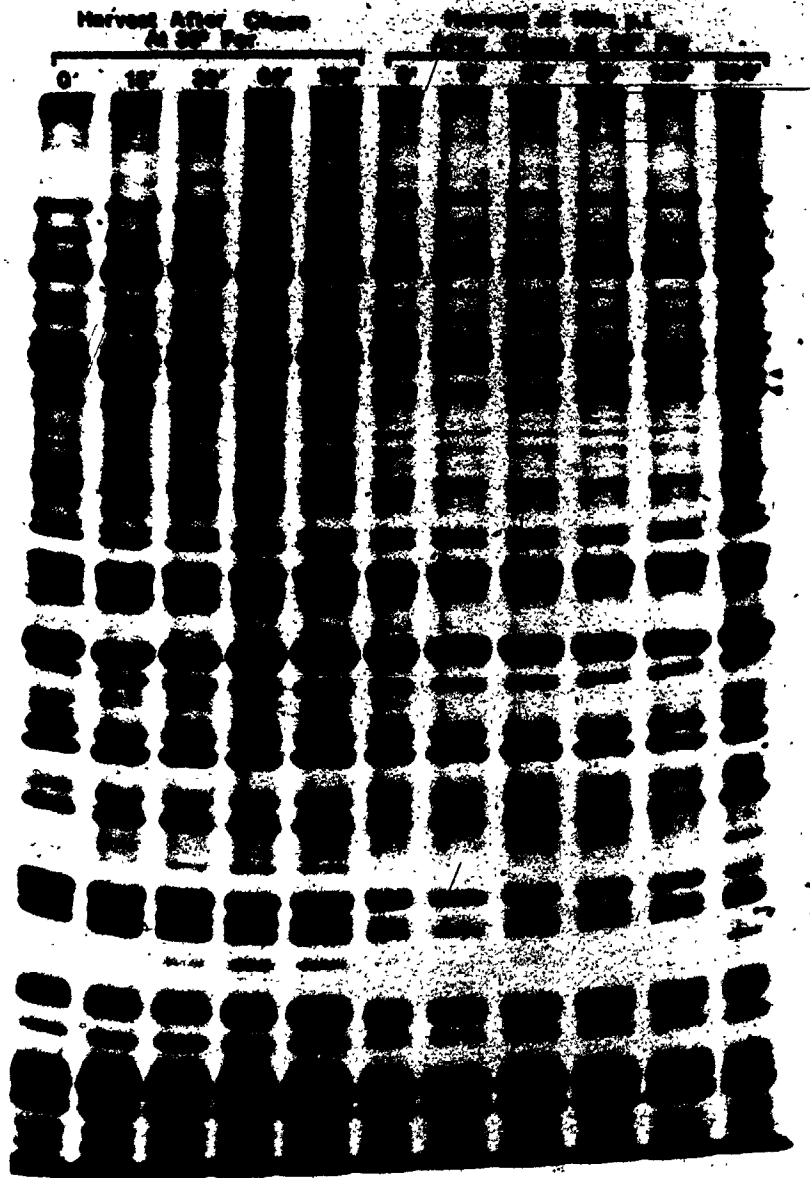


4.1.4 Instability of Factor(s) Required for PTC

Since the defect in processing associated with the ts1085 mutation was readily reversible after temperature shift-down, the question arose whether PTC, once it had been initiated for brief periods under permissive conditions, continued after return to the restrictive environment. The answer was sought by means of a series of studies employing pulse-labeling with ^{35}S methionine and pulse-chases during infection with ts1085 at 33° and $39-40^{\circ}$. Following a 1 hr labeling interval at 39° , commencing 9 hr p.i., individual cultures were placed in chase medium and either incubated at 33° for 15-360 min. before harvesting or at 33° for 15-360 min. then shifted to 39° and incubated until 16 hr p.i. had elapsed. The results, illustrated in the autoradiogram (Fig. 13), suggested, first of all, that the quantity of material migrating as the p62 and p60 bands was directly proportional to the duration cells were held at the permissive temperature before sampling (compare channels 6 with 10 and 11 or Fig. 13). Secondly, they revealed that during prolonged incubation under restrictive conditions, after shift-up from varying periods at 33° , no further processing of p94 and p65 into the products p62 and p60 had occurred (channels 6-10 of Fig. 13). Therefore, the extent of PTC was directly related to the duration of incubation at 33° , implying that any induced protease factor(s) involved was either thermolabile or active for only brief periods.

Because in the case of ts1085, coincident with activation of PTC, the assembly-arrest phenotype can be reversed (Stern et al., 1973), experiments were conducted to examine effects of temperature shifts,

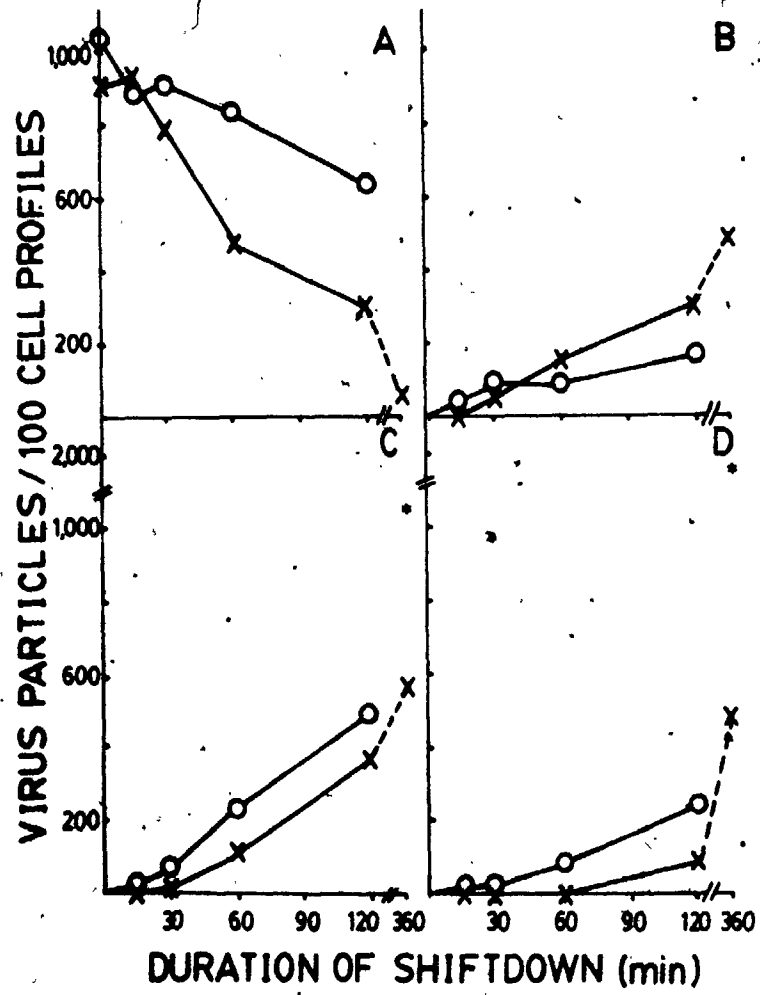
Figure 13. Autoradiogram showing effect of incubating ts1085 infected cells for varying periods at the permissive temperature. Cultures, maintained at 39°, were pulse-labeled with ^{35}S methionine 9-10 hr p.i., placed in chase medium, and shifted to 33° for the duration indicated. Samples were then either collected forthwith (5 left-hand channels) or following shift-up to 39° and further incubation for a total of 16 hr p.i. (6 right-hand channels). Arrows indicate positions of p62 and p60.



D

like those described in Figure 13, on virus morphogenesis. The virus-related structures, evident by electron microscopic examination of thinly sectioned cells, were grouped and enumerated as aberrant factories, incomplete normal envelopes, complete immature forms, and mature virions. A summary of these data is presented in Figure 14. Examination of panel A of this figure reveals that the number of aberrant envelopes decreased in direct proportion to the duration of incubation at 33°. Coincidentally, the number of incomplete (panel B) or complete immature progeny virions with spherical envelopes of normal appearance (panel C) increased in relation to the duration of the shift-down period. Likewise, as shown in panel D of Figure 14, there was a gradual increase in the number of mature virions as the duration of incubation at 33° was extended. In cultures kept for 60-120 min. at 33° then returned to 39° for another 4-5 hr, the number of incomplete particles decreased (Fig. 14B), while the number of complete, immature particles increased by about 50% (Fig. 14C). There was an approximately three-fold elevation in the number of mature virions during resumed incubation at 39°, compared with the number observed in cells preserved at the end of incubation at 33° (Fig. 14D). Regardless of the duration of the shift-down period or extent of incubation at the nonpermissive temperature, the number of immature and mature virions observed was far lower than that seen with the ts1085 infection maintained throughout the 16-hour period at the permissive temperature (asterisk in Fig. 14C and D). The results revealed that conversion from the aberrant to normal envelopes is connected directly to the morphological defect in ts1085. In agreement with the data on PTC presented in Figure 13, particle

Figure 14. Enumeration by quantitative electron microscopy of stages in ts1085 development following incubation for various periods at the permissive temperature. Temperature shifts, commencing at 10 hr p.i. were carried out according to the schedule outlined in the legend to Figure 13. Each point in a cumulative count derived from 100 thinly sectioned cell profiles. Samples were taken either when incubation at 33° was ended (X) or after an additional incubation at 39° for a total of 16 hr from the time of inoculation (O). (A) Aberrant envelopes; (B) incomplete normal envelopes; (C) complete spherical immature particles; (D) mature virions. The number of progeny virions present in equivalent samples of cultures incubated throughout at the permissive temperature is indicated by an asterisk.

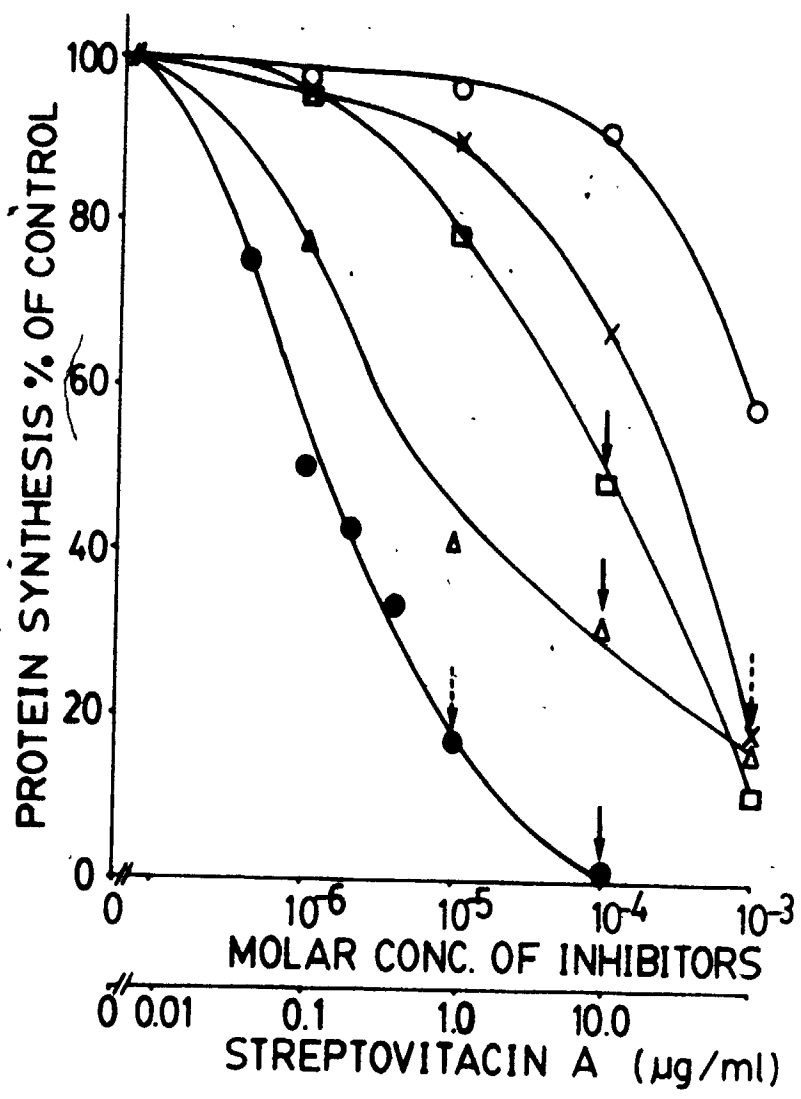


counting revealed that the extent of normal assembly of incomplete, immature particles and their envelopes during brief intervals at 33° was directly proportional to duration of the ts1085 infection under permissive conditions (Fig. 14B). On the other hand, conversion of some incomplete, immature particles into the complete, spherical immature form could occur at 39°, as evident by the decrease of the former (Fig. 14B) and increase of the latter (Fig. 14C), following temperature shift-up and further incubation. These data indicate that both the extent of PTC and assembly of normal, immature virions including envelopes may be subject to a factor(s) which functions briefly and/or has a brief half-life at 33°.

4.1.5 Identification of Factor(s) Involved in PTC

The active site-specificity of the proteolytic factor(s) was tested with several protease inhibitors possessing known specificity for their native substrates. Compounds such as TLCK and TPCK have affinities for and are recognized as substrates by trypsin-like and chymotrypsin-like proteases, respectively, to which they bind covalently and inhibit at the active site (reviewed by Shaw, 1975). However, since these inhibitors also suppress protein synthesis at higher concentration (Pong et al., 1975) it was necessary to determine the molarity at which these compounds acted specifically as proteolytic, rather than translational, inhibitors. Streptovitacin A (Str A) was used to check the effects of a specific inhibitor of translation. The results, summarized in Figure 15, revealed that TLCK at 10⁻⁴ M inhibited protein synthesis by about 25% without affecting

Figure 15. Relationship between concentration of streptoviticin A (Str A) or protease inhibitors and inhibition of protein synthesis. At 9 hr p.i. wt-infected cultures were treated for 1 hr with varying concentrations of one of the following: Str A (●); PMSF (○); TLCK (X); TPCK (△) or ZPCK (□), then were pulse-labeled for 1 hr at 37° with ^{35}S methionine prior to sampling for determination of incorporation of the radioisotope into protein. Each point represents an average value obtained from duplicate cultures. The broken arrow indicates the concentration of the inhibitors which suppressed PTC partially; solid arrow, the concentration which blocks PTC entirely, as judged from the data in Figures 16 and 17.



detectably PTC, as illustrated in the autoradiogram of Figure 16. At 10^{-3} M, TLCK, which inhibited protein synthesis by over 80%, did not interfere with conversion of p94 and 65 to p62 and 60, but reduced the amounts of p23, 18.5, and 18.0 product polypeptides. These findings imply that the proteolytic factor involved in cleaving core polypeptides does not have the specificity of trypsin. Application of TPCK, at 10^{-4} M, which reduced protein synthesis by about 65% (Fig. 15), completely blocked processing of core polypeptides (Figures 16 and 17) indicating that a protease with the specificity of chymotrypsin was involved. This result was substantiated using ZPCK, another compound with affinity for chymotrypsin-like proteases, which at 10^{-4} M reduced translation by only 50% (Fig. 15) but PTC completely (Fig. 17). To prove that reduced protein synthesis in the presence of TPCK and ZPCK was not per se responsible for suppression of processing, Str A was added in varying concentrations to vaccinia infected cultures during the chase period, following pulse-labeling with ^{35}S methionine. It was evident that at 0.1 ug/ml of this drug, which reduced protein synthesis by about half (Fig. 15), PTC occurred normally (Fig. 16) and at 1.0 ug/ml, which lowered the protein synthesis rate by 82%, appreciable cleavage was evident in the autoradiogram (Fig. 16). Another protease inhibitor, PMSF, was less potent in affecting translation and even at 10^{-3} M had no influence on PTC. Taken together, these data revealed that proteolytic factor(s) which may bring about processing of the viral core precursor proteins possessed the specificity of a chymotrypsin-like protease.

Figure 16. Effect of varying concentrations of protease inhibitors and streptoviticin A (Str A) on PTC. The autoradiogram was prepared from cytoplasmic extracts of wt vaccinia virus-infected cells. Cultures were treated as indicated above each channel, following pulse-labeling with ^{35}S methionine for 1 hr commencing 9 hr p.i. (P), and incubated in chase medium in the presence of inhibitor for a further 8-hr period (C). Molar concentrations of protease inhibitors which were 10^{-6} , 10^{-5} , 10^{-4} , and 10^{-3} are labeled respectively as -6, -5, -4, and -3. Str A concentrations in ug/ml were 0.04, 0.1, and 1.0. Arrowheads indicate positions of p62, p60, p23, p18.5, and p18, respectively. Channel V: purified vaccinia virus.

DMSO TPCK TLCK PMSF STRA
P C -3 -3 -4 -4 -4 -4 -3 -3 -3 -4 -3 M A 1.0 V

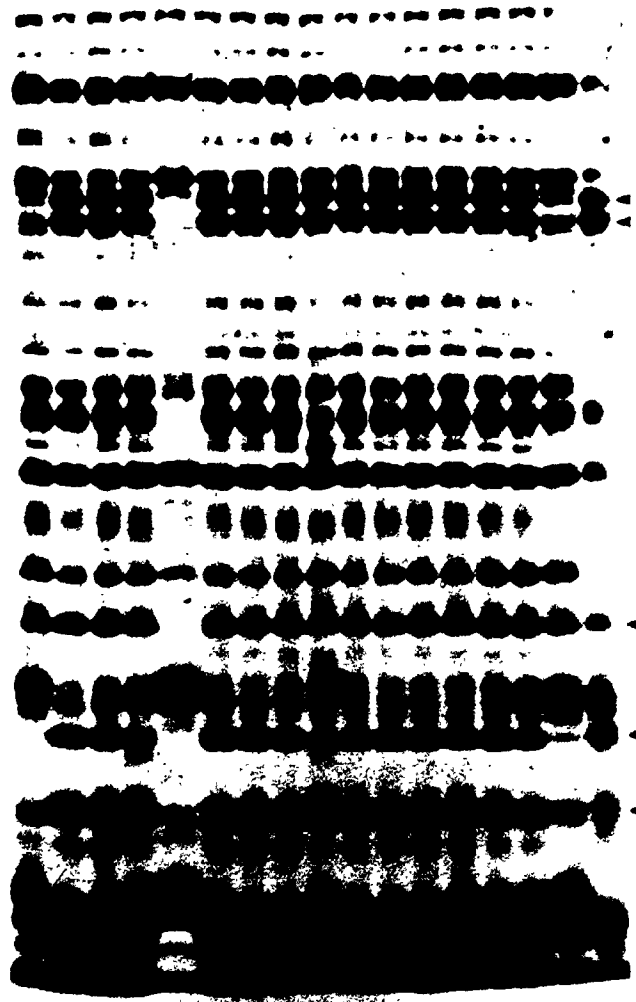
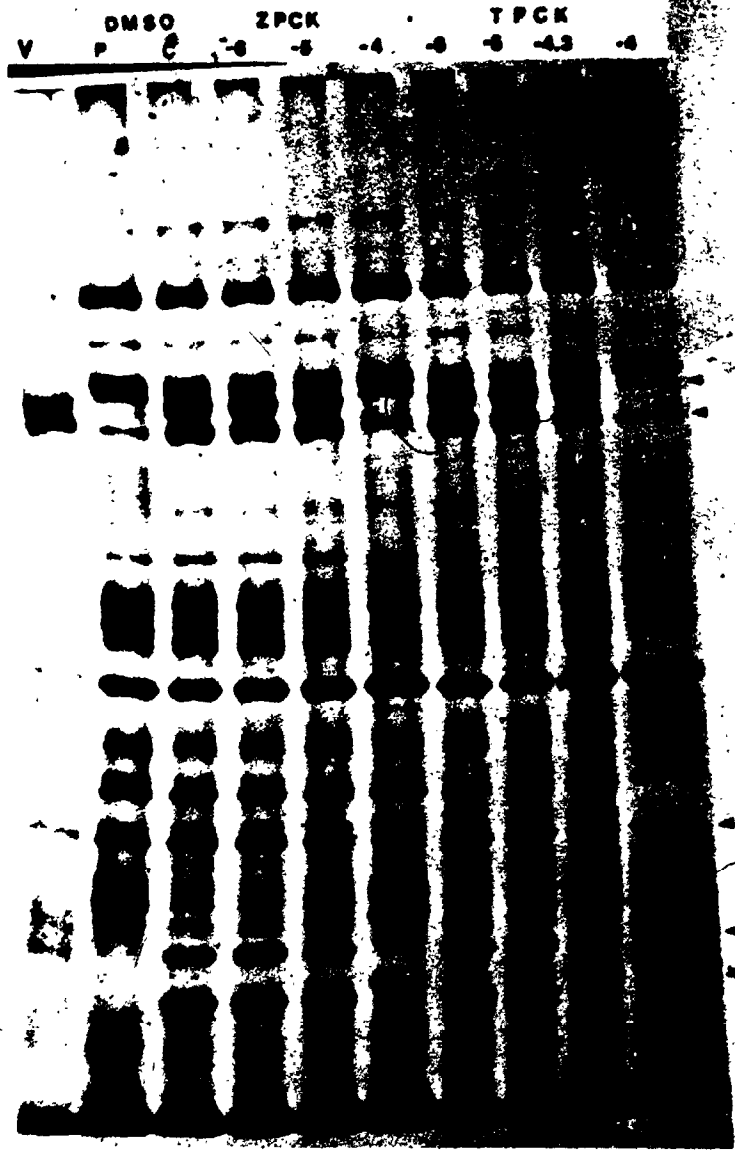


Figure 17. Effect of varying concentrations of protease inhibitors TPCK and ZPCK on PTC. The autoradiogram was prepared from cytoplasmic extracts of wt vaccinia virus-infected cells labeled with ^{35}S methionine and chased as described in the legend to Figure 16. Molar concentrations of protease inhibitors which were 10^{-6} , 10^{-5} , 5×10^{-5} , and 10^{-4} are labeled as -6, -5, -4.3, and -4, respectively. Arrowheads indicate positions of p62, p60, p23, p18.5, and p18, respectively. Channel V: purified vaccinia virus.



4.1.6 ^3H ZPCK Labeling of the Protease Involved in PTC

Evidence that the protease inhibitors have affinity for and form covalent bonds with chymotrypsin-like protease(s) involved in vaccinia virus development, suggested that such protease(s) might be identified by means of ZPCK carrying a suitable radiolabel. To this end, cultures infected with wt vaccinia or mock infected cultures were incubated in the presence of ^3H ZPCK for 3 hr commencing 10 hr p.i. As a further test of the origin of the protease(s), cells intensely γ -irradiated with 70 kilorad from a ^{60}Co source prior to infection were analyzed for ZPCK binding proteins because such irradiation had been shown to suppress profoundly or abolish transcription of host cell genes (see sections 4.3.2 and 4.3.4). Therefore, any polypeptide induced after infection binding ZPCK would most likely be a virus-specified function. When extracts of ^3H ZPCK-labeled cells were subjected to SDS-PAGE analysis, the autoradiograms revealed the presence, exclusively in material from infected cells, of a prominent band migrating with an apparent molecular weight of 12,500 (Fig. 18, channels 6 and 7). This band was entirely absent from uninfected, γ -irradiated or unirradiated cells (Fig. 18, channels 8 and 9). Several identical minor polypeptides were also evident in PAGE of extracts from both virus-infected and uninfected cells. None of these corresponded to the prominent polypeptide presumed to have been labeled by ^3H ZPCK. The major, virus-specified polypeptide, marked by addition of ^3H ZPCK, did not match in molecular weight any of the ^{35}S methionine-labeled proteins from purified virus, also displayed in channel V of Figure 18. These results suggested that a

Figure 18. Identification of a polypeptide with presumed affinity for ZPCK. The autoradiogram prepared from cytoplasmic fractions isolated from cells infected with wt or ts1085 vaccinia virus and incubated in the presence of 10^{-4} M [3 H] ZPCK for 3 hr commencing at 10 hr p.i. Channels (1-5): ts1085 incubated at 33° (1) and at 39° (2); shifted after 10 hr at 39° to 33° during exposure to ZPCK (3); as in (3) but with Str A, 10 ug/ml, added (4); as in (3) but with actinomycin D, 4 ug/ml, added (5). Channels (6 and 7): infected with wt virus; Channels (8 and 9): mock-infected. Channels (7 and 9): cells exposed to γ -irradiation 1 hr prior to inoculation. Channel V: purified vaccinia virus.



virus-specified p12.5 protein, which has affinity for ZPCK, was induced during vaccinia virus infection, but was absent from mature virions. A reasonable hypothesis is that this polypeptide is related to the protease factor(s) involved in PTC.

To ascertain whether the same polypeptide was present or absent during ts1085 infection, which is blocked in processing at the restrictive temperature, infected cultures were labeled as above with ^3H ZPCK. It was evident from the autoradiogram (Fig. 18, channels 1-5) that regardless of the conditions prevailing during the infection, i.e., whether labeling occurred at 33 or 39-40°, or whether following temperature shift-down transcription was inhibited with actinomycin D or translation with Str A at concentrations which block PTC (Nagayama et al., 1970), the p12.5 polypeptide was induced. Therefore, synthesis of the ZPCK-binding protein presumably occurred under circumstances which were nonpermissive for processing and maturation of vaccinia virus. This finding was supported by results obtained during infection with wt vaccinia virus in the presence of 100 ug/ml of rifampicin, an antibiotic which simultaneously blocks morphogenesis and PTC in a manner closely mimicked by ts1085 and other mutants of this phenotype. When assayed during rifampicin treatment or during arrest of transcription and translation, following removal of rifampicin, there was synthesis of the polypeptide marked by ^3H ZPCK (Fig. 19, channels 1-5).

Possible identity of the ^3H ZPCK-binding p12.5 protein with any labeled by ^3H amino acids or ^{35}S methionine was investigated using SDS-PAGE analysis of cytoplasmic extracts. It appeared from the

Figure 19. Characterization of factor with binding affinity for ZPCK. Labeling with ^3H ZPCK as described in legend of Figure 18. Channels (1-5): infected with wt vaccinia virus at 37° throughout; untreated (1); exposed to 100 ug/ml rifampicin throughout (2); treated with 100 ug/ml rifampicin for 40 hr p.i. then drug was washed out and incubation continued with ^3H ZPCK in the absence (3), or presence of 10 ug/ml Str A (4), or presence of 4 ug/ml actinomycin D (5).

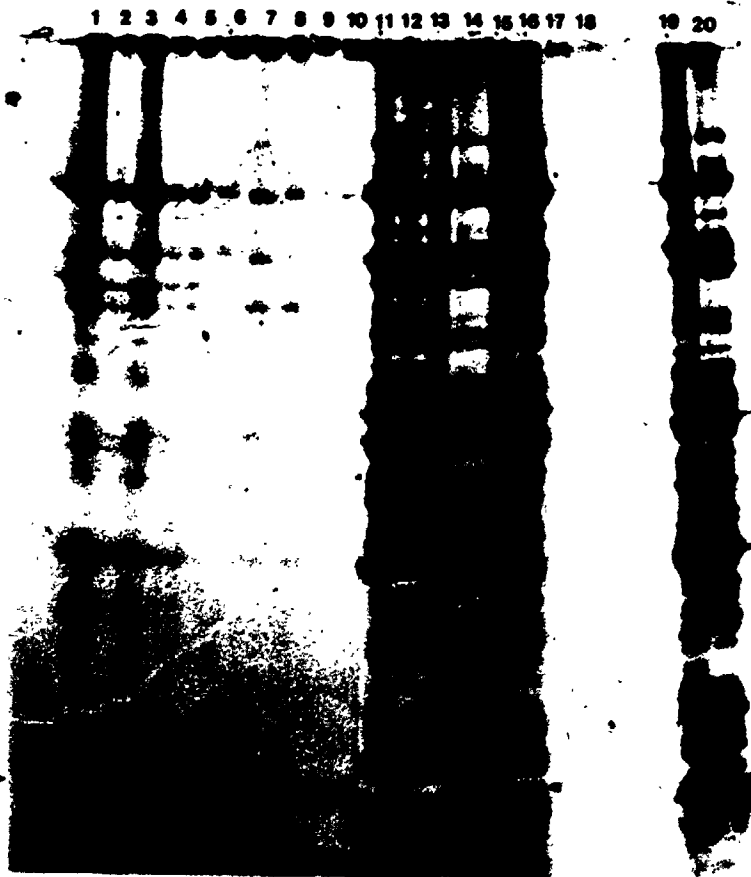
Channels (6-10): infected with ts1085 and incubated at 33° (6); 39° (7); 39° for 10 hr after inoculation then shifted to 33° during exposure to ^3H ZPCK in the absence (8), or presence of 10 ug/ml Str A (9), or the presence of 4 ug/ml actinomycin D (10).

Channels (11-16): infected with wt virus or ts1085 and pulse-labeled with ^3H amino acid mixture (10 uCi/ml) for 1 hr commencing 9 hr p.i. and where indicated, incubated for a further 8 hr in chase medium. Channels (11-14): ts1085 infected, and sampled after pulse at 33° (11); pulse and chase at 33° (12); pulse at 39° (13); pulse and chase at 39° (14). Channels (15 and 16): wt inoculated cells maintained throughout at 37° ; pulse (15); pulse and chase (16).

Channels (17 and 18): concentrated factories from

wt infected cells prepared as described in Material and Methods, incubated in vitro at 37° for 3 hr with 10⁻⁴ M |³H|ZPCCK without (17), or with 1% NP-40 (18).

Channels (19 and 20): wt infected cells maintained at 37° throughout, labeled for 1 hr commencing 9 hr p.i. with |³⁵S|methionine (10 uCi/ml), sampled after pulse (19); or after pulse and chase (20).



autoradiogram in Figure 19 that there were bands labeled with ^3H amino acids or ^{35}S methionine (channels 11-16 and 19,20) of molecular weight corresponding to that of the p12.5 polypeptide (Fig. 19, channels 1-10). Therefore, during vaccinia virus infections, when host-related protein synthesis is inhibited (Moss and Salzman, 1968), and induction of host-related functions blocked by γ -irradiation, a p12.5 polypeptide, with presumed affinity for chymotrypsin protease inhibitors, may be induced under circumstances either permissive or restrictive for PTC and envelope assembly and maturation.

4.1.7 Summary

Five temperature-sensitive (ts) mutants of vaccinia virus, which mimic the effects of rifampicin, being defective in assembly of the envelope and maturation, were characterized. Although the mutants showed a similar morphological phenotype when examined by electron microscopy (Figures 1-4), analysis by complementation titrations between the mutants (Table II) indicated that each mutation produced a distinct defect. This result was supported by SDS-PAGE analysis of cytoplasmic extracts of virus-infected cells (Figures 5 and 6) whereby each mutant produced a distinct cleavage pattern in its protein profile (Fig. 7). Detailed complementation analysis of ts1085 and a DNA(-) mutant, ts6389, revealed that PTC of precursor proteins occurred during co-infection (Fig. 8) and that progeny of both parental phenotypes were produced (Fig. 9). However, PTC of ts1085-specified precursor proteins did not occur following syncytiogenesis involving cells singly inoculated with wt vaccinia

virus and cleavage defective ts1085 (Fig. 11). Extracts from infected cells were also unable to bring about in vitro cleavage of precursor polypeptides (Fig. 12). Data from temperature shift experiments with ts1085-infected cells (Fig. 13) indicated that the extent of PTC was directly related to the duration of incubation at the permissive temperature. Similarly, the extent of assembly of normal envelopes was also proportional to the duration of incubation of the ts1085-infected cells at 33° (Fig. 14). The use of affinity labeling protease inhibitors indicated that the factor(s) for proteolysis was a virus-induced chymotrypsin-like non-virion polypeptide of 12,500 molecular weight (Figures 15-19). This factor was found in wt and ts1085-infected cells under both permissive and nonpermissive conditions for PTC and virus maturation (Figures 18 and 19).

4.2 Vaccinia Virus Replication Examined by Means of Host Cell Mutants and α -Amanitin

The finding that viral DNA synthesis occurred in either cells pre-treated with the DNA cross-linking antibiotic mitomycin C (Magee and Miller, 1962; Kajioka et al., 1974) or in enucleated cells (Prescott et al., 1971) proved that events leading to the replication of the viral genome are under viral as opposed to cellular control. There has been, however, a debate regarding the autonomy of later events based primarily on the work of Pennington and Follet (1974) who showed that virus maturation is inhibited in cytoplasts. In addressing this question of the requirement of the nucleus, a study of the role of host transcriptional function(s) in vaccinia virus

biogenesis was undertaken.

The highly active toxin α -amanitin, derived from the toadstool Amanita phalloides, has been recognized as a specific inhibitor of DNA-dependent RNA polymerase II (polymerase II) of animal cells (Roeder, 1976). By virtue of its inhibitory specificity, α -amanitin was used to demonstrate that certain DNA and RNA agents, such as papovaviruses, adenoviruses, and influenza viruses, having an obligatory developmental stage in the host nucleus, most probably require polymerase II activity for replication (Amanti et al., 1975; Ledinko, 1971; Lamb and Choppin, 1977). By contrast, the poxviruses, which develop in the cytoplasm, contain in the virion core an α -amanitin-insensitive DNA-dependent RNA polymerase (Costanzo et al., 1970). The availability of the rat myoblast L6H9 cell line and a mutant line derived therefrom having a polymerase II resistant to α -amanitin (Somers et al., 1975a) prompted the examination of the possible role of host transcriptional function(s) in the life cycle of vaccinia virus.

4.2.1 Effect of α -Amanitin on Virus Replication in

BSC-1 and Rat Myoblast Cells

To investigate the role of host transcription into mRNA involving polymerase II, α -amanitin, which specifically inhibits polymerase II of animal cells when used at appropriately low concentrations (Roeder, 1976), was used. The host used was a myoblast cell line in which the wild-type cell is Ama^+ and the mutant, containing α -amanitin-resistant polymerase II, is designated Ama 102 (Somers et al., 1975a). Because

the permeability and time required to inhibit polymerase II activity varies depending on the cell (Ingles et al., 1976), it was necessary to ascertain the duration of treatment with α -amanitin to effect an inhibition of vaccinia replication. The data in Table IV show that treatment of Ama^+ cells for 5 or 10 hr abolished the capacity of the host to support production of infectious progeny, while shorter treatment resulted in fractional yields of PFU. Because the inhibitor was also kept in the culture medium throughout the growth cycle, it may be concluded that suppression of replication was elicited gradually and progressively. This is consistent with the slow inhibition of polymerase II activity by α -amanitin demonstrated biochemically (Somers et al., 1975b) and implies that this host enzyme or a subcomponent of it is necessary for formation of infectious vaccinia virus. In sharp contrast to the above findings, treatment of Ama 102 cells with the drug had no inhibitory effect on the yield of vaccinia PFU (Table IV), also indicating the involvement of polymerase II in the vaccinia virus cycle.

As a control of the specificity of α -amanitin on vaccinia, replication of vesicular stomatitis virus (VSV) was tested under parallel circumstances. The data shown in Table IV revealed that the drug did not suppress VSV production in either Ama^+ or Ama 102 cells. It is interesting to note that yields in Ama 102 cells were usually almost an order of magnitude greater than in Ama^+ cells.

It seemed appropriate to examine, as a further control, replication of another DNA agent, herpes simplex virus, type 1 (HSV-1), known to pass through a replication phase during which

TABLE IV

Virus replication in BSC-1 cells and in rat myoblasts
Ama⁺ and resistant Ama 102
treated with α -amanitin^a

Host	Untreated		Hours before inoculation when treatment commenced ^c				
	Following adsorption ^b	Following replication ^c	0	-2	-5	-10	-24
IHD-W VACCINIA VIRUS ^d							
Ama ⁺	6	1,000	62	39	6	6	ND ^f
Ama 102	6	700	570	510	630	680	ND
VESTICULAR STOMATITIS VIRUS ^d							
Ama ⁺	4	860	1,100	ND	ND	1,200	ND
Ama 102	3	7,700	16,000	ND	ND	18,000	ND
HERPES SIMPLEX VIRUS TYPE 1 ^e							
BSC-1	0.5	1,400	ND	200	ND	ND	0

- a, α -amanitin, 10 ug/ml for BSC-1 cells and 2 ug/ml for both rat myoblast lines, was present prior to and throughout the infection as indicated.
- b, sampled 2 hr p.i.
- c, sampled 24 hr p.i. for vaccinia virus and VSV; 16 hr p.i. for HSV-1.
- d, virus replication in monolayers of 10^6 cells/plate inoculated with 10 pfu/cell, expressed as 10^4 pfu/plate.
- e, virus replication in monolayers of 1.2×10^6 cells/plate inoculated with 0.2 pfu/cell, expressed as 10^4 pfu/plate.
- f, ND, not done.

transcription into early functions is blocked by α -amanitin (Ben-Zeev and Becker, 1977). In agreement with previous findings of others (Costanzo et al., 1977), exposure of BSC-1 cells to the drug profoundly inhibited HSV-1 replication implying that polymerase II is intimately involved in the life cycle of this virus (Table IV).

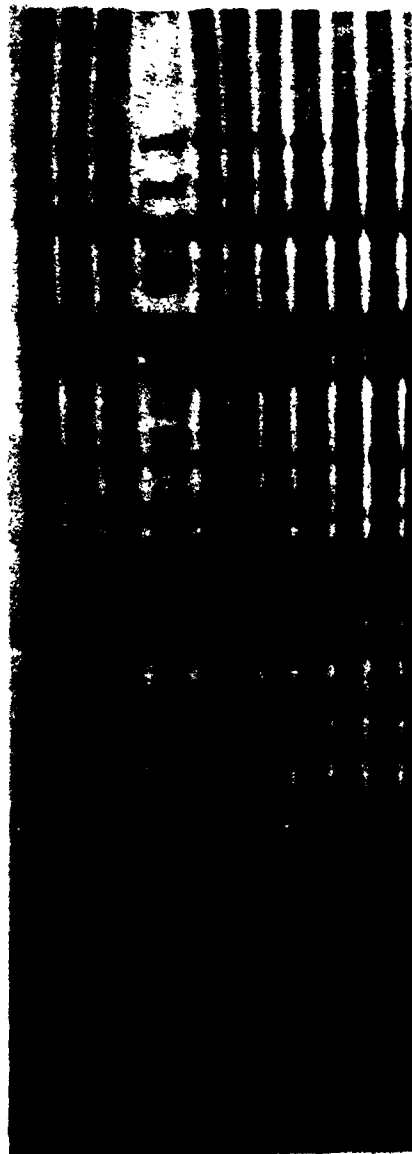
4.2.2 Effects of α -Amanitin on Synthesis of Vaccinia Virus-Specified Products

The effects of α -amanitin on synthesis of vaccinia virus-specified products, including the synthesis of DNA and polypeptides in the cytoplasm, were monitored by isotopic labeling, SDS-PAGE and enumeration of progeny particles by quantitative electron microscopy.

Analysis of cytoplasmic extracts from untreated and drug treated cells labeled with ^3H thymidine, conducted by G. McFadden, showed that, in Ama^+ , in the absence of α -amanitin about 3300 cpm per 10^6 cells were incorporated and, in cells pretreated for 10 hr and maintained in the presence of the drug during infection, 2600 cpm per 10^6 cells were converted into an acid-precipitable product. In Ama^{102} cells, cytoplasmic extracts from both untreated and treated cells contained about 23,000 cpm of ^3H thymidine per 10^6 cells incorporated into a macromolecular product. These data revealed that α -amanitin either does not inhibit or inhibits only partially vaccinia-specified DNA synthesis. As with VSV replication, the much greater rate of vaccinia-related DNA synthesis in Ama^{102} cells remains unexplained.

Figure 20. Autoradiogram of polypeptide profiles from untreated and α -amanitin-treated cultures in a SDS-PAGE slab gel prepared from wt vaccinia virus-infected L-2, Ama⁺ and Ama 102 whole cell extracts. After pulse-labeling with [³⁵S] methionine and chasing, cells were disrupted in lysis buffer. Treatment with 50 ug of pancreatic deoxyribonuclease I per ml for 15 min. at 4⁰ was used to hydrolyze the DNA. Arrows indicate the positions of p 94, p65, p62, p60, p23, p18.5, and p18, respectively. (P), pulse; (C), pulse and chase. Channels: (1-4), Ama⁺ extracts; (5-8), Ama 102 extracts; (9 and 10), L-2 extracts. Channels 3, 4, 7, and 8 show samples from drug-treated cells.

P C P C P C P C P C
1 2 3 4 5 6 7 8 9 10



← 2

← 23

← 25

← 30

MW X 10⁻⁴

← 23

← 25

← 30

The influence of α -amanitin on the spectrum of vaccinia-specified polypeptides synthesized in Ama^+ and Ama 102 cells was ascertained by pulse-chase experiments and SDS-PAGE analysis. The results illustrated in Figure 20 showed that when Ama^+ was the host, the quantity of [^{35}S] methionine-labeled polypeptides formed in the presence of the toxin was less than in its absence (channels 3 and 4). However, the inhibitor did not abolish the synthesis of the p94 and p65 precursors nor their processing to the p62 and p60 protein products. Most or all of the other identifiable polypeptides, including p23, 18.5 and 18.0 were also synthesized, as evident in channels 3 and 4 of Figure 20. In infection of Ama 102, α -amanitin did not reduce the quantity of vaccinia polypeptides produced or affect the normal PTC during the chase (Fig.20, channels 7 and 8). The pattern of bands and processing of vaccinia-induced polypeptides were identical whether the host was the wild-type Ama^+ , Ama 102 polymerase II mutant, or mouse L-2 cells. The comparative data on L-2 cells shown in channels 9 and 10 emphasize the uniformity of the spectrum of vaccinia-induced polypeptides, regardless of the cell type used for virus replication.

Results using electron microscopy to quantitate the formation of vaccinia-related structures (Table V) corroborated the findings from SDS-PAGE and PFU assays. Only in Ama^+ cultures treated with α -amanitin was the appearance of "factories" and assembly of immature and mature progeny virus severely curtailed. The small number of mature progeny evident in this sample was obviously insufficient to alter the titer, expressed in Table IV in terms of the relatively less

TABLE V

Quantitative electron microscopy of Vaccinia Virus
replication in wild-type Ama⁺ and resistant
Ama 102 rat myoblasts treated with α -amanitin

Host Cell and Treatment	Vaccinia Virus Products			% Cell Profiles with Mature Progeny Virions
	"factories"	immature particules	mature virions	
Ama ⁺ without drug	41	436	325	35
Ama ⁺ with α -amanitin	8	23	13	1 - 2
Ama 102 without drug	56	600	332	30
Ama 102 with α -amanitin	78	946	844	56

The data are normalized as counts per 100 profiles of thinly sectioned cells. In the Ama⁺ with α -amanitin sample over 200 profiles were examined.

sensitive PFU. Electron microscopic examination of 210 cell profiles in the drug-treated Ama^+ sample revealed the presence of 27 mature virions, all situated in only 3 cell profiles, and 48 immature particles, observed in only 6 profiles. This finding demonstrated that after treatment with the toxin, when rates of virus DNA synthesis were only partially reduced, vaccinia development was arrested to a varying degree, so that in some cells there was a complete absence of any virus structure while in a very small percentage of others, virus assembly had been completed. Presumably the few mature progeny virions observed in this sample accounted for the processing of p94 and p65 polypeptides to their p62 and p60 products, evident in Figure 20 and shown previously to be an obligatory step in the vaccinia maturation process (Stern *et al.*, 1977). The occurrence of mature progeny in treated Ama^+ cells also implied that, in the occasional host cell, inhibition of polymerase II may have been only partial or nonexistent.

4.2.3 Summary

An investigation employing the toadstool toxin, α -amanitin, a specific inhibitor of DNA-dependent RNA polymerase II, was undertaken to examine the possible role of host transcriptional function(s) in vaccinia biogenesis. Treatment of sensitive rat myoblasts, Ama^+ cells, abolished the ability of the host to support production of infectious progeny while the α -amanitin resistant mutant, Ama^{102} remained fully competent to produce infectious vaccinia in the presence of the toxin (Table IV). Synthesis of virus-specified DNA

occurred at a high rate (80% of control) in infected cells exposed to the drug but virus polypeptide synthesis was reduced. The toxin did not abolish their synthesis of precursor polypeptides nor their processing to their products (Fig. 20). Electron microscopic analysis (Table V) revealed that a profound inhibition of vaccinia-related structures in treated sensitive cells with the occasional production of mature particles. Thus experiments using, as the host, the α -amanitin sensitive Ama^+ and the drug resistant Ama 102 mutant rat myoblasts clearly demonstrated the involvement of a host function related to polymerase II in the life cycle of vaccinia virus.

4.3 Involvement of Host Transcription in the Replication of Vaccinia Virus

The finding that the host transcriptional factor DNA-dependent RNA polymerase II is a requisite function for vaccinia replication presented the possibility that transcription of the host genome is involved in vaccinia biogenesis. Alternatively, polymerase II may be involved, in some manner, in viral genome transcription. The hypothesis regarding a requirement for ongoing host transcription was tested in experiments using cells exposed to intense γ -irradiation prior to infection. It was hoped that such treatment would render the cells transcriptionally dysfunctional as a consequence of template damage (Okada, 1970). Gene expression in irradiated cells was measured by rates of macromolecular synthesis, including putative mRNA formation, as well as gene function assessed in terms of viral hemagglutinin activity and the induction of the interferon-mediated

antiviral state.

4.3.1 Effect of γ -Irradiation on Virus Replication

In testing the hypothesis that continuous transcription of the host DNA is required for completion of vaccinia virus replication, the use of intense γ -irradiation was invoked because it is known that DNA is damaged upon exposure to ionizing radiation (Okada, 1970). Cells of both primate and rodent origin were exposed to high doses of γ -irradiation from a ^{60}Co source one hr prior to infection. The results, given in Table VI, indicated that vaccinia virus replication is undiminished in BSC-1 or L6H9 cells pre-irradiated with 40 krad (1 rad = 1.0×10^{-2} J/kg) and only marginally reduced in L-2 cells treated similarly. Indeed, exposure to as much as 70 krad had little inhibitory effect on vaccinia progeny yield in the three cell lines tested as indicated by yields ranging from 75 to 90% of unirradiated control samples. In the case of an RNA virus without nuclear requirement, exemplified by VSV (Follet et al., 1970), virus production also occurred at a high rate in BSC-1 cultures previously irradiated with 40 krad. A further control, that of testing the ability of irradiated cells to support HSV-1 replication, was chosen because here, as in the case of vaccinia virus, host polymerase II was required for herpes virus replication (Costanzo et al., 1977; Ben-Zeev and Becker, 1977; Table IV of this thesis). The data (Table VI) revealed that pre-irradiated (40 krad) BSC-1 cells were able to support HSV-1 replication as evident by the large yield of infectious progeny. Therefore for the three viruses tested, intense

TABLE VI

Virus replication in γ -irradiated and unirradiated Primate
and Rodent cell lines

γ -Irradiation dose (kilorad)	Virus Yield ($\times 10^{-4}$ pfu/plate) from Pre-Irradiated Cells ^a				
	L-2 ^b	L6H9(Ama ⁺) ^c	BSC-1 ^c		
	Vaccinia ^d	Vaccinia ^e	Vaccinia ^f	HSV-1 ^g	VSV ^h
0	5,200	1,000	3,200	1,400	1,800
40	4,800	1,000	3,200	650	700
55	5,000	900	3,000	ND ⁱ	ND
70	4,800	900	2,400	ND	ND

a, cells were irradiated 1 hr prior to inoculation.

b, 1×10^6 cells/plate.

c, 1.2×10^6 cells/plate.

d, m.o.i. = 10 pfu/cell; samples harvested 24 hr p.i.; background titer at 2 hr p.i. was 2×10^4 pfu.

e, as in d except background titer at 2 hr p.i. was 6×10^4 pfu.

f, as in d except background titer at 2 hr p.i. was 3×10^4 pfu.

g, m.o.i. = 0.2 pfu/cell; samples harvested 16 hr p.i.; background titer at 2 hr p.i. was 5×10^3 pfu.

h, m.o.i. = 0.1 pfu/cell; samples harvested 10 hr p.i.; background titer at 2 hr p.i. was 3×10^3 pfu.

i, ND, not done.

γ -irradiation of the host cell prior to infection had little or no inhibitory effect on the competence of the host to support virus replication. Moreover, the finding that vaccinia virus replication was not influenced appreciably or at all in any of the three cell lines exposed to as much as 70 kilorad of γ -irradiation prior to infection suggested that transcription of the host genetic information might not be essential in the life cycle of vaccinia virus.

4.3.2 Effect of γ -Irradiation on Cellular Macromolecular Synthesis

The ability of intensively pre-irradiated cells to function as fully permissive hosts for the growth of vaccinia virus raised the question as to whether intense γ -irradiation affected host cell macromolecular synthesis. This was assessed by measuring the effects of exposure to various doses of γ -irradiation on synthesis of DNA, RNA, and protein. For this purpose, cultures of BSC-1 and L-2 cells were placed in MEM containing isotopically labeled thymidine, uridine, and methionine and incorporation of precursors into TCA-precipitable material was ascertained. Data, summarized in Table VII, showed that 10 krad of irradiation only moderately reduced synthesis to 88.8% of unirradiated BSC-1 cells for DNA, to 73.3% for RNA, and to 67.9% for protein. Exposure of BSC-1 cells to 40 krad^a reduced protein synthesis to almost half the control rate but had a more profound effect on nucleic acid production which was lowered to only 1.5 and 3.0% in the case of DNA and RNA respectively. After irradiation with 70 krad, the remaining rates for DNA and RNA were, respectively, 0.7 and 1.2%, but the level of protein synthesis remained at 36.2% that of the controls.

TABLE VII

Macromolecular synthesis in γ -irradiated and unirradiated cells^a

Cell Line	Dose in kilorads	DNA		RNA		PROTEIN	
		³ H Thymidine (cpm X 10 ⁻²) ^b	%	³ H Uridine (cpm X 10 ⁻³) ^b	%	³⁵ S Methionine (cpm X 10 ⁻¹) ^b	%
BSC-1	0	452	100	124	100	333	100
	10	401	88.8	91.0	73.3	227	67.9
	40	7	1.5	3.7	3.0	145	43.5
	70	3	0.7	1.5	1.2	121	36.2
L-2	0	802	100	32.3	100	207	100
	70	97	12.1	3.5	10.8	159	76.8

a, Monolayers in 35-mm Petri dishes of L-2 cells (1 X 10⁶ cells/plate) or

BSC-1 cells (1.2 X 10⁶ cells/plate) were labeled as described in Materials and Methods.

b, Averages were determined from duplicate cultures.

L-2 cells exposed to γ -irradiation under identical conditions appeared to be less sensitive than BSC-1 cells. Nevertheless, with this cell type also, DNA and RNA synthesis was affected much more than the synthesis of protein (Table VII).

The severe reduction of overall RNA formation by γ -irradiation made it imperative to determine whether transcription into messenger RNA (mRNA) was affected differently or to the same extent. As a measure of putative mRNA synthesis, the formation of the poly-adenylated (poly(A)) RNA fraction was measured (Lanyon *et al.*, 1972). To this end, RNA from ^3H uridine-labeled cells, BSC-1, L-2, and L6H9, was separated by means of oligo(dT)-cellulose chromatography. The data, summarized in Table VIII, revealed that the degree of inhibition of RNA synthesis in BSC-1 cells was a function of the dose of radiation applied. Furthermore, synthesis of poly(A)-containing RNA was inhibited to about the same extent as that of the bulk RNA. These relationships were more easily recognized in the graphic presentation of the results, as shown in Figure 21. Of particular interest were data pertaining to the effects produced by 40 and 70 kilorad which inhibited labeling of both the total and poly(A) RNA components by 90-94%.

With L-2 cells, 70 krad inhibited formation of poly(A) RNA by 92% while the overall production of RNA was reduced by 87%, as shown in Table VIII. Similarly, total RNA and poly(A) RNA synthesis in the rat myoblast line, L6H9, were inhibited by 88 and 90%, respectively, when these cells were exposed to the equivalent dose of radiation (Table VIII). Thus, in the three cell strains tested, there appeared to be no

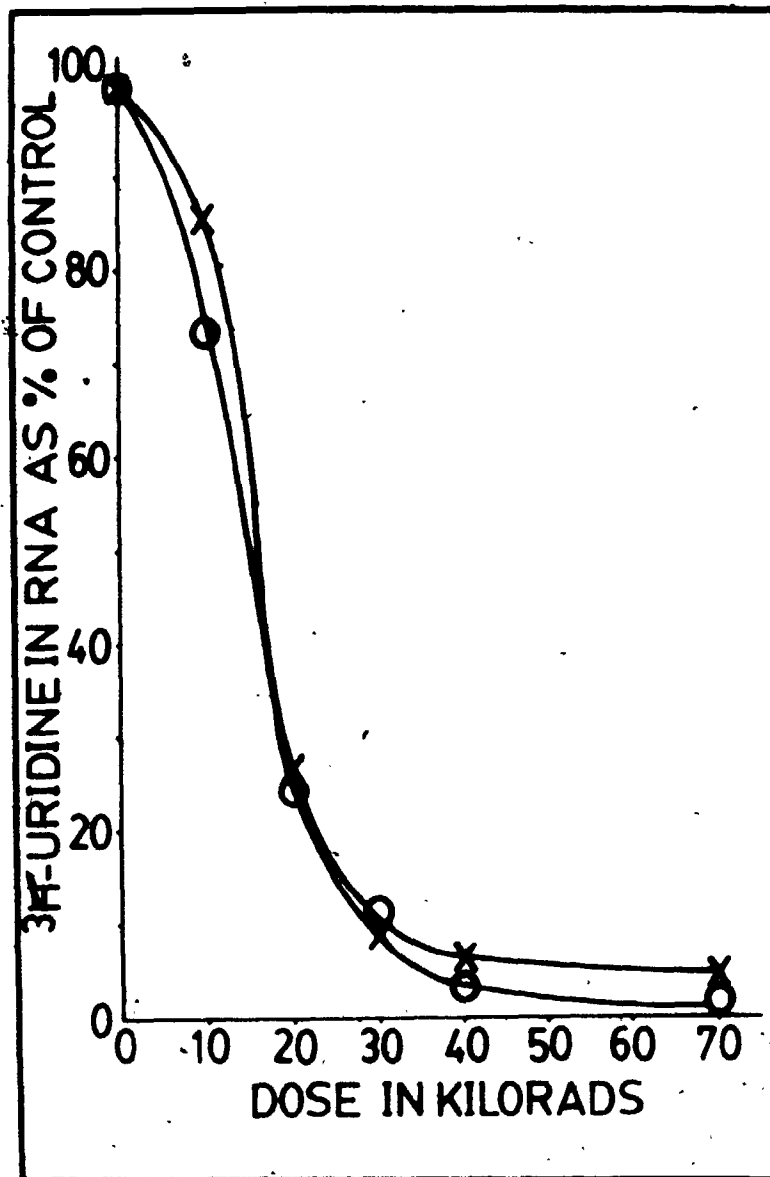
TABLE VIII

Effect of γ -irradiation on synthesis of RNA in Primate and Rodent cell lines

Dose in kilorads	BSC-1			L-2			L6H9(Ama) ⁺		
	Total RNA ^a cpm-3 X 10 ⁻³	Poly(A) RNA ^a cpm-1 X 10 ⁻¹	%	Total RNA ^a cpm-3 X 10 ⁻³	Poly(A) RNA ^a cpm-1 X 10 ⁻¹	%	Total RNA ^a cpm-3 X 10 ⁻³	Poly(A) RNA ^a cpm-1 X 10 ⁻¹	%
0	2,990	7,850	100	3,800	2,120	100	2,030	1,640	100
10	2,200	6,500	82.8	-	-	-	-	-	-
20	734	1,390	17.7	-	-	-	-	-	-
30	337	868	11.1	-	-	-	-	-	-
40	319	683	8.7	-	-	-	-	-	-
70	278	448	5.7	509	183	8.6	239	158	9.6

a, calculated per 1 X 10⁷ cells.

Figure 21. Effect of γ -irradiation on synthesis of RNA. BSC-1 cells were labeled with ^3H uridine for 2 hr commencing 1 hr post-irradiation. The total RNA was extracted and the poly(A)-containing fraction was selected as described in Materials and Methods. The cpm in total RNA (O) and poly(A) RNA (X) are expressed as percent of unirradiated control cells.



differential effect of intense γ -irradiation on transcription of total RNA versus putative mRNA. Moreover, these data revealed that host-related RNA synthesis and transcription into mRNA were profoundly suppressed following γ -irradiation with doses which did not influence appreciably if at all the replication of vaccinia virus.

4.3.3 Effect of γ -Irradiation on Incorporation of [3 H] Uridine into RNA of Vaccinia-Infected Cells

The finding that high doses of γ -irradiation were able to suppress cellular RNA synthesis made it possible to dissociate host-related from virus-specified transcription following infection with vaccinia virus. To this end, either pre-irradiated or unirradiated cultures of L-2 cells were inoculated and "pulse" labeled for 15 min. with [3 H]uridine at 8 hr p.i., when virus transcription is at a maximum rate (Oda and Joklik, 1967), but host-related transcription has been reduced to its lowest rate (Becker and Joklik, 1964). Labeled cells were then divided into nuclear and cytoplasmic fractions and the [3 H]uridine incorporation measured as described in Materials and Methods. As evident from Table IX, there was an inhibition of uridine incorporation into nuclei of infected but unirradiated cells, confirming the previously published findings. By contrast, there was an approximately 15-fold increase in cytoplasmic RNA synthesis in infected cells as compared with uninfected cells, presumably due to vaccinia virus-specified transcription. A similar increase was also evident in the cytoplasm of companion irradiated and infected cultures, indicating that pretreatment with 70 kilorad had

TABLE IX

Incorporation of ^3H uridine into nuclear or cytoplasmic
RNA of infected and uninfected L-2 cells^a

		Whole cells ^b	Nuclear Fraction ^b	Cytoplasmic Fraction ^b
Mock Infected	Unirradiated	31,460	27,460	1,290
	γ -Irradiated ^c	2,875	2,710	70
Vaccinia Infected	Unirradiated	22,020	3,890	19,130
	γ -Irradiated ^c	18,540	1,035	17,250

- a, 1×10^6 cells were "pulse" - labeled for 15 minutes with ^3H uridine at 8 hr p.i.
- b, disintegrations per minute; averages were determined from duplicate cultures.
- c, cells were exposed to 70 kilorad of γ -irradiation 1 hr prior to inoculation.

little or no influence on virus transcription. It should be noted from Table IX that after 15 min. pulse-labeling, ^3H uridine incorporation into the cytoplasm of uninfected cells was, as anticipated, very low. In γ -irradiated cultures the quantity of RNA label was reduced by more than 90% in both the cytoplasmic and nuclear fractions (Table IX), substantiating the data presented in Table VIII. The combined results of Tables VIII and IX revealed that pre-irradiation did not affect appreciably either viral mRNA synthesis or replication.

4.3.4 Effect of γ -Irradiation on Biological Function of RNA

To ascertain whether high doses of γ -irradiation can cause the abolition of gene expression, infection by IHD-J vaccinia virus was selected and studied, in collaboration with G. McFadden, as the first model of transcription and translation. This model was selected because (1.) cytoplasmic virus DNA replication occurs synchronously 1-4 hr after infection and (2.) the IHD-J strain induces production of hemagglutinin as one of the late, late viral functions (Ichihashi et al., 1971), for which the requisite transcription and translation commences at approximately 4 hr after infection, when late virion-related polypeptide synthesis is already well underway (Dales and Mosbach, 1968). The results (Table X) revealed that cells irradiated with 50 krad immediately after completion of viral DNA synthesis were able to produce only small quantities of hemagglutinin. Exposure to 5 krad caused only a partial inhibition of virus-specified synthesis. Application of inhibitors, used as the controls, revealed

TABLE X

Comparison between effect of γ -irradiation
and inhibitors of synthesis on
vaccinia virus hemagglutinin production

Treatment	Hemagglutinin end-point titer
None	1024
5 kilorad	512
50 kilorad	64
Actinomycin D	64
Streptoviticin A	16

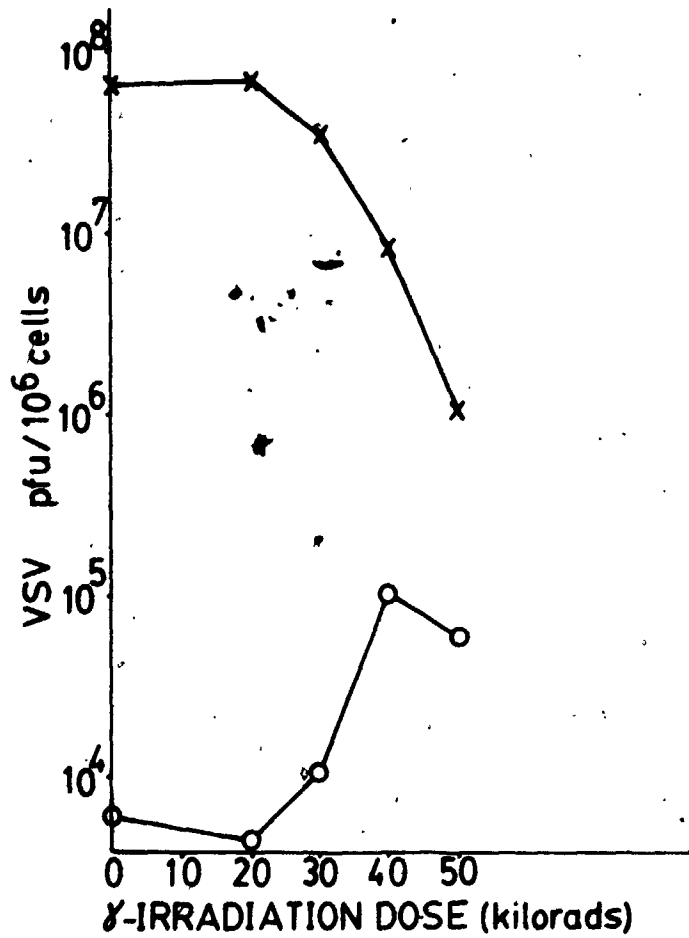
L-2 cell monolayers were inoculated with IHD-J vaccinia virus and incubated for 4 hr at 37°. Individual cultures were either irradiated or placed in medium containing actinomycin D or streptoviticin A, then incubated for an additional 20 hours. Hemagglutinin (HA) was assayed as previously described (Ichihashi and Dales, 1971), with the exception that calcium and magnesium were omitted from the phosphate buffered saline. HA titers were calculated per 1×10^6 cells.

that actinomycin D, if added after DNA synthesis at a concentration sufficient to block transcription rapidly (Dales and Mosbach, 1968), caused reduction of hemagglutinin formation to approximately the same low level as a dose of 50 krad. As anticipated, streptovitacin A, an inhibitor of translation, also inhibited the expression of the virus-specific activity. Thus, viral gene expression, as measured by the production of hemagglutinin, was severely inhibited by intense γ -irradiation treatment late in the infectious cycle.

In order to assess the influence of γ -irradiation on some defined cellular activity, the manifestation of the interferon-mediated antiviral state was selected as the second model of gene expression. This function was selected because, first of all, it is an inducible one, secondly, because it is amenable to quantitative assay, and finally, because it manifests or is related to the induction and expression of a multicomponent cellular system involving a number of enzymatic functions (Baglioni, 1979).

The antiviral state was induced in BSC-1 cells following various doses of γ -irradiation 1 hr prior to the addition to monolayer cultures of 100 units/ml of human leukocyte interferon (INF), kindly provided by Dr. C. Tan, University of Calgary. After an 8-hr period of INF treatment, the monolayers were inoculated with vesicular stomatitis virus (VSV) at a multiplicity of infection of 0.1 pfu/cell. Following incubation for 10 hr p.i., the infectious virus formed was assayed as plaque-forming units on monolayers of BSC-1 cells. Cell monolayers untreated with INF, but otherwise irradiated and incubated under identical circumstances, were used as the controls for

Figure 22. Effect of γ -irradiation on the INF-induced antiviral state. BSC-1 cells were incubated in the absence (X) or presence (O) of human leukocyte interferon (INF) for 8 hr commencing 1 hr after γ -irradiation. Following removal of INF, the cultures were inoculated with VSV and incubated for 10 hr until harvest for PFU assays.



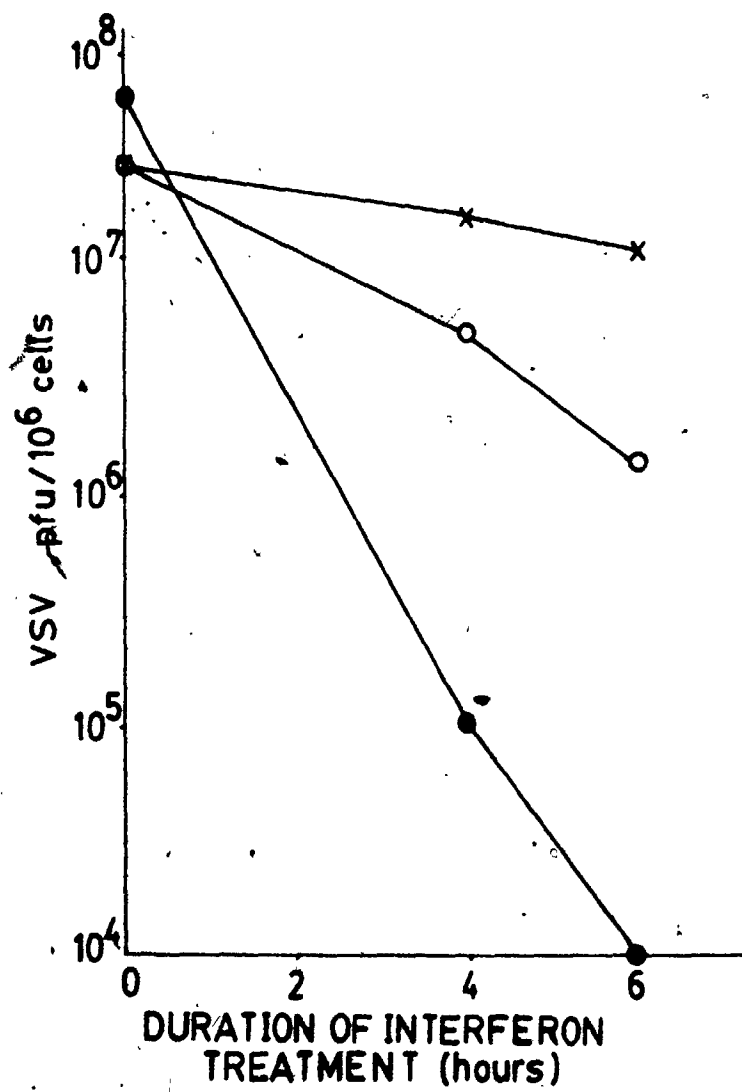
replication of VSV. The results, summarized in Figure 22, indicated that induction of the antiviral state by the INF treatment used, was effective as evident by the reduction in the PFU of about 4 log units. Irradiation of monolayers with 20 kilorad prior to INF treatment appeared to have little or no effect on the induction of the antiviral state. However, at doses of 40-50 krad, there was over 1 log increase in VSV production in the irradiated and INF-treated cultures compared to the unirradiated INF-treated culture. However, since irradiation per se at 30-50 krad adversely affected VSV replication in BSC-1 cells, it may be assumed that the actual difference in titer between INF-treated and untreated cells exposed to 30 krad was only 3 log units, at 40 krad about 2 log units, and at 50 krad about 1 log unit. These data are consistent with the view that the biochemical sequence of events related to induction of the antiviral state by INF was being effectively suppressed by high doses of γ -irradiation. Therefore, host-specified functions, expression of which is dependent on ongoing transcription, were effectively suppressed by 40-50 kilorad, doses which have little or no effect on vaccinia virus replication.

Since intense irradiation, followed by prolonged incubation decreased the capacity of BSC-1 cells to support VSV replication, INF treatment over shorter durations was instituted. Thereby, it was possible to enhance the replicating capacity of the irradiated host while allowing sufficient time for induction of the antiviral state. Cells exposed to 40 krad were incubated for 1 hr before commencing INF treatment for 4 to 6 hr as described above. As evident from the data in Figure 23, INF-untreated cells irradiated 5 hr prior to inoculation

Figure 23. Effect of γ -irradiation and duration of INF

treatment on the induction of the antiviral state.

BSC-1 cells were γ -irradiated (40 kilorad) 1 hr prior to addition of INF, as described in Figure 22. After incubation for the periods indicated, the monolayers were washed, inoculated with VSV, and harvested 10 hr p.i. for PFU assays. γ -Irradiated (X); INF treated (●); γ -irradiated, then INF treated (○).



produced about half a log less virus (1.5×10^7 PFU) than the unirradiated and untreated cells (5×10^7 PFU), hereafter referred to as controls. By contrast, the unirradiated cells treated with INF for 4 hr yielded 2.5 log units less (1×10^5 PFU) of VSV than the controls. Following γ -irradiation and INF treatment for 4 hr, the yield of VSV (4.7×10^6 PFU) was only 1 log less than obtained with the controls. With more prolonged treatment cultures irradiated 7 hr prior to infection replicated approximately a half a log unit less VSV (1.1×10^7 PFU) than the controls. A 6-hr period of INF treatment of unirradiated cells caused an approximate 3.5 log unit reduction in titer (Fig. 23), but pre-irradiation enabled cells treated with INF to produce 1.3×10^6 PFU of VSV, i.e., a reduction in yield of only 1.5 log units compared with the controls. Therefore, γ -irradiation enhanced VSV replication in INF-treated cells by over 2 log units.

These combined data in Figures 22 and 23 indicated that pre-irradiation with 40 or more kilorad can effectively inhibit induction of the antiviral state and must, therefore, cause suppression of gene expression manifested as a specific biological function.

4.3.5 Summary

The finding that high doses of γ -irradiation did not affect appreciably the ability of a variety of cells types to support herpes simplex virus and vesicular stomatitis virus (Table VI) and vaccinia virus mRNA synthesis (Table IX) and replication (Table VI) prompted an investigation into the effects of intense radiation on gene

expression. While protein synthesis continued at a relatively high rate, nucleic acid synthesis in irradiated cells was much more adversely affected (Table VIII). There appeared to be no differential effect of high doses of γ -irradiation on total versus putative messenger RNA transcription in that both are extensively suppressed in a parallel fashion (Table VIII and Figure 21). Gene expression, measured in terms of vaccinia hemagglutinin production (Table X) and induction of the interferon-mediated cellular antiviral state (Figures 22 and 23) was severely inhibited by γ -irradiation. Taken together, these data indicated that intensely γ -irradiated cells, which can support vaccinia replication, are severely inhibited in expression of their genetic information as assessed both as putative mRNA synthesis and cellular activity.

Chapter 5

DISCUSSION, CONCLUSIONS AND PERSPECTIVE

The efforts described in this dissertation have been directed towards the elucidation of the roles, first, of post-translational cleavage (PTC) and envelope assembly and, secondly, of the host DNA-dependent RNA polymerase II (polymerase II) and ongoing host gene expression in vaccinia virus biogenesis.

Previously published investigations with inhibitors (Nagayama et al., 1970; Katz and Moss, 1970a and 1970b) and temperature-sensitive (ts) mutants (Stern et al., 1977) revealed that post-translational cleavage (PTC) of virion core polypeptides is a necessary step for the development of infectious, mature vaccinia virus. Present studies have focused on a group of ts mutants, group E (Dales et al., 1978), which are defective in envelope assembly and maturation. Characterization of this group of mutants and experimentation directed towards understanding the nature of the cleavage process has culminated in a revised model of vaccinia morphogenesis.

The investigation into the function of polymerase II in the life cycle of vaccinia virus has centered on two major questions: Is this host function required for virus replication and, if so, is continuous transcription of the host genome also required? To this end, the use of the toadstool toxin α -amanitin, a specific inhibitor of polymerase II in animal cells (Roeder, 1976), in sensitive and resistant cell lines, and studies employing intense γ -irradiation, known to damage DNA (Okada, 1970), have resolved this recently controversial subject. A discussion of the findings presented in this work and of others on this topic has led to the formulation of a hypothesis concerning the role of polymerase II in the life cycle of a poxvirus.

5.1 Characterization of Group E Mutants

Members of the group E mutants, ts1085, ts7743, ts9203, ts9251, and ts9383; were initially categorized by their morphological similarities observed during their arrested development (Dales et al., 1978). A more detailed characterization of individual phenotypic defects has confirmed that these mutants are blocked at about the same stage, that which mimics the effect of rifampicin on virus morphogenesis (Nagayama et al., 1970), to a lesser or greater degree. Generally, it was observed that numerous viroplasmic foci were formed, each surrounded by flexible envelope segments but which are devoid of spicules, and DNA paracrystals accumulate. This was most evident in infections with mutants ts1085 and ts9203 but was less so in the case of ts7743 where both normal partially complete and even spherical particles were also detected. Mutants ts9251 and ts9383 behaved in an

anomalous manner under nonpermissive conditions in that the development of mature virions was observed in addition to the formation of aberrant membranes described above. This morphological "leakiness" could not be explained by occasional break-through of the mutant culminating in normal progeny development because, when measured in terms of production of infectious progeny, both these mutants are tight. Since group E mutants mimic the morphological abnormalities observed in wild-type (wt) infected cells treated with rifampicin and since this antibiotic also affects normal PTC (Nagayama et al., 1970), it was necessary to determine whether the late proteins were processed normally at the restrictive temperature. All five mutants were found to be unique in their cleavage profile, ranging from a complete block in the case of ts1085, confirming the results of Stern et al. (1977), to apparently normal PTC in the case of ts9251. Thus, although assembly appeared to be a tightly coupled process (reviewed in Dales and Pogo, 1981), proteolytic cleavage of one precursor was not necessarily dependent on the prior processing of another precursor polypeptide. Since reversal of incubation conditions, from restrictive to permissive, resulted in normal PTC leading to production of core protein products, it followed that the cleavage defect was indeed related to a temperature sensitivity. Furthermore, the appearance of apparently normal virus structures, as in the case of ts7743 and ts9383, without formation of all cleavage products, indicated that assembly of immature virion envelopes with normal morphology was not contingent upon PTC, in support of previous data of Stern and Dales (1976b) and Stern et al. (1977). Conversely, normal envelope assembly was not a precondition for PTC of certain

precursors, as evident by production of p23 in ts9203 infections where the defect in envelope assembly is morphologically tight. Thus the formation of normally enveloped immature particles and cleavage of precursor polypeptides to core proteins were not necessarily interdependent processes.

The finding that each mutant of the E group displayed a unique cleavage pattern indicated that the mutations occurred in distinct loci. This result was supported by the checkerboard series of complementation experiments where all but one member of the group was able to complement each other. The notable lack of complementation between ts7743 and the four other mutants, and positive complementation with the DNA(-) mutant, ts6389 (Dales et al., 1978), implied that ts7743 could be a regulatory mutant rather than a "trans-acting dominant mutant" (Carstens and Weber, 1977). Recombination mapping studies conducted by J.L. Lake (Lake et al., 1979) and K. Essani (doctoral dissertation, 1982) have confirmed that each mutation has a distinct locus. Because ts9251 appeared to have no cleavage defect even though aberrant membranes were formed, exclusion of this mutant from the group would indicate that as many as four gene functions may be involved in the PTC process.

5.2 Nature and Role of PTC in Vaccinia Virus Assembly

In an endeavor to elucidate further the connection between processing and virus assembly, experiments were formulated to identify proteolytic factor(s) which might be involved. As one approach, complementary infections were initiated from which it became evident that the dual infection at 39 with ts1085 and ts6389, a DNA(-) mutant, resulted in enhanced production of infectious mature virus of both parental phenotypes as well as rescue from the processing defect inherent in ts1085. This experiment demonstrated that an active proteolytic factor, once induced, could bring about cleavage of core polypeptide precursors specified by a cleavage defective mutant. Therefore, the defect in ts1085 probably involves proteolysis itself, not the protein substrates being cleaved. By contrast, with mutants like ts7743, in which some precursor core proteins are processed, the uncleaved precursor proteins may themselves be mutated, as demonstrated by McFadden *et al.* (1980) for a p37 core polypeptide of ts9251. Such proteins might, therefore, not be amenable to PTC. Data from complementation experiments also ruled out the possibility that an inhibitor of PTC was acting during ts1085 infections. In contrast to the above data on complementation by dual infection, results from cell-cell fusion experiments designed to assess the mobility of protease factor(s) showed that PTC was not rescued after coalescence of cytoplasm of cells infected by wt virus permissive for PTC with cells infected by cleavage-defective ts1085. This result indicates that the proteolytic factor(s) involved may not be readily diffusible between factories within a continuous cytoplasmic compartment, perhaps

because the site of synthesis and activity in PTC must be somehow spatially coordinated.

A series of experiments on cells infected with ts1085, in which cultures were shifted for short periods to the permissive temperature then returned to the restrictive one, provided results consistent with the view that PTC, once initiated under permissive conditions, does not continue to function after "shift-up" to the restrictive temperature. This evidence is consistent with the finding that continuous transcription and translation are necessary to maintain PTC, even in the case of wt vaccinia infections (present data and Stern and Dales, 1976a) and implies that the proteolytic activity required to process vaccinia core polypeptides and facilitate the maturation process can be active for only short periods. Data from quantitative electron microscopic analysis are entirely consistent with the idea that control of PTC determines also the assembly and maturation process. With ts1085, this is evident from the direct relationship between time spent at 33° and the number of immature and mature progeny formed. Continuation of virus development following shift-up to 39°, albeit limited in extent, presumably involves those immature virions which can utilize the preformed protease factor(s) available to them. This suggestion is in agreement with the finding by Dales and Mosbach (1968), that after rapid suppression of translation with Str A, during infection with wt vaccinia, assembly of a small quantity of mature, infectious progeny may continue.

Present studies, employing specific protease inhibitors, were useful in characterizing the vaccinia virus protease factor(s). Both

TPCK and ZPCK, used at concentrations which have been shown to inhibit in vivo processing of polioviral polypeptides in HeLa cells (Sommers et al., 1972), were effective in blocking PTC of vaccinia virus-specified precursor polypeptides, implying a chymotrypsin-like active site on the protease. Binding studies, employing isotopically labeled ZPCK, although not definite because dissociation of H from the inhibitor molecule was not ruled out, suggested that this proteolytic factor may reside in a p12.5 virus-specified polypeptide, which is absent from the mature virion itself. Thus it is unlikely that the vaccinia virion-associated alkaline protease reported by Arzoglou et al. (1979) is the same activity involved in PTC. In terms of molecular weight, the p12.5 protein is substantially smaller than the p24 MW of chymotrypsin, but approximates the p15 size of the protease within avian sarcoma virus, which is involved in PTC of the virion pr76 precursor polyprotein and maturation (von der Helm, 1977).

While the p12.5 polypeptide appeared to have a greater affinity for the labeled ZPCK probe as compared to any other polypeptide, as shown in Figure 18, the possibility that a polypeptide other than p12.5 is the proteolytic factor cannot be discounted.

Synthesis of a viral protein with affinity for ZPCK, under a variety of circumstances permissive or restrictive for PTC and maturation, suggests that it is not the inhibited synthesis of the factor per se which is connected with the arrest in proteolysis. Indeed, the ability of [³H]ZPCK to bind to the putative protease under conditions nonpermissive for PTC in ts1085 infections would argue against the ts1085-specified protease itself being temperature

sensitive. Rather, the basis for the defects may result from (1) unavailability at the time and place required, of nascent protease, as evident with wt infection, or (2) protease interactions with abnormal products or aberrantly constructed immature particles. Thus, in the case of mutants like ts1085 and ts7743, the conformational state of each polypeptide precursor within the assembling immature virion and spatial relationship to the envelope could conceivably determine whether PTC can occur. With wt vaccinia infections, it might be assumed that the protease factor has to be in the appropriate place and active state within assembling immature forms to permit PTC to occur.

5.3 Role of Polymerase II and Host Cell Gene Expression in Vaccinia Biogenesis

Current experiments, using as the host the α -amanitin-sensitive Ama⁺ and drug resistant Ama 102 mutant rat myoblasts, clearly demonstrate the involvement of a host function related to polymerase II in vaccinia biogenesis. Since virus-specified DNA synthesis occurred at near normal rates (80%), while synthesis of late proteins and formation of vaccinia-related structures were curtailed to varying degrees, polymerase II may be involved in expression of late viral functions. These findings are essentially in agreement with the data of Wiruby et al. (1979b) which document the sensitivity of vaccinia virus replication to α -amanitin.

The two candidates for the obligatory requirement of polymerase II involve transcription of either vaccinia or host cell genetic

information. The observation that intensively γ -irradiated cells were fully competent to produce infectious vaccinia progeny, as well as HSV-1 which is known to be sensitive to α -amanitin during its replicative cycle (Costanzo et al., 1977; Ben-Zeev and Becker, 1977; this study), imply that polymerase II may be involved in viral transcription of both these agents (Watson and Clements, 1980) but that host-specified transcription is probably not involved in this process. The data produced by Hruby and co-workers (1979b) concerning the ability of exposure of BSC-40 cells to ultraviolet (UV) irradiation, prior to infection, to inhibit production of infectious vaccinia virus appears to be in conflict with the results of γ -irradiation studies in this dissertation. While comparisons of UV and γ -irradiation experiments are difficult because of the mode of measuring exposure, Joules per square millimeter and Joules per kilogram respectively, one might relate the data based on multiples of lethal doses, measured in terms of the dose that will kill 67% of the mammalian cells in culture, i.e., Do (Okada, 1970). Energies used by Hruby and co-workers, $4.8 \times 10^{-5} \text{ J/mm}^2$ correspond to 5.7 times Do for mouse L cells (Chiu and Rauth, 1971), while γ -irradiation studies conducted herein employed 4 to $7 \times 10^2 \text{ J/kg}$ or 235 to 410 times Do for the same strain of mouse fibroblasts (Whitmore and Till, 1964). An explanation for the apparent inability of UV-irradiated cells to support vaccinia virus even though the doses, when converted to Do units, were less than that used to γ -irradiate cells, which retain the capacity to support the production of the virus, may lie in the ability of UV irradiation to induce stable cross-links between nucleic acids and proteins. Strniste and Smith (1974) reported that

prokaryotic DNA-dependent RNA polymerase could be induced to form stable binary complexes with a synthetic DNA polymer when exposed to UV irradiation and more recently van Eekelen and van Venrooij (1981) have shown that by UV irradiation of intact HeLa cells, proteins tightly associated with heterogeneous nuclear RNA can be induced to cross-link with RNA. Thus it is conceivable that the inability of UV-irradiated cells to support vaccinia virus replication may be due to sequestration of polymerase II within the nucleus, where it is concentrated (Schartz et al., 1974; Seifart et al., 1972), and is therefore unable to interact with vaccinia virus-specified DNA, located in the cytoplasmic factories (Cairns, 1960) and bring about formation of late vaccinia transcripts. Similar reasoning may be invoked to explain the dramatic inhibition of vaccinia replication in enucleated cells (Pennington and Follett, 1974; Hruby et al., 1979a).

Data from the current investigation of the effects of intense γ -irradiation on mammalian cultured cells reveal that synthesis of nucleic acid is much more sensitive than that of protein and in this sense the formation of the two kinds of macromolecules may be uncoupled. The fact that RNA agents of the picorna group, such as mengovirus (Tobey et al., 1970) and the enveloped rhabdovirus VSV, as well as DNA viruses, exemplified by vaccinia and HSV-1, are able to replicate efficiently in cells pre-irradiated with doses as high as 70 kilorad implies that the host translational apparatus escapes the effects of γ -irradiation relatively intact. By contrast, replication of DNA and transcription into putative mRNA, ascertained in terms of the polyadenylated RNA fraction, is profoundly inhibited. Gene

expression, whether measured in terms of a viral activity, vaccinia virus-specified hemagglutinin, or in the manifestation of an inducible cellular function, namely the interferon-mediated antiviral state, was also efficiently suppressed by high doses of γ -irradiation. Taken together, these data indicate that intensively γ -irradiated cells, which can readily support vaccinia virus replication, are severely inhibited in expression of their genetic information, as assessed both as putative mRNA synthesis and cellular activity.

Synthesis in the cytoplasm of infected, pre-irradiated cells of near normal levels of vaccinia-specified RNA shows, on the one hand, that irradiation does not affect pools of metabolites and any host functions which might be required for virus-specified RNA synthesis and, on the other, that host-related transcription is most probably not required for optimum viral transcription. Thus it is unlikely that the observed decrease in RNA synthesis due to γ -irradiation is the consequence of either a decrease in [^3H]uridine uptake into the cell or dilution of the isotope within an enlarged uridine pool. It is more likely that the inhibition of [^3H]uridine incorporation into RNA, either the bulk material or the poly(A) fraction, reflects the damage by γ -irradiation on the transcription process from the DNA template.

Use of intense γ -irradiation as applied here to mammalian cells in culture has not, to our knowledge, been reported previously. However, numerous studies have found little or no inhibitory effect of irradiation on ribosomal, transfer, heterogeneous nuclear, and mRNA when doses lower than 2 kilorad are applied (reviewed by Walters and

Enger, 1976; Hopwood and Tolmach, 1979). Thus HeLa cells irradiated with 500 rad incorporate isotopic uridine during the first 10 hr after treatment at the same rate as the unirradiated controls (Hopwood, 1974). Similarly, Enger and Campbell (1974) found that the rate of mRNA synthesis in Chinese hamster ovary cells exposed to 800 rad was equal to that of the control over a 7-hr period post-irradiation. In support of the above findings, this study shows that irradiation with doses over 10 kilorad is necessary to substantially inhibit total or poly(A) RNA synthesis.

5.4 Conclusions and Perspective

Despite the large size of the poxvirus genome, approximately 123×10^6 daltons (Muller et al., 1977), and highly complex series of regulated events involved in the replication and maturation cycle (Dales, 1963; Moss, 1974; Stern and Dales, 1976b; Dales and Pogo, 1981) more detailed knowledge of the system may be acquired through studies using conditional-lethal mutations of the type previously described (Sambrook et al., 1966; Padgett and Tomkins, 1968; Basilico and Joklik, 1968; Stern et al., 1977; Drillien et al., 1977; Chernos et al., 1978). Combined data from current and previous investigations, employing inhibitors and more recently ts mutants, has led to the following proposed model of vaccinia virus biogenesis: The envelopes, assembled de novo and coated externally by spicules which determine the spherical form of the immature particles (Dales and Mosbach, 1968; Essani et al., 1982), are formed around quanta of DNA and polypeptides, among them proteins destined as enzymatic or

structural components of the core (Pogo and Dales, 1971; Nagayama et al., 1970; Stern and Dales, 1976b; Stern et al., 1977). The internal components then acquire precise conformation with respect to the envelope, an event which becomes critical for subsequent PTC controlled by the virus-specified protease. The protease or a factor required for activity, with a rapid turnover rate or short half-life, has to be incorporated as a nascent polypeptide into immature particules before the envelope has been completed or sealed. Once PTC is initiated, all precursors are processed, although cleavage of one is not contingent on that of another. PTC is obligatory for progression from immature into the mature form. As a consequence, PTC, induction of core enzymes, internal differentiation into the core and lateral bodies, and acquisition of infectiousness are temporally controlled, tightly coupled events.

Concerning the role of the DNA-dependent RNA polymerase II activity in vaccinia biogenesis, the most plausible hypothesis, which takes into consideration all the available information, should assume that early vaccinia functions, expressed initially from the virion core, then after uncoating, and including those required for DNA replication, are catalyzed by a virus DNA-dependent RNA polymerase (Kates and McAuslan, 1967a and 1967b), whereas some or all of the late functions involve host polymerase II. Whether the entire polymerase II is monopolized in this process or perhaps only one of the enzyme's subunits is unknown. It should, however, be remembered that both the vaccinia RNA polymerase and polymerase II are multicomponent enzymes (Nevins and Joklik, 1977; Roeder, 1976). Because in vitro experiments

demonstrate specificity of low concentrations of toxin for polymerase II of *Ama* cells (Ingles et al., 1976) and experiments on attachment with radioactive α -amanitin by use of a cross-linking agent suggest, but by no means prove, that the toxin acts by specific binding to the p140 subunit of polymerase II (Brodaner and Wieland, 1976), it is not inconceivable that this subunit functions in concert with vaccinia RNA polymerase subunits in the late transcription process. Precedents for this notion have been established in the prokaryotes, as documented on the modification of host RNA polymerase by some bacteriophages of *Escherichia coli* and *Bacillus subtilis* (reviewed by Losik and Pero, 1976). The possibility that viral and host polymerase enzyme subunits can be assembled into a hybrid transcriptional enzyme may be testable if, in the future, host mutants become available in which different polymerase II subunits are genetically altered.

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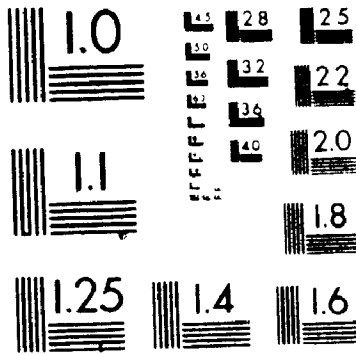
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Microbiology

Biogenesis of poxviruses: Role for the DNA-dependent RNA polymerase II of the host during expression of late functions

(effects of α -amanitin on development/host polymerase II mutant in replication/role of polymerase II in late transcription)

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ABSTRACT The participation of host RNA polymerase II in the vaccinia life cycle was examined by comparing efficiency of multiplication after treating the Ama⁺ sensitive and Ama 102 drug resistant lines with α -amanitin. In the latter, resistance is due to a mutation in RNA polymerase II. The toxin profoundly reduces synthesis of virus-specified polypeptides and morphopoeisis in Ama⁺ but not in Ama 102 rat myoblasts without appreciably altering vaccinia DNA replication in either cell type. This implicates RNA polymerase II in the expression of late virus functions. Circumstantial evidence from a model system indicates that γ irradiation of the host prior to infection might disrupt transcription into functional mRNA from the nucleus. Irradiation does not, however, alter the capability of the host to support vaccinia multiplication fully. Therefore, ongoing host nuclear transcription may not be required by this virus. The above results are consistent with the ability of cytoplasts to produce small quantities of mature progeny. Our studies lead us to hypothesize that RNA polymerase II or a subunit of the host enzyme may participate directly in late transcription of the vaccinia genome.

The highly active toxin α -amanitin, derived from the toadstool *Amanita phalloides*, has been recognized as a specific inhibitor of DNA-dependent RNA polymerase II, hereafter referred to as polymerase II, of animal cells (1). By virtue of its inhibitory specificity, α -amanitin was used to demonstrate that certain DNA and RNA agents, such as papovaviruses, adenoviruses, and influenza viruses, having an obligatory developmental stage in the host nucleus, most probably require polymerase II activity for replication (2-4). By contrast, the poxviruses, which develop in the cytoplasm, were reported to be insensitive to this toxin (as cited in ref. 5) and to contain in the virion core an α -amanitin-insensitive DNA-dependent RNA polymerase (5). However, some requirement for the host nucleus is implied in the replication of vaccinia because virus development in cytoplasts is incomplete (6, 7). The availability of the rat myoblast L₃ cell line, which our initial experiments showed can support the growth of vaccinia and from which a mutant was derived having a polymerase II resistant to α -amanitin (8), prompted us to examine the possible role of host transcriptional function(s) in the life cycle of poxviruses.

MATERIALS AND METHODS

Cells and Viruses. Monolayers of L₂ mouse fibroblasts were used for virus propagation and assays of plaque-forming units (PFU) in nutrient medium and under culture conditions described (9). The viruses used were the hemagglutinin-inducing parental IHD-J or the syncytogenic IHD-W variant of vaccinia (10) and the Indiana strain of vesicular stomatitis virus (VSV). For inoculation, 10 PFU/cell usually were added as reported elsewhere (11).

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To investigate the role of host-derived functions in development of vaccinia we used (i) a clone L6H9, designated Ama⁺, and an α -amanitin-resistant mutant Ama 102 derived from this clone of a rat myoblast line (8), both kindly provided by M. E. Pearson (University of Toronto), and (ii) temperature-sensitive mutant 422E derived from hamster BHK21 fibroblasts, which is conditional-lethal for 28S ribosomal RNA formation and assembly of the 60S ribosomal subunit (12), provided by H. E. Meiss (New York University Medical School).

Synthesis and Labeling. Cytoplasmic DNA synthesis in IHD-W vaccinia-infected Ama⁺ and Ama 102 cells was measured by continuous labeling, at 37°C for 4 hr after inoculation, in the presence of 1 μ Ci of [methyl-³H]thymidine per ml (New England Nuclear) as described (13). Briefly, labeled cells were allowed to swell in hypotonic saline/buffer solution, then were disrupted in a Dounce homogenizer. The radioactivity of trichloroacetic acid-precipitable material was measured in a scintillation counter. Procedures used by this laboratory for labeling and characterizing nascent polypeptides have been described (14, 15). Briefly, at 9.5 hr after inoculation, monolayers were exposed for 60 min to 20 μ Ci of [³⁵S]methionine per ml (New England Nuclear) added to methionine-free nutrient medium. Samples for preparing cytoplasmic extracts were taken either at the end of the pulse or after 8 hr of incubation in chase medium. Aliquots were used for determination of trichloroacetic acid-precipitable radioactivity and for polyacrylamide gel electrophoresis.

Inhibitors. Synthesis of RNA was suppressed by adding to the nutrient medium 4 μ g of actinomycin D per ml (Sigma); protein was inhibited with 10 μ g of streptovivacin A per ml (gift from Upjohn) as described (11). In the experiments involving Ama⁺ and Ama 102, rat myoblast cultures were treated with 2 μ g of α -amanitin per ml (Sigma) according to ref. 8.

Electron Microscopy. The methods for collecting and preparing cell samples for thin sectioning and examination by transmission electron microscopy were the same as those described in previous studies (11).

RESULTS

Vaccinia Virus Replication Examined by Means of Host Cell Mutants and α -Amanitin. To investigate the role of host transcription into mRNA involving polymerase II (1), we used α -amanitin, which specifically inhibits polymerase II of animal cells when used at appropriately low concentrations (1). The host used was a myoblast cell line in which the wild-type cell is Ama⁺ and the mutant, containing α -amanitin-resistant polymerase II, is designated Ama 102 (8). Because the permeability and time required to inhibit polymerase II activity varies depending on the cell (16), it was necessary to ascertain the

Abbreviations: polymerase II, DNA-dependent RNA polymerase II; PFU, plaque-forming units; VSV, vesicular stomatitis virus.

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duration of treatment with α -amanitin to effect an inhibition of vaccinia replication. The data in Table 1 show that treatment of Ama^+ cells for 5 or 10 hr abolished the capacity of the host to support production of infectious progeny, while shorter treatment resulted in fractional yields of PFU. Because the inhibitor was also kept in the culture medium throughout the growth cycle, it may be concluded that suppression of replication was elicited gradually and progressively. This is consistent with the slow inhibition of polymerase II activity by α -amanitin demonstrated biochemically (17) and implies that this host enzyme or a subcomponent of it is necessary for formation of infectious vaccinia virus. In sharp contrast to the above findings, treatment of Ama 102 cells with the drug had no inhibitory effect on the yield of vaccinia PFU (Table 1), also indicating the involvement of polymerase II in the vaccinia virus cycle.

As a control of the specificity of α -amanitin effects on vaccinia, replication of VSV was tested under parallel circumstances. The data shown in Table 1 revealed that the drug did not suppress VSV production in either Ama^+ or Ama 102 cells. Yields in Ama 102 were usually almost an order of magnitude greater than in Ama^+ , an observation which deserves further attention.

The effects of α -amanitin on synthesis of virus-specified products, including the synthesis of DNA and polypeptides in the cytoplasm, were monitored by isotopic labeling, polyacrylamide gel electrophoresis, and enumeration of progeny particles by quantitative electron microscopy.

Analysis of cytoplasmic extracts from untreated and drug-treated cells labeled with [3H]thymidine showed that, in Ama^+ , in the absence of α -amanitin about 3300 cpm per 10^6 cells were incorporated and, in cells pretreated for 10 hr and maintained in the presence of the drug during infection, 2600 cpm per 10^6 cells were converted into an acid-precipitable product. In Ama 102, cytoplasmic extracts from both untreated and treated cells contained about 23,000 cpm of [3H]thymidine per 10^6 cells incorporated into a macromolecular product. These data revealed that α -amanitin either does not inhibit or inhibits only partially vaccinia-specified DNA synthesis. As with VSV replication, the much greater rate of vaccinia-related DNA synthesis in Ama 102 cells remains unexplained.

The influence of α -amanitin on the spectrum of vaccinia-specified polypeptides synthesized in Ama^+ and Ama 102 cells was ascertained by pulse-chase experiments and polyacrylamide gel electrophoresis. According to the usage adopted in this laboratory, individual polypeptides in gels are identified by their molecular weight so that, for example, p94 is a polypeptide of M_r 94,000. The results illustrated in Fig. 1 show that when Ama^+ was the host, the quantity of [^{35}S]methionine-labeled polypeptide formed in the presence of the toxin was

Table 1. Virus replication in wild-type Ama^+ and resistant Ama 102 rat myoblasts treated with α -amanitin

Host	Untreated	Hr before inoculation when treatment commenced			
		0	-2	-5	-10
IHD-W vaccinia virus					
Ama^+	1000*	62	39	6	6
Ama 102	700	570	510	630	680
Vesicular stomatitis virus					
Ama^+	860	1,100	ND†	ND	1,200
Ama 102	7700	16,000	ND	ND	18,000

* Virus titers are expressed as 10^4 PFU/ 10^6 cells

† ND, not done. At 3 hr after inoculation the background titer for vaccinia virus-infected Ama^+ and Ama 102 cells was $\approx 6 \times 10^4$ PFU

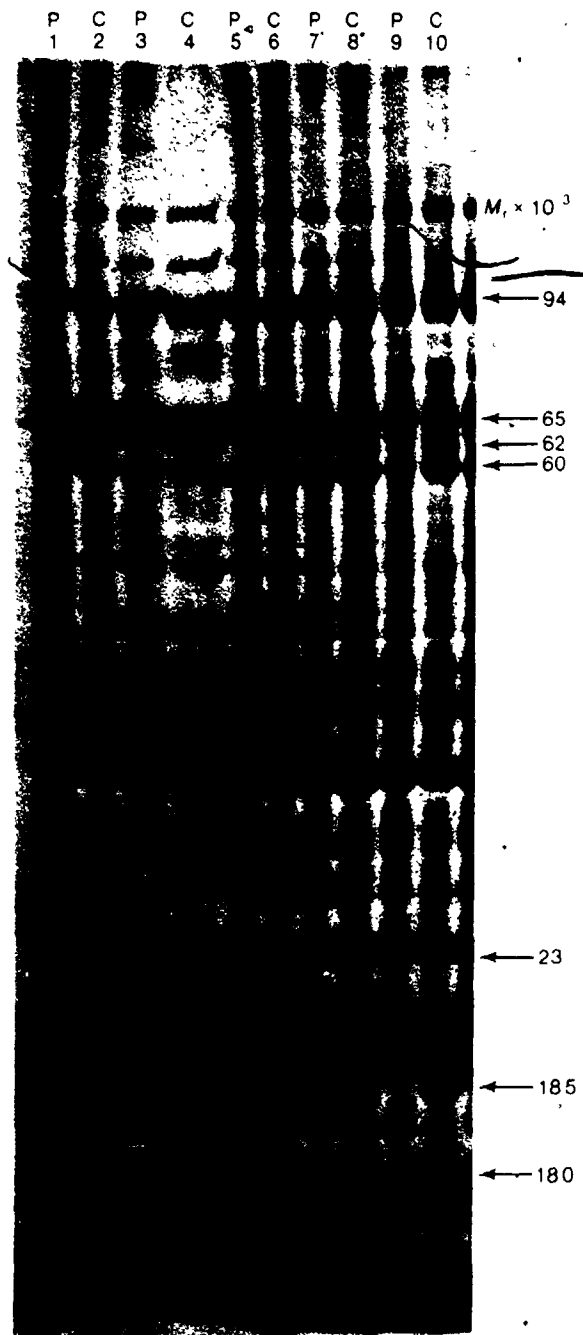


FIG. 1. Fluorogram of polypeptide profiles from untreated and α -amanitin-treated cultures in a slab gel prepared from infected L_2 Ama^+ and Ama 102 whole cell extracts according to refs. 14 and 15. After pulse labeling with [^{35}S]methionine and chasing, cells were disrupted by sonication in the presence of a nonionic detergent as described (14, 15), with the exception that the lysate buffer solution contained 1 mM $MgCl_2$. Treatment with 50 μg of pancreatic deoxyribonuclease I per ml (Worthington) for 15 min at 4°C was used to hydrolyze the DNA. Into each channel was placed 10–50 μg of protein and the film was exposed for 3 days. The vertical scale showing $M_r \times 10^{-3}$ was calculated from polypeptides used as M_r standards. Arrows denote the positions of seven polypeptides: p94, p65, p62, p60, p23, p185, and p18. P, pulse; C, chase. Channels: (1–4) Ama^+ extracts; (5–8) Ama 102 extracts, (9 and 10) L_2 extracts. Channels 3, 4, 7, and 8 show samples from drug-treated cells.

much less than in its absence (channels 3 and 4). However, the inhibitor did not abolish the synthesis of the p94 and p65 precursors nor their processing to the p62 and p60 products. Most or all of the other identifiable polypeptides, including p23, p18.5, and p18, were also synthesized, as evident in channels 3 and 4 of Fig. 1. In infection of Ama 102, α -amanitin did not reduce the quantity of vaccinia polypeptides produced or affect the normal posttranslational cleavages during the chase (channels 7 and 8, Fig. 1). The pattern of bands and processing of vaccinia-induced polypeptides were identical whether the host was the wild-type Ama⁺, Ama 102 polymerase II mutant, or mouse L₂ cells. The comparative data on L₂ cells shown in channels 9 and 10, which are identical to our previous findings (18), emphasize the uniformity of the spectrum of vaccinia-induced polypeptides, regardless of the cell type used for virus replication.

Results using electron microscopy to quantitate the formation of vaccinia related structures (Table 2) corroborated the findings from polyacrylamide gel electrophoresis and PFU assays. Only in Ama⁺ cultures treated with α -amanitin was the appearance of "factories" and assembly of immature and mature progeny virus severely curtailed. The small number of mature progeny evident in this sample was obviously insufficient to alter the titer, expressed in Table 1 in terms of the relatively less sensitive PFU. Electron microscopic examination of 210 cell profiles in the drug-treated Ama⁺ sample revealed the presence of 27 mature virions, all situated in only 3 profiles, and 48 immature particles, observed in only 6 profiles. This finding demonstrated that after treatment with the toxin, when rates of virus DNA synthesis were only partially reduced, vaccinia development was arrested to a varying degree, so that in some cells there was a complete absence of any virus structure while in a very small percentage of others virus assembly had been completed. Presumably the few mature progeny virions observed in this sample accounted for the processing of p94 and p65 polypeptides to their p62 and p60 products, evident in Fig. 1 and shown previously to be an obligatory step in the vaccinia maturation process (18). The occurrence of mature progeny in treated Ama⁺ cells also implies that, in the occasional host cell, inhibition of polymerase II may have been only partial or nonexistent.

To compare the influence of the polymerase II function in vaccinia replication with another possible nuclear function, we examined vaccinia virus development in a temperature-sensitive mutant 422E derived from BHK21 cells. The 422E cell is conditional-lethal for 28S ribosomal RNA formation and, as a consequence, also for the assembly of the 60S ribosomal subunit. With this host, preincubation at the restrictive temperature of

39°C for 24 hr or longer followed by infection with IHD-W vaccinia, also at 39°C, failed to suppress virus development, as judged by a comparison of the amount of virus formed at 39°C and at the permissive temperature of 33°C. This finding implies that formation of nascent ribosomes prior to or during infection was not required for vaccinia replication.

Effects of γ Irradiation on Vaccinia Replication. Published evidence implicating a role of the host nucleus in vaccinia biogenesis (6, 7) suggested that the continued template activity of the host DNA might be required for completion of the virus life cycle. This idea was tested by exposing monolayers of L₂ cells to intense γ irradiation from a ⁶⁰Co source 4 hr prior to inoculation. Treatment with 70,000 rads (1 rad = 1.0 × 10⁻² J/kg) was lethal for these cells, the majority of which died within 48 hr. Nevertheless, when infected 4 hr after exposure they remained fully competent to produce infectious vaccinia, as evident by the burst size, ≈ 100 PFU/cell, obtained from both control and irradiated cultures. In these cells mitosis can be abolished by <5000 rads. Our observation suggests that extensive damage to the host DNA by γ irradiation did not impair vaccinia production.

To ascertain whether high doses of γ irradiation can cause the abolition of gene expression, we used as a model of transcription and translation infection by IHD-J vaccinia. This model was selected because cytoplasmic virus DNA replication occurs synchronously 1-4 hr after infection and because the IHD-J strain induces production of hemagglutinin as one of the late, late viral functions (19), whereby the requisite transcription and translation commences at approximately 4 hr after infection, when late virion-related polypeptide synthesis is already well underway (11). The results (Table 3) revealed that cells irradiated with 50,000 rads immediately after completion of virus DNA synthesis were able to produce only small quantities of hemagglutinin and infectious particles. Exposure to 5000 rads caused only a partial inhibition of virus-specified synthesis. Application of inhibitors, used as the controls, revealed that actinomycin D if added after DNA synthesis at a concentration sufficient to block transcription rapidly, caused reduction of hemagglutinin and virus formation to approximately the same low level as a dose of 50,000 rads. As anticipated, streptovitacin A, an inhibitor of translation, also inhibited the formation of virus materials. These combined data imply that intense irradiation of the host before infection probably caused severe damage to nuclear DNA and affected transcription into functional mRNA without reducing synthesis of infectious vaccinia virus.

Table 2. Quantitative electron microscopy of vaccinia virus replication in wild-type Ama⁺ and resistant Ama 102 rat myoblasts treated with α -amanitin

Host cell and treatment	Vaccinia virus products			% cell profiles with mature progeny virions
	"Factories"	Immature particles	Mature virions	
Ama ⁺ , no drug	41	436	325	35
Ama ⁺ + α -amanitin	8	23	13	1-2
Ama 102, no drug	56	600	332	30
Ama 102 + α -amanitin	78	946	844	56

The data are normalized as counts per 100 profiles of thinly sectioned cells. In the sample of Ama⁺ with α -amanitin, over 200 profiles were examined.

Table 3. Comparison between effect of γ irradiation and inhibitors of synthesis on vaccinia virus replication and hemagglutinin production

Treatment	% virus produced	HA end-point titer
None	100	1024
5000 rads	26	512
50,000 rads	16	64
Actinomycin D	6	64
Streptovitacin	6	16

L-cell monolayers were inoculated with IHD-J vaccinia and incubated for 4 hr at 37°C. Individual cultures were either irradiated by a ⁶⁰Co γ source (calibrated to deliver 120 rads/sec) or placed in medium containing actinomycin D or streptovitacin A, then incubated for an additional 20 hr. Hemagglutinin (HA) was assayed as described (10), with the exception that Ca²⁺ and Mg²⁺ were omitted from the phosphate-buffered saline. Virus yields in PFU and HA titers were calculated per 10⁶ cells. In untreated cultures ~300 PFU/cell were formed.

DISCUSSION

Current experiments using as the host the α -amanitin-sensitive Ama⁺ and drug-resistant Ama 102 mutant rat myoblasts clearly demonstrate the involvement of a host function related to polymerase II in the life cycle of a poxvirus. The question as to whether ongoing transcription from the nucleus of the host is absolutely required has not been resolved unequivocally. Our observations with host cells extensively γ irradiated before infection favor the idea that transcription from the nucleus into functional mRNA may not be obligatory for completion of the vaccinia cycle of development. This presumption might appear to be paradoxical in relation to data indicating that vaccinia fails to complete its maturation in infected cytoplasts (6). However, upon careful examination of the article detailing the results with cytoplasts (6), it becomes clear that occasionally enucleated cells can produce mature vaccinia progeny. Because polymerase II occurs in and is able to be isolated from the cytoplasm, it could be available to the virus even in the absence or dysfunction of the cell nucleus. Turnover would, of course, deplete the pool of this enzyme with time.

Concerning pulse-chase experiments and polyacrylamide gel electrophoresis of the synthesis of vaccinia-specified polypeptides, once again information derived from infection of cytoplasts (6, 7) might appear to be in conflict with our previous findings which indicated that posttranslational cleavage, including that of p94 and p65 precursors to the p62 and p60 products, is obligatory for completion of virion maturation (18). However, if one keeps in mind the results of Pennington and Follett (6) concerning the variable virus development among cytoplasts sometimes culminating in the formation of mature progeny, then our present findings with Ama⁺ cells treated with α -amanitin as the host are quite consistent with observations made on cytoplasts. In both systems the mass of isotopically labeled virion proteins is reduced but processing occurs normally, accounting for the presence of a few mature virions.

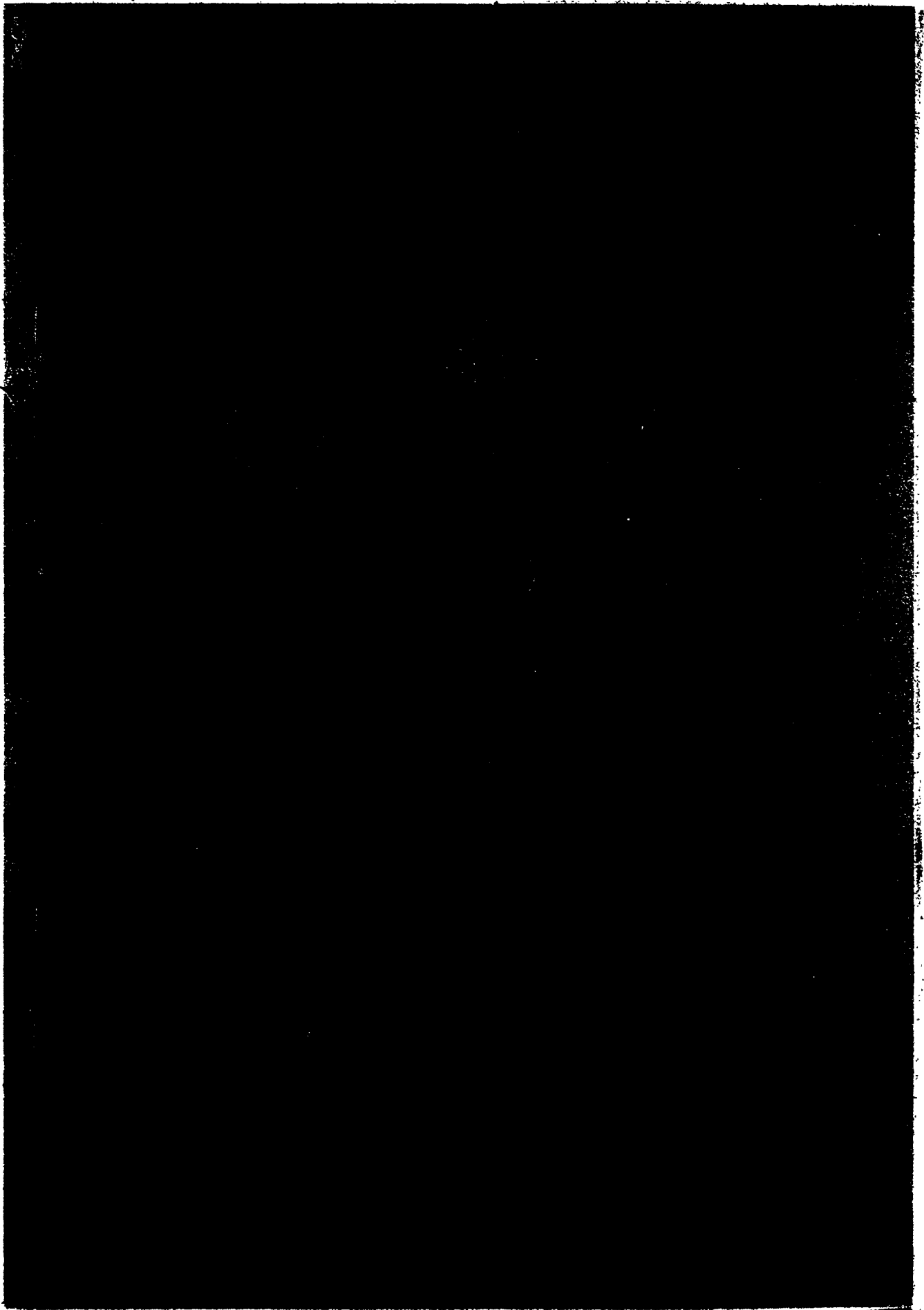
From the above, the most plausible hypothesis, which takes into consideration all the available information, should assume that early vaccinia functions, expressed initially from the virion core, then after uncoating, and including those required for DNA replication, are catalyzed by a virus DNA-dependent RNA polymerase whereas some or all of the late functions involve host polymerase II. Whether the entire polymerase II is monopolized in this process or perhaps only one of the enzyme subunits is unknown. It should, however, be remembered that both the vaccinia RNA polymerase and polymerase II are multicomponent enzymes (1, 20). Because *in vitro* experiments demonstrate specificity of low concentrations of toxin for polymerase II of Ama⁺ cells (16) and experiments on attachment with radioactive α -amanitin by use of a cross-linking agent suggest, but by no means certify, that the toxin acts by specific binding to the p140 subunit of polymerase II (21), it is not inconceivable that this subunit functions in concert with vaccinia RNA polymerase subunits in the late transcription process. Precedents for this notion have been established in the prokaryotes, as documented on the modification of host RNA polymerase by some bacteriophages of *Escherichia coli* and *Bacillus subtilis* (reviewed in ref. 22). The possibility that viral

and host polymerase enzyme subunits can be assembled into a hybrid transcriptional enzyme may be testable if, in the future, host mutants become available in which different polymerase II subunits are genetically altered.

After the initial submission of this article a paper appeared dealing with the role of the host cell nucleus in vaccinia replication (23). The data of Hruby *et al.* (23), like our own, document the sensitivity of vaccinia virus replication to α -amanitin. However, our hypothesis and that of the other workers diverge in that Hruby *et al.* implicate direct involvement of the host nucleus in the vaccinia cycle.

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COLOURED COVER

Potential of intense γ -irradiation of host cells for analysis of virus-specified transcription and replication¹

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Intense γ -irradiation from a cobalt source differentially affects macromolecular synthesis of cultured mammalian cells. Exposure of monkey BSC-1 or murine fibroblastic L₂ cells to 40 or 70 krad (1 rad = 1×10^{-2} J/kg) abolishes DNA and RNA synthesis almost entirely but reduces the formation of protein much less. A dose-response analysis of irradiation shows that synthesis of total RNA and the messenger component thereof, measured as the poly(A)-containing fraction, are equally diminished. Host cells in which formation of DNA and RNA are minimal can support normal or nearly normal replication and transcription rates of vesicular stomatitis and vaccinia viruses. Therefore, use of pretreatment with γ -irradiation, as employed here, should prove to be generally useful in examining virus-related transcription under circumstances in which application of drugs affecting gene expression, such as actinomycin D, is deemed undesirable.

Silver, M. & Dales, S. (1982) Potential of intense γ -irradiation of host cells for analysis of virus-specified transcription and replication. *Can. J. Biochem.* 60, 279-283

Une intense irradiation γ , provenant d'une source de cobalt, affecte de façon différentielle la synthèse macromoléculaire dans les cellules mammaliennes cultivées. L'exposition des cellules BSC-1 du singe ou des cellules L₂ fibroblastiques de murinés à 40 ou 70 krad (1 rad = 1×10^{-2} J/kg) abolit presque entièrement la synthèse du DNA et du RNA, mais elle réduit beaucoup moins la formation des protéines. Une analyse dose-réponse de l'irradiation montre que les synthèses du RNA total et du RNA messager de ce RNA total, mesuré en tant que fraction contenant le poly(A), sont également diminuées. Les cellules hôtes, où la formation du DNA et du RNA est minimum, peuvent supporter la réplication normale ou presque normale et les vitesses de transcription des virus de la stomatite vésiculaire et de la vaccine. Donc, l'irradiation γ utilisée comme prétraitement, tel que nous le faisons ici, pourrait s'avérer une méthode généralement utile pour examiner la transcription de virus apparentés dans des conditions où l'usage de drogues affectant l'expression génique, telle l'actinomycine D, est jugée indésirable.

[Traduit par le journal]

Introduction

During lytic infection by eukaryotic DNA and RNA agents, autonomy of virus-specified macromolecular syntheses, separable from those of host cells, is well recognized. However, since recent findings reveal an obligatory requirement in the pox virus life cycle for transcription factor(s) associated with the host (1, 2), the autonomy is not as complete as had been assumed. The *in vivo* or *in vitro* application of transcriptional inhibitors such as actinomycin D proved to be informative in demonstrating noninvolvement of host functions of

certain RNA viruses including those of the picorna group (3, 4). Unfortunately these drugs have a more limited usefulness in cell-virus systems which may involve at some stage DNA-dependent transcription, as is the case with the myxo- and retro-viruses, and all DNA viruses (4, 5, 6).

Our initial demonstration that extensively γ -irradiated cells are able to support undiminished vaccinia virus replication (1) prompted us to examine further the influence of this type of irradiation upon macromolecular synthesis, particularly transcription, in mammalian cells. The results obtained make it evident that exposure of cells to high doses of γ -irradiation prior to infection may be used to bring about transcriptional arrest under circumstances in which use of inhibitors such as actinomycin D is deemed to be undesirable or inadequate.

ABBREVIATIONS: multiplicity of infection; pfu, plaque-forming units; pi, postinfection; poly(A) RNA, polyadenylated RNA; TCA, trichloroacetic acid; VSV, vesicular stomatitis virus; FCS, fetal calf serum; NM, nutrient medium; MEM, minimum essential medium; DEP, diethyl pyrocarbonate; NP₄₀, nonidet P₄₀; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)amino ethane.

¹This work was supported by the Medical Research Council of Canada.

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Materials and methods

Cells and virus

Monolayers of L₂ mouse fibroblasts (7) were used for virus propagation and determination of infectious particles in terms

of pfu, according to previously described culture methods (8). The monkey BSC-1 cell line was obtained from Connaught Laboratories, Toronto. The NM consisted of Eagle's MEM (9), supplemented with 10% FCS. To initiate infection with the syncytiogenic substrain IHD-W of vaccinia (10), virus was added at an input multiplicity (moi) of 10 pfu/cell. Virus replication was measured on samples harvested 24 h pi. In the case of VSV of the Indiana subtype, cultures were inoculated at a moi of 0.01 pfu/cell and harvested for assays of replication at 10 h pi.

Reagents

[Methyl-³H]thymidine (specific activity 77.0 Ci/mmol (1 Ci = 37 GBq)), [5-³H]uridine (25.0 Ci/mmol), [U-¹⁴C]uridine (350 mCi/mmol), and L-[³⁵S]methionine (1010.6 Ci/mmol) were purchased from New England Nuclear. Oligo(dT)-cellulose, type 3, was obtained from Collaborative Research Inc., DEP from Sigma Chemical Company, and NP₄₀ from Shell.

γ-Irradiation

Confluent or nearly confluent monolayers grown for 3 days after subculture were placed on ice and irradiated using a ⁶⁰Co source (Gamma cell 220, Atomic Energy Canada, Ltd. calibrated to deliver 110 rad/s; 1 rad = 1 × 10⁻² J/kg). The nutrient medium was then replaced and cultures were returned to incubators and kept at 37°C for at least 1 h prior to experimentation.

Determination of macromolecular synthesis using isotopic labelling

Rates of host cell DNA, RNA, and protein syntheses were measured as follows. The NM was removed; the monolayers were washed with PBS and incubated at 37°C for 1 h in MEM containing [methyl-³H]thymidine (1 μCi/mL), [5-³H]uridine (1 μCi/mL), or [³⁵S]methionine (1 μCi/mL). The monolayers were placed on ice, and isotopes were removed by washing twice with cold PBS. Cells were harvested by scraping into 2 mL of PBS and following addition of SDS at a final concentration of 1%; the cell suspension was agitated on a Vortex mixer to effect cell rupture. Macromolecules were precipitated by addition of TCA at a final concentration of 10%. In some instances, cell material was kept frozen at -20°C, then thawed prior to precipitation with TCA.

To facilitate mRNA selection on oligo(dT)-cellulose columns, the nascent RNA was labelled with [5-³H]uridine or [U-¹⁴C]uridine (2.5 μCi/mL) at 37°C for 2 h as described above. Cell cultures were washed once with ice-cold PBS before RNA extraction.

To ascertain rates of [5-³H]uridine (10 μCi/mL) incorporation into RNA of preirradiated (70 krad), vaccinia virus-infected cells, 60-mm monolayers of L₂ cells were pulse labelled for 15 min at 37°C commencing 8 h pi. The labelled cultures were immediately chilled to 0°C, washed twice in PBS, and lysed according to published methods (11). The lysis buffer contained 1% NP₄₀, 10 mM Tris-HCl (pH 7.4) and 1 mM MgCl₂. Nuclei freed by the procedure were centrifuged into pellets at 800 × g for 5 min. The pellets were washed with PBS and resuspended in lysis buffer. SDS was added at a final concentration of 1% to both the nuclear and supernatant cytoplasmic fractions. Before precipitating cell material labelled for DNA and RNA with TCA at 0°C, calf thymus DNA was

added as carrier (final concentration 40 μg/mL). The precipitates were trapped on glass fiber filters (Reeve-Angel 934AH), washed sequentially with 10% TCA and 95% ethanol, and placed in vials containing toluene-based scintillation fluid. For determining counts per minute a Beckman LS-350 scintillation counter, with an efficiency for ³H of 46%, was employed.

RNA extraction and oligo(dT)-cellulose chromatography

RNA was extracted using the guanidine-HCl technique of Strohman et al. (12). To facilitate comparisons between different samples, aliquots of [U-¹⁴C]uridine-labelled material, derived from unirradiated L₂ cells, were mixed with samples of the [5-³H]uridine-labelled BSC-1 cells prior to commencement of RNA extraction. The purified RNA was washed twice in 95% ethanol and applied to oligo(dT)-cellulose columns for chromatography by a modified method of Avim and Leder (13). Approximately 0.3 mg of RNA, dissolved in binding buffer containing 0.01 M Tris-HCl (pH 7.5) - 0.5 M KCl, was applied to 0.1 g of oligo(dT)-cellulose packed into columns, previously washed with binding buffer. Elution of the unadsorbed material by continuous washing with binding buffer was followed by elution of bound material using 0.01 M Tris-HCl (pH 7.5). Calf thymus DNA was added as carrier to eluted fractions and the samples were precipitated with TCA as described above. All reagents used in RNA extraction and oligo(dT)-cellulose chromatography were previously treated with DEP to minimize activity of pancreatic RNase.

Results

γ-Irradiation and macromolecular synthesis

Despite requirement for host polymerase II (pol II) activity for completion of the replicative cycle of vaccinia, intensely preirradiated L₂ cells function as a fully permissive host (1). This raised the question as to whether intense γ-irradiation affected macromolecular synthesis. This was assessed by measuring the effects of exposure to various doses of γ-irradiation on synthesis of DNA, RNA, and protein. For this purpose, cultures of BSC-1 and L₂ cells were placed in MEM containing isotopically labelled thymidine, uridine, and methionine and incorporation of the precursors into TCA-precipitable material was ascertained. Data, summarized in Table 1, showed that 10 krad of irradiation only moderately reduced synthesis to 88.8% of unirradiated BSC-1 cells for DNA, to 73.3% for RNA, and to 67.9% for protein. Exposure of BSC-1 cells to 40 krad reduced protein synthesis to almost half the control rate but had a more profound effect on nucleic acid production which was lowered to only 1.5 and 3.0% in the case of DNA and RNA, respectively. After irradiation with 70 krad, the remaining rates for DNA and RNA were, respectively, 0.7 and 1.2%, but the level of protein synthesis remained at 36.2% that of the controls.

L₂ cells exposed to γ-irradiation under identical conditions appear to be less sensitive than BSC-1 cells (Table 1). Nevertheless, with this cell type also DNA and RNA synthesis was affected much more than the synthesis of protein (Table 1). The capacity of L₂ cells,

TABLE 1. Macromolecular synthesis in γ -irradiated and unirradiated cells^a

Cell line	Dose, krad	DNA		RNA		Protein	
		³ H]thymidine, cpm × 10 ⁻² , ^b %	%	³ H]uridine, cpm × 10 ⁻³ , ^b %	%	³⁵ S]methionine, cpm × 10 ⁻¹ , ^b %	%
BSC-1	0	452	100	124	100	333	100
	10	401	88.8	91.0	73.3	227	67.9
	40	7	1.5	3.7	3.0	145	43.5
	70	3	0.7	1.5	1.2	121	36.2
L ₂	0	802	100	32.3	100	207	100
	70	97	12.1	3.5	10.8	159	76.8

^aMonolayers in 35-mm Petri dishes of L₂ cells (1 × 10⁶ cells/plate) or BSC-1 cells (1.2 × 10⁶ cells/plate) were labelled as described in Materials and methods

^bAverages determined from duplicate cultures

preirradiated with 70 krad, to produce vaccinia virus in high yields (1) may be explained by the relatively low impairment of translation caused by the γ -irradiation.

Effect of γ -irradiation on transcription, particularly into mRNA

The severe reduction of overall RNA formation by γ -irradiation made it imperative to determine whether transcription into mRNA was being affected differentially or to the same extent. As a measure of putative mRNA synthesis we determined formation of the poly(A)-RNA fraction (14). To this end, RNA from [³H]uridine-labelled BSC-1 cells was separated by means of oligo(dT)-cellulose chromatography. The data presented in Fig. 1 revealed, first of all, that inhibition of poly(A) RNA synthesis by different doses of irradiation paralleled closely the formation of total RNA. Secondly, the data showed that the amount of mRNA formed was a function of the dose of irradiation applied. Thus pretreatment of BSC-1 cells with 10 krad 1 h prior to labelling with [³H]uridine affected poly(A) RNA synthesis only marginally, but irradiation with 20 krad caused 73% inhibition, while treatment with 30, 40, or 70 krad inhibited synthesis by 90, 94, and 96%, respectively.

With L₂ cells, 70 krad inhibited formation of poly(A) RNA by 92% (data not shown) while the overall production of RNA was reduced, as shown in Table 1, by 89%. Thus in both cell strains tested, there appears to be no differential effect of intense γ -irradiation on total RNA vs. putative mRNA transcription. The combined data indicate that at irradiation doses of 40 and 70 krad gene expression, i.e., transcription, from cellular DNA is virtually abolished.

Effect of γ -irradiation on virus transcription and replication

Almost complete abolition of host cell transcription following exposure to 40 or 70 krad γ -irradiation made it feasible to examine virus-specified transcription and

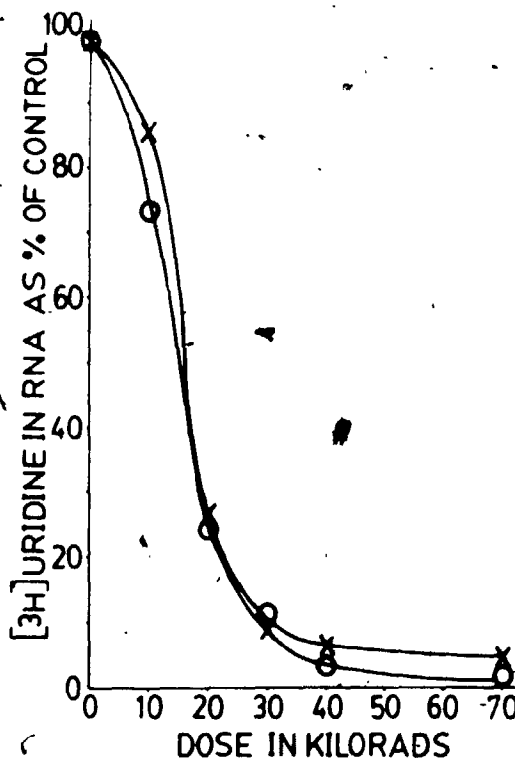


FIG. 1. Effect of γ -irradiation on synthesis of RNA. BSC-1 cells were labelled with [³H]uridine for 2 h commencing 1 h postirradiation. The total RNA was extracted and the poly(A)-containing fraction was selected as described in Materials and methods. The cpm in total RNA (O) and poly(A) RNA (X) are expressed as percent of unirradiated control cells.

replication in the virtual absence of host-related gene expression. In the case of an RNA virus without nuclear requirement (15), exemplified by VSV, virus production occurred at an undiminished rate in BSC-1 cultures previously irradiated with 40 krad (Table 2). Such preirradiated cultures were also able to support formation of vaccinia virus, as also shown by the data in Table

TABLE 2. Virus replication in γ -irradiated and unirradiated BSC-1 cells^a

When sampled	Virus titer ($\times 10^{-4}$ pfu./p/late)	
	Vaccinia	VSV
After adsorption ^b	6	1
After replication, control cells	10 000	5000
After replication, irradiated cells ^d	10 000	2000

^aBSC-1 cells (3.6×10^6) infected with vaccinia (moi = 10) or VSV (moi = 0.01)

^bSamples harvested 2 h pi

^cVaccinia samples harvested 24 h pi, VSV samples harvested 10 h pi

^dCells exposed to 40 krad 1 h prior to infection

2. Normal yields of vaccinia virus could be generated from irradiated cells in which mRNA production was inhibited by at least 94%. This finding is in line with our previous suggestion that requirement for host DNA dependent RNA polymerase II activity of this virus may not be directly connected with transcription from the host DNA (1).

To demonstrate clearly the use of high doses of γ -irradiation for dissociating host-related from virus-specified transcription we examined cytoplasmic RNA synthesis following infection with vaccinia virus. For this purpose, either preirradiated or unirradiated cultures of L₂ cells were inoculated and "pulse" labelled for 15 min with [³H]uridine at 8 h pi, when virus transcription is at a maximum rate (16), but host-related transcription has been reduced to its lowest rate (17). As evident from Table 3, there was an inhibition of uridine incorporation into nuclei of infected but unirradiated cells, confirming the previously published findings. By contrast, there was an approximately 15-fold increase in cytoplasmic RNA synthesis in infected cells as compared with uninfected cells, presumably due to vaccinia virus-specified transcription. A similar increase was also evident in the cytoplasm of companion irradiated and infected cultures, indicating that pretreatment with 70 krad had little or no influence on virus transcription. It should be noted from Table 3 that after 15 min pulse

labelling, [³H]uridine incorporation into the cytoplasm of uninfected cells was, as anticipated, very low. In γ -irradiated cultures the quantity of RNA label was reduced by more than 90% in both cytoplasmic and nuclear fractions (Table 3), substantiating the data presented in Table 1. The above combined results in Tables 1-3 reveal that preirradiation did not affect appreciably either viral mRNA synthesis or replication.

Discussion

Data from the current investigation on the effects of intense γ -irradiation on mammalian cultured cells reveal that synthesis of nucleic acid is much more sensitive than that of protein and in this sense the formation of the two kinds of macromolecules may be uncoupled. The fact that RNA agents of the picorna group such as mengovirus (18) and the enveloped rhabdovirus VSV as well as DNA viruses, exemplified by vaccinia, are able to replicate normally in cells preirradiated with doses as high as 70 krad (1) implies that the host translational apparatus escapes the effects of γ -irradiation relatively intact. By contrast replication of DNA and transcription into putative mRNA, ascertained in terms of the polyadenylated RNA fraction, is profoundly inhibited. Equivalent doses of γ -irradiation when applied to vaccinia-infected cells are able to effectively suppress viral gene expression, measured in terms of biological functions such as the viral hemagglutinin (1); this demonstrates, as expected, that intense γ -irradiation can interrupt equally gene expression of both a virus and its host. The mechanism of action is undoubtedly through primary damage to the DNA.

Synthesis in the cytoplasm of infected, preirradiated cells of near normal levels of vaccinia-specified RNA shows, on the one hand, that irradiation does not affect pools of metabolites and any host functions which might be required for virus-specified RNA synthesis and on the other, that host-related transcription is most probably not required for optimum viral transcription. Thus it is unlikely that the observed decrease in RNA synthesis due to γ -irradiation is the consequence of either a decrease in [³H]uridine uptake into cells or dilution of

TABLE 3. Incorporation of [³H]uridine into nuclear or cytoplasmic RNA of infected and uninfected L₂ cells^a

		Whole cells ^b	Nuclear fraction ^b	Cytoplasmic fraction ^b
Mock infected	Unirradiated	31 460	27 460	1 290
	Irradiated	2 875	2 710	70
Vaccinia infected	Unirradiated	22 020	3 890	19 130
	Irradiated	18 540	1 035	17 250

^aExperimental protocol as described in Materials and methods.

^bDisintegrations per minute originating from 1×10^6 cells

the isotope within an enlarged uridine pool. It is more likely that inhibition of [³H]uridine incorporation into RNA, either the bulk material or the poly(A) fraction, reflects the damage by γ -irradiation on the transcription process from the DNA template.

Use of intense γ -irradiation as applied here to mammalian cells in culture has not, to our knowledge, been reported previously. However, numerous studies have found little or no inhibitory effects of irradiation on ribosomal, transfer, heterogeneous nuclear, and mRNA when doses lower than 2 krad are applied (reviewed in Refs. 19, 20). Thus HeLa cells irradiated with 500 rad incorporate isotopic uridine during the first 10 h after treatment at the same rate as the unirradiated controls (21). Similarly, Enger and Campbell (22) found that the rate of mRNA synthesis in Chinese hamster ovary cells exposed to 800 rad was equal to that of the control over a 7-h period postirradiation. In support of the above findings, our present study shows that irradiation with doses over 10 krad is necessary to substantially inhibit total or poly(A) RNA synthesis.

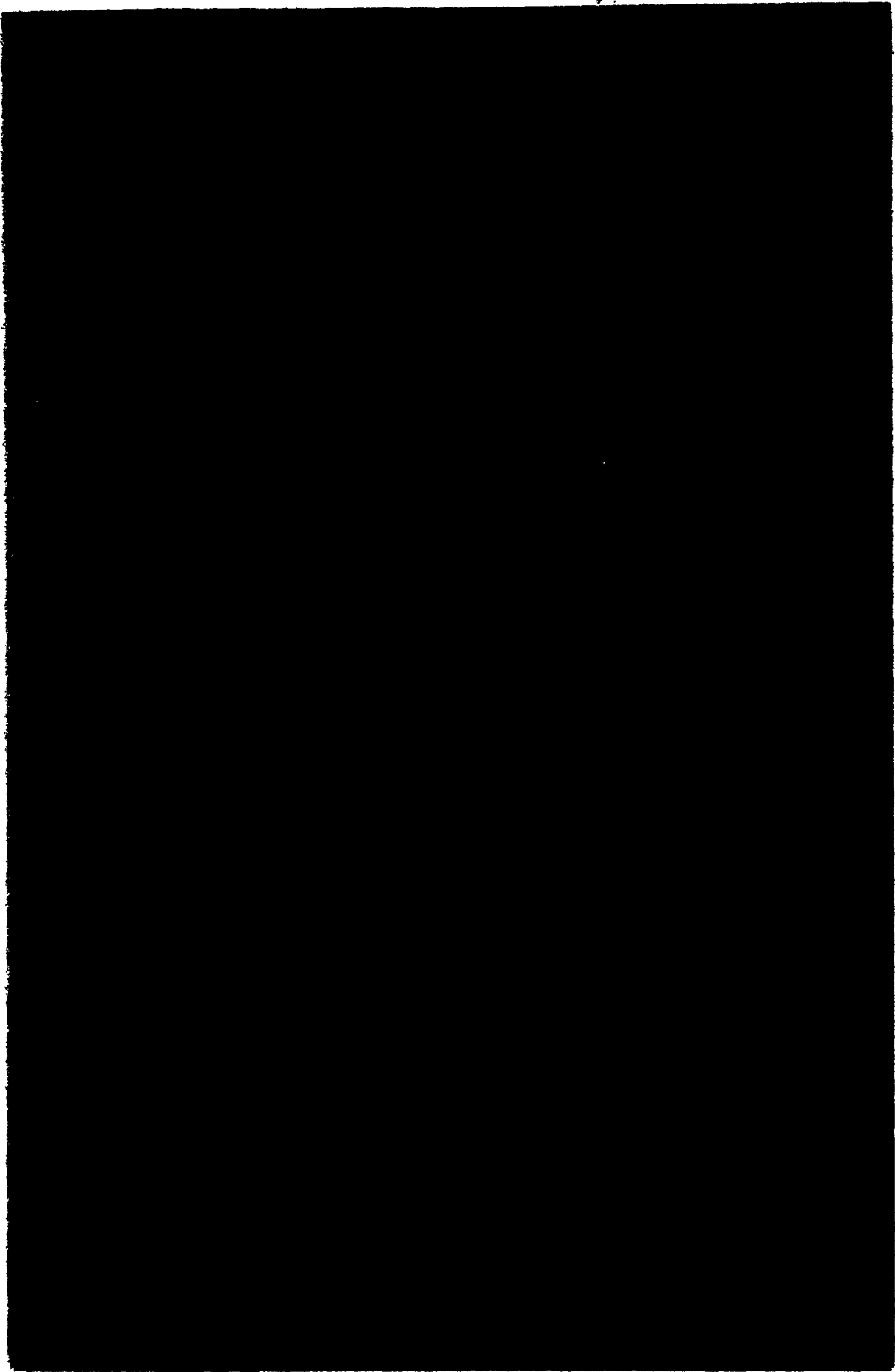
The ability of intense γ -irradiation to suppress mRNA synthesis by over 95% while sparing a major fraction of protein synthesis offers a system favourable to biochemical investigation of virus infections. This procedure permits an examination of virus replication and transcription in a host cell in which the equivalent functions have ceased, thereby obviating the use of drugs such as actinomycin D which affect equally cellular and viral transcription.

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COLOURED COVER

Biogenesis of Vaccinia: Complementation and Recombination Analysis of One Group of Conditional-Lethal Mutants Defective in Envelope Self-assembly¹

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Five temperature-sensitive (*ts*) mutants of vaccinia virus, which mimic the effects of rifampicin, being defective in assembly of the envelope and maturation, were characterized. Although the mutants showed a similar morphogenetic phenotype when examined by electron microscopy, analyses by isotopic pulse-chase experiments in which the viral polypeptides were separated by polyacrylamide slab gels, and complementation titrations between the mutants, indicated that each mutation produced a distinct defect. Recombination experiments suggested a close relationship between resistance to the antibiotic rifampicin and the *ts* mutations studied. This result was supported by the apparent covariance of the drug resistance and *ts* markers.

INTRODUCTION

Despite the large size of the poxvirus genome, approximately 123×10^6 daltons (Müller *et al.*, 1977), and highly complex series of regulated events involved in the replication and maturation cycle (Dales, 1963; Stern and Dales, 1976b; Moss, 1974), more detailed knowledge of the system may be acquired through studies using conditional-lethal mutations of the type previously described (Sambrook *et al.*, 1966; Padgett and Tomkins, 1968; Basilico and Joklik, 1968; Stern *et al.*, 1977; Drillien *et al.*, 1977; Chernos *et al.*, 1978). Recently, we reported the isolation of over 90 temperature-sensitive (*ts*) mutants from vaccinia virus strain IHD-W, which could be arranged into 17 distinct groups on the basis of their phenotypically expressed defects at the restrictive temperature (Dales, *et al.*, 1978).

In this paper, the characterization of five such mutants belonging to group E is described. Virus development with these

mutants is blocked at about the same stage; numerous viroplasmic foci are formed, each surrounded by flexible envelope segments but which are devoid of spicules, and DNA paracrystals accumulate. On morphological grounds, the defects mimic those effected by the antibiotic rifampicin (Nagayama *et al.*, 1970). Moreover, Stern *et al.* (1977) reported that for one member, *ts* 1085, the defect was related, as with rifampicin (Moss and Rosenblum, 1973), to a block in post-translational cleavage required for virus maturation. Experiments were therefore directed at determining the number of genes involved with this proteolytic processing, since a defect in any one gene could result in a common phenotypic expression. We wished also to ascertain whether the locus for rifampicin resistance is closely associated with these *ts* mutations.

MATERIALS AND METHODS

Viruses and cells. The IHD-W subtype of vaccinia (Hanafusa, 1960), designated the wild-type (WT) parent, and the six temperature-sensitive (*ts*) mutants (*ts* 1085; *ts* 7743; *ts* 9203; *ts* 9383; *ts* 9251; *ts* 6389) derived from it, have been previously described

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(Dales *et al.*, 1978). All the viruses were propagated and titrated by plaque assay on monolayer cultures of the L2 strain of mouse L929 fibroblasts (Rothfels *et al.*, 1959). These cultures were maintained in minimal Eagle's medium (MEM) containing 10% fetal calf serum (FCS).

Conditions of incubation. Monolayer cultures on plastic petri dishes (Falcon Plastics) were incubated in Hotpack incubators gassed with a humidified mixture of air and 5–10% CO₂. A permissive temperature of ~33° and a restrictive temperature of ~40° were used for all experiments (Dales *et al.*, 1978).

Selection of rifampicin-resistant ts mutants. Resistance to the antibiotic rifampicin, an inhibitor of virus maturation (Nagayama, 1970) was chosen as the second marker for the three-factor cross experiments. Confluent L cell monolayers (4 × 10⁶ cells) were inoculated with *ts* 7743 and *ts* 9383 at 1 PFU/cell. Following adsorption at 33° for 1 hr, the unadsorbed virus was removed with repeated washing, and the cells overlaid with MEM containing 5% FCS and 100 µg/ml rifampicin (Sigma) and incubated at 33°. Areas of limited cell fusion were observed in some cultures at 3–4 days postinfection. The putative resistant virus was enriched by two further passages in the presence of rifampicin before being cloned by two plaque purifications. The two double mutants were designated *ts* 7743R and *ts* 9383R. Because of cytotoxicity difficulties with rifampicin at 40°, the concentration of the antibiotic was reduced to 50 µg/ml in all titrations at the restrictive temperature. The replication of wild-type virus was still inhibited 10-fold.

Genetic studies with the ts mutants. In order to investigate the genetic nature of each *ts* mutation, a series of complementation and recombination experiments were undertaken.

Complementation. Complementation assays were done in monolayer cultures of L2 cells grown in flat-bottomed glass tubes (3 × 10⁵ cells). The five assembly-defective *ts* mutants (*ts* 1085; *ts* 7743; *ts* 9203; *ts* 9383; *ts* 9251) and the *ts* mutant defective in viral DNA synthesis (*ts* 6389) were diluted to deliver 40 PFU/cell. The mutants were

mixed in every pairwise combination before inoculation. The mixed infections received 20 PFU/cell of each mutant (total of 40 PFU/cell), and the single infections 20 PFU/cell. Following an adsorption period of 1 hr at 4° (Pogo and Dales, 1971), the unadsorbed inoculum was removed with repeated washings with prewarmed MEM (40°) and the cultures incubated at 40° for 24 hr. The virus yield from each infection was titrated at 33°, and the complementation index (CI) for each cross calculated as follows:

$$CI = \frac{(A \times B)^{33}}{A^{33} + B^{33}}$$

where $(A \times B)^{33}$ is the yield of the mixed infection titrated at 33°, and A^{33} and B^{33} are the yields of the single infections titrated at 33°. A complementation index greater than 3 was considered a positive indication of complementing gene functions.

Recombination. All recombination experiments were done in the flat-bottomed glass tubes. A total multiplicity of 40 PFU/cell was used for the mixed infections, and 20 PFU/cell for the single infections. Monolayers were inoculated at 4° for 1 hr, after which the unadsorbed virus was removed with repeated washings with prewarmed MEM (33°). The cultures were incubated for 24 hr at 33°. Virus yields were titrated at 33° to assay the total progeny virus, at 40° to measure the *ts*⁺ recombinant virus, and at 40° in the presence of 50 µg/ml rifampicin to measure the *ts*⁺R recombinant virus. Percentage recombination frequencies (RF) were calculated as follows:

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

where $(A \times B)^{40}$ and $(A \times B)^{33}$ are the mixed infection yields titrated at 40 and 33°, and A^{40} , A^{33} , B^{40} , B^{33} are the single infection yields at the designated temperatures (Lake *et al.*, 1975).

Isotopic labeling and virus purification. To prepare isotopically labeled virus, monolayer cultures of L2 cells grown in 100-mm

petri dishes were inoculated with 10–20 PFU/cell at 4° for 1 hr. The cultures were then incubated for 24 hr in the presence of 5 μ Ci/ml [³⁵S]methionine in MEM containing 5% FCS and one-third normal concentration of methionine. The cells were harvested, and mixed with 2 \times 10⁷ unlabeled infected cells, which acted as carrier material. The virions were then purified as described by Stern and Dales (1974).

Labeled cytoplasmic extracts were prepared by inoculating monolayer cultures (35-mm plates containing 1 \times 10⁶ cells) with 10 PFU/cell at 4° for 1 hr. Unadsorbed virus was removed with repeated washings, and the cultures incubated at 33 or 40°. Virus polypeptides were labeled by incubating the infected cells for 30 min in 1 ml methionine-free MEM medium (MFM) and subsequently for 1 hr in 0.2 ml of 25 μ Ci/ml [³⁵S]methionine in MFM. The pulse was terminated by washing, and the label chased in MEM containing 3 \times normal concentration of methionine. The cells were gently scraped away from the plastic, washed in 10 mM Tris [tris(hydroxymethyl)aminoethane]-HCl (pH 7.2), and lysed in 100 μ l of 0.1% Nonidet P.40 (Shell) in 10 mM Tris-HCl (pH 7.2). The nuclei and cellular debris were removed by centrifugation (800 *g* for 3 min), and the supernatant stored at -20° as the cytoplasmic fractions. Aliquots of 5 μ l were applied to filter paper disks (Whatmann 3MM), washed sequentially in 10% trichloroacetic acid (TCA) and 95% ethanol, and counted in toluene scintillation fluid. The isotope L-[³⁵S]methionine (100–300 Ci/mmol) was purchased from New England Nuclear.

Electrophoresis of cytoplasmic extracts and virus proteins. Cell extract and purified virus were prepared for polyacrylamide gel electrophoresis (PAGE) by mixing 1 vol with an equal volume of dissociating buffer (70 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% mercaptoethanol, and 0.001% bromophenol blue). The samples were boiled for 2 min. The dissociated proteins were separated by electrophoresis on 15-cm 11% polyacrylamide slab gels of 0.375 M Tris-HCl (pH 8.8) and 1% sodium dodecyl sulfate (SDS). The ratio of acrylamide to bisacrylamide was 30/0.8. The

stacking gel consisted of 4% acrylamide in 0.125 M Tris-HCl (pH 6.8) and 1% SDS, and the electrophoresis buffer was 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. Samples were adjusted to give comparable amounts of radioactive label and loaded into 8-mm-wide wells. The gels were run at a constant current of 25 mA until the tracking dye reached the bottom of the gel (Studier, 1973).

Following electrophoresis, the gel was fixed for 30 min in 50% methanol, 7% acetic acid, treated for fluorography by the method of Bonner and Laskey (1974), dried on Whatman 3MM filter paper, and exposed to Kodak X-Omat R film.

Electron microscopy. The procedure used for propagating cells and virus to be used in samples for electron microscopy was essentially the same as those previously described (Dales *et al.*, 1978).

RESULTS

Morphological Characterization

The five isolates employed in this study belong to the E category in the spectrum of assembly mutants selected previously (Dales *et al.*, 1978). When replicating at 40° this group is characterized by the aberrant formation of virus envelopes (Figs. 1–3). Usually these envelopes consist of a bilayer, unit membrane backed externally by a layer of spicules which endow the envelope with rigidity and a curvature so as to form spheres enclosing immature particles (Dales and Mosbach, 1968). When the *ts* defect was expressed in the extreme only flexible sheets of unit membrane, like those identified by arrows in Figs. 2 (inset) and 3, were evident while the rigid, curved segments were almost entirely missing. More usually, however, the envelopes of normal structure, containing spicules, were contiguous with sheets of flexible membrane as in *ts* 1085 described previously (Stern *et al.*, 1977) and in *ts* 9203, illustrated in Fig. 1. The accumulation of DNA paracrystals like those shown in Fig. 3 was another common finding with group E mutants. The aberrations with *ts* 1085 and 9203 are very similar to those that develop in the presence of the

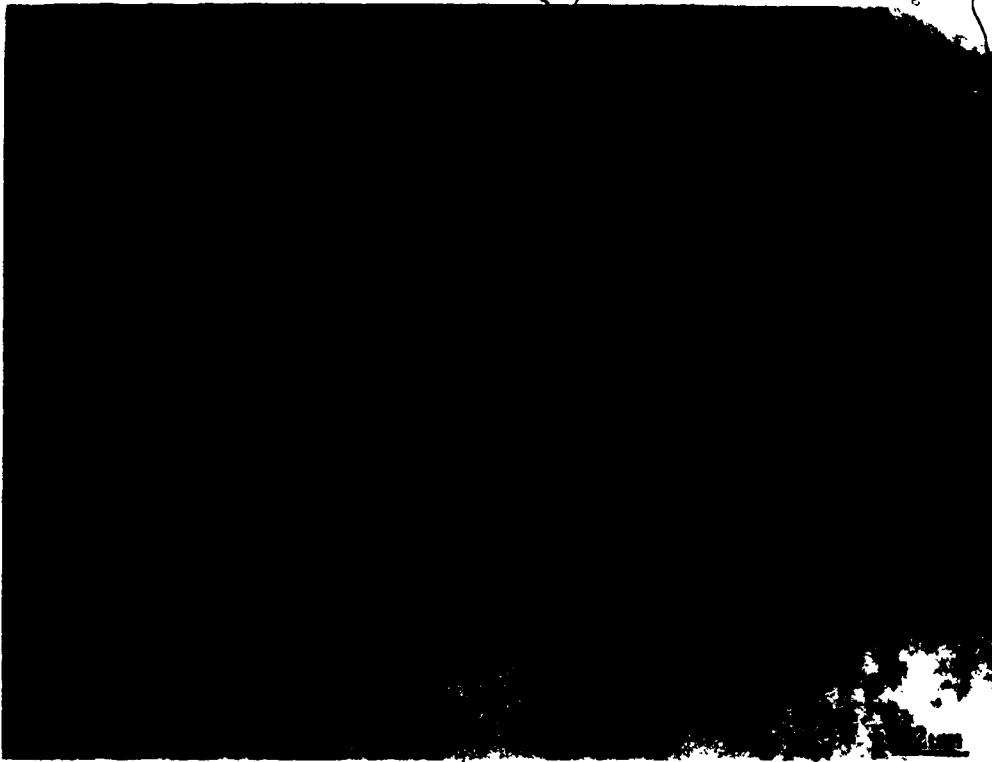


FIG. 1. Electron micrograph of a selected cytoplasmic area of a cell from a culture inoculated with *ts* 9203. The extensive viroplasmic matrix, occupying the center of the field, enclosed discrete foci of denser material each surrounded by aberrant virus envelopes (arrows). In Figs. 1-3, *v* identifies a vacuole, *m* a mitochondrion, and *VM* viroplasmic matrix, $\times 50,000$.

drug rifampicin (Nagayama *et al.*, 1970; Moss, 1974).

With one of the mutants under study here, *ts* 7743, envelope formation conformed more with the normal process, as evident by the presence of numerous partially complete envelopes and even some spherical particles (Fig. 3). Another mutant, *ts* 9251 behaved in an anomalous manner at 40°, because both abnormal envelope malforma-

tion and development of mature virions were observed (Fig. 2). Therefore, from the point of view of morphogenesis this mutant must be termed leaky. However in terms of infectivity, as measured by PFU *ts* 9251 is a tight mutant (Dales *et al.*, 1978).

Virus-Specified Polypeptides

Previous studies with *ts* 1085 (Stern *et al.*, 1977) indicated that the defects, as

FIG. 2. Other examples showing selected areas of cytoplasm from cells infected with *ts* 9251. In the inset the viroplasmic matrix is enclosed by aberrant envelopes indicated by arrows. The extensive viroplasmic matrix occupying the central area contains aberrant as well as normal (I) immature forms of vaccinia virus. The mature particles, identified by arrows, illustrate that occasionally there was a breakthrough whereby morphogenesis progressed all the way to maturation. $\times 27,500$. Inset $\times 47,000$.

FIG. 3. Similar example of Fig. 2 illustrating the appearance of virus structures following infection with *ts* 7743. Development generally stopped prior to the assembly of complete, rigid, spherical envelopes, as evident from the presence of flexible membranes (arrows) and DNA paracrystals. However a few immature particles of normal appearance (I) were visible. $\times 45,000$.



with rifampicin (Moss and Rosenblum, 1973), were related to a block in post-translational cleavage. Since the five *ts* mutants studied appeared to be arrested at about the same stage of development (Dales *et al.*, 1978), it was necessary to determine whether the late proteins were processed normally at the restrictive temperature.

The polypeptide patterns for the five *ts* mutants along with an identically treated DNA defective mutant, *ts* 6389, and the WT-infected cells are shown in Figs. 4a and b. As in the previous article (Stern and Dales, 1976a) polypeptides mentioned below will be identified by their molecular weight, so that p94 is a polypeptide migrating in the described system with an approximate molecular weight of 94,000. The polypeptide patterns produced by all the *ts* mutants at 33° were essentially similar to the profile obtained with WT virus at 40°. More specific differences were identified in the cytoplasmic extracts prepared from *ts* mutant infections at 40°. The processing of the major core polypeptides, p62 and p60, from the higher molecular weight precursors, p94 and p65, respectively, has been shown to be defective for *ts* 1085 (Stern *et al.*, 1977). In the current experiments, the precursor p94 was present in all the mutant infections at 40°. Processing of this precursor to the core polypeptide p62 was affected in infections with *ts* 1085, *ts* 7743, and *ts* 9203, but not with *ts* 9383. Accumulation of the precursor p65 was also evident at 40° in all the cytoplasmic extracts from infections with the 5 *ts* mutants, but processing to p60 was blocked or reduced in quantity in the case of *ts* 1085 and *ts* 9203.

In the case of three other polypeptides p23 was reduced or absent from *ts* 1085 and *ts* 7743, while p18.5 and p18 were missing or present in small quantities in all but *ts* 9251. These results indicated that the defect in the post-translational cleavage was different in the case of each mutant, diagrammatically presented in Fig. 5. The polypeptide pattern for infections with *ts* 9251 appeared to be an anomaly to the proposed grouping of the *ts* mutants on the basis of their morphological appearance under the EM, since some morphologically mature progeny were found and both precursors were apparently processed with equal efficiency at 33 and 40°. However, it is not known whether one or both products are fully functional, because little infectious progeny appeared at 40° despite the presence of mature-like particles (Fig. 2). The variability in processing indicates that either precursor can be cleaved in the absence of the other, so that the enzymology of the reaction is not as tightly coupled as the assembly processes.

If the infected cells were labeled at 40°, but incubated for a further 14 hr at 33°, all the precursors were processed normally, indicating that cleavage was related to a temperature sensitivity.

The pattern of polypeptide bands produced by *ts* 6389 was typical of that expected in a mutant defective in DNA synthesis. Only the early, prereplicative polypeptides were evident.

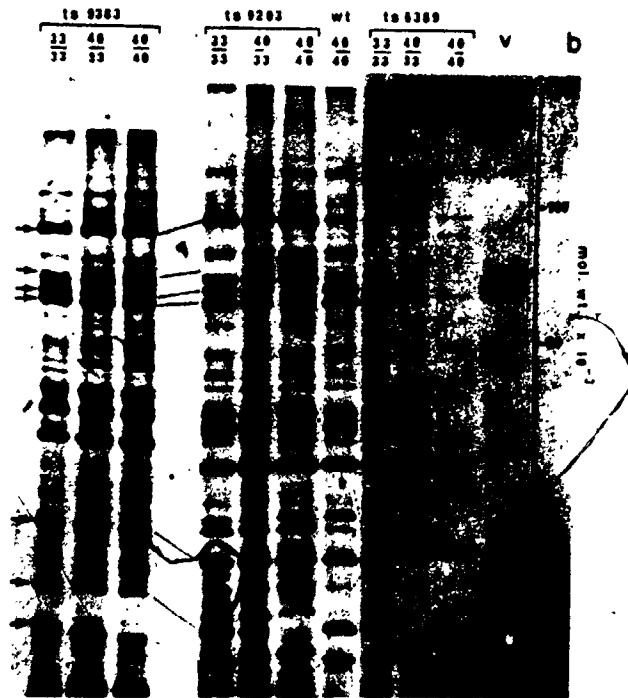
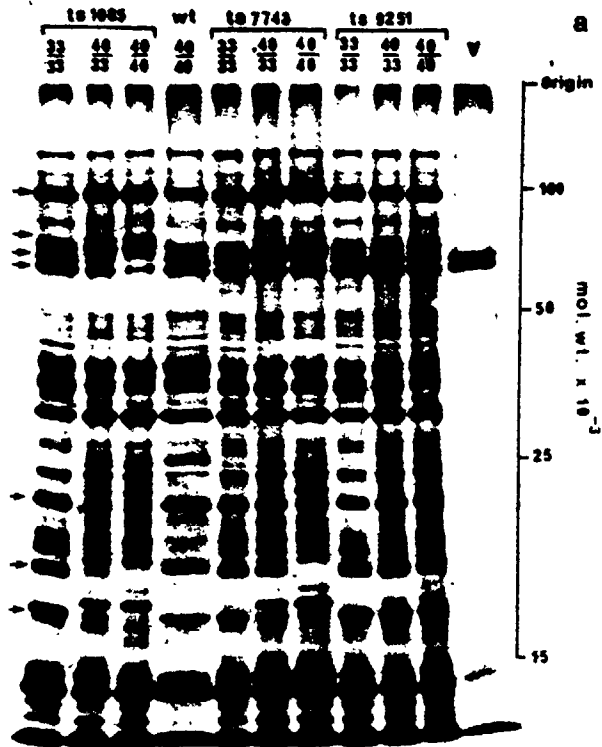
Complementation between the Mutants

To determine whether the *ts* mutations were localized in different or identical genes,

FIG. 4a. Autoradiogram of a slab gel prepared from infected cytoplasmic extracts of L cells and purified virus. Cultures were inoculated with WT, *ts* 1085, *ts* 7743, or *ts* 9251 vaccinia virus and incubated at the designated temperatures. Each culture was pulse-labeled 10 hr postinfection with 25 μ Ci/ml [³⁵S]methionine and further incubated for 8 hr in chase medium. The influence of temperature shifts on the processing of individual polypeptides is evident. In each case the temperature during labeling is the upper number of the ratio and temperature during the chase is the lower number. The vertical scales showing molecular weight $\times 10^{-3}$ was calculated from polypeptides used as MW standards and is also drawn on the basis of known molecular weights of several major virion polypeptides (Stern *et al.*, 1977). The arrows denote the positions of the seven polypeptides considered in this analysis: p94, p65, p62, p60, p23, p18.5, and p18. Channel marked V = purified vaccinia virus.

FIG. 4b. Autoradiogram of a similar slab gel showing labeled polypeptides occurring in the cytoplasmic extracts of cells infected with *ts* 9383, *ts* 9203, and *ts* 6389.

BIOGENESIS OF VACCINIA



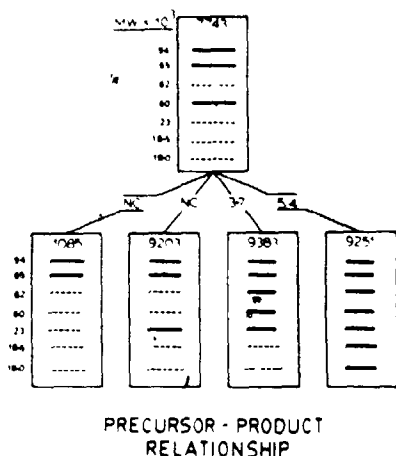


FIG. 5. Schematic representation of the processing of virion polypeptide precursors p94 and p65 during infection with individual *ts* mutants. —, Normal or accumulated amount of polypeptide; ----, polypeptide absent or present in reduced amount. The rates of complementation between *ts* 7743 and the four other mutants ranged from 5.4 to no complementation (N.C.)

a checkerboard series of complementation experiments was undertaken. Although large variability was recorded from experiment to experiment involving specific crosses, complementation was repeatedly observed with most of the mutants. A complementation index greater than 3 was taken as positive evidence that genetic interaction had occurred. The mutants *ts* 1085, *ts* 9203, *ts* 9383, and *ts* 9251 readily complemented each other, and all gave high values when crossed with the DNA(-) *ts* 6389. Since

ts 9251 was apparently not defective in proteolytic cleavage, the crosses with this mutant were discounted as concerns determination of the number of genes involved in group E defects. Most dual infections yielded good complementation, ranging from 8.8 to 143 (Table 1). The striking high value obtained in the cross *ts* 9251 × *ts* 9383 may in some way be related to the leakiness of the phenotype, described above.

In mixed infections of group E mutants, involving *ts* 7743 complementation was either very poor or nonexistent. On the other hand, a cross between *ts* 7743 and *ts* 6389 outside this group gave high complementation values. Since *ts* 7743 was able to both complement outside group E defectives and did not interfere with replication of wild-type vaccinia virus, there was no evidence to indicate that it might be a "trans-acting dominant mutant" (Castens and Weber, 1977). Our observations could be explained if one assumes that *ts* 7743 mutation was being expressed in a polar fashion with respect to the functions necessary for post-translational cleavage.

Recombination Experiments

It was possible that the group E *ts* mutations were closely linked on the vaccinia genome. This idea was analyzed by means of systematic three-factor crosses involving the 5 *ts* mutants and *ts* 7743R and *ts* 9383R, two of the *ts* mutants into which a marker for resistance to rifampicin (R) was introduced. It should be noted that

TABLE 1
COMPLEMENTATION BETWEEN THE MUTANTS

Mutant	<i>ts</i> 1085	<i>ts</i> 7743	<i>ts</i> 9203	<i>ts</i> 9383	<i>ts</i> 9251	<i>ts</i> 6389
<i>ts</i> 1085	—	N.C. ^a	8.8 ^b	62	3.2	33
<i>ts</i> 7743		—	N.C.	3.7	5.4	52
<i>ts</i> 9203			—	49	8.4	50
<i>ts</i> 9383				—	143	33
<i>ts</i> 9251					—	38
<i>ts</i> 6389						—

^a No complementation detected.

^b The complementation indexes (average of two replicates) were calculated by dividing the mixed infection yield with the sum of the corresponding single infections.

after incorporating the mutation for rifampicin resistance the tightness of the *ts* trait was altered and the mutants became about 1000-fold more leaky, so that the difference in PFU 44/33° was reduced from 10⁻⁵ to 10⁻². Furthermore, the plaque morphology of *ts* 7743R and *ts* 9383R was altered from a minute to larger plaque typifying wild-type virus assayed at 40°.

The leakiness of *ts* 7743R and *ts* 9383R made the reproducibility of the data on recombination frequencies (*RF*) more variable from experiment to experiment, thereby introducing some difficulties in the interpretation of the results. Similar difficulties have been encountered with leaky *ts* mutants of rabbitpox (P. D. Cooper, personal communication). Nevertheless, a tentative map for group E mutations was established by crossing each *ts* mutant with *ts* 7743R and *ts* 9383R as illustrated in Fig. 6. Each cross was made on two occasions. Each time two replicate cultures were employed and the progeny of crosses of an entire experiment were assayed at the same time. The cross *ts* 7743R × *ts* 9383R was always included, so as to standardize the data from different experiments and the PFU assays, conducted on different days.

Thereby, any differences in the efficiency of plating of the *ts*⁺R recombinants in the presence of rifampicin at 40° could be standardized (i.e., *ts*⁺ %*RF* must be equal to *ts*⁺R %*RF*). The *ts*⁺ %*RF*, which represents the total *ts*⁺ was ascertained, without regard to rifampicin selection and includes both the *ts*⁺R⁺ and *ts*⁺R⁻. Analyses of these data gave a measure of the distance between *ts* lesion of the unknown mutant and *ts* lesion of the *ts*R mutant. Concerning the position of the R marker, three situations could be predicted. In the first case R is placed between two of the *ts* mutations used in the cross. In the other two cases both *ts* mutations in the cross exist on one side of R whereby the *ts*⁺ recombinants produced are either all R⁺ or all R⁻, depending on the proximity of the particular *ts* markers to R. Thus *ts*⁺R %*RF* measured the distance between the *ts* defect of the *ts*R and the rifampicin-resistance locus.

Since only five group E mutants were examined, and high multiplicities of infection were employed, the low *RF* values calculated may not reflect the actual linkage distances. This is especially true in view of our ignorance about the extent of the vaccinia map as a whole. On the basis of

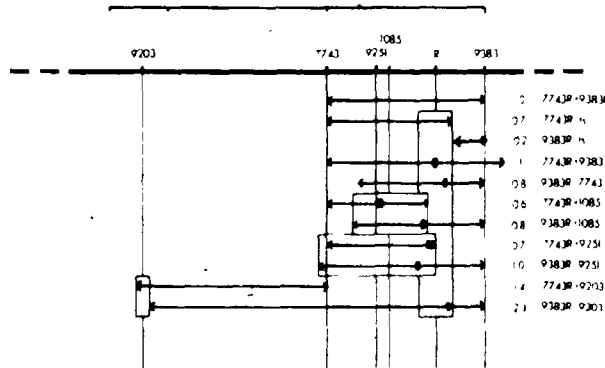


FIG. 6. Recombination map of five *ts* mutants of vaccinia virus based on experiments using three-factor crosses among temperature-sensitive (*ts*) and rifampicin-resistant (R) mutants. %*RF* above the diagram represents the percentage recombinants which have the *ts*⁺ character. The numbers shown on the right hand side next to the recombinants participating in each particular cross are the %*RF* values. The framed-in areas contain data on %*RF* which in some cases deviated from the expected value if the *RF*s were strictly additive. The positions of *ts* mutants on the map were related in each case to *ts* 7743R and *ts* 9383R. The bar with a dot is the frequency of *ts*⁺R recombinants from which the locus for rifampicin resistance was calculated.

data from several experiments it is, however, significant that %RF between *ts* 9383, one of group E mutants and the DNA(-) *ts* 6389, used as an external marker, was approximately three times higher than the highest %RF calculated for any crosses using mutants within this group. In the case of *ts* 9203, defective in processing of the p94 and p65 precursors, a close link to the other four mutants was established with uncertainty because the %RF calculated from two-factor crosses (unpublished data) placed *ts* 9203 in a different position from that shown on the map in Fig. 6. The locus for rifampicin resistance, R was shown to be closely associated with group E *ts* defects, as emphasized by the apparent covariance of the *ts* and R markers. There was reasonable additivity of the distances between markers, and two-factor cross experiments supported the mutation sequence shown in Fig. 6.

DISCUSSION

Among 90 *ts* vaccinia mutants selected previously, 78 were grouped on morphological grounds into categories according to an ascending order of complexity reached in development at the restrictive temperature (Dales *et al.*, 1978). Present experiments on group E mutants have permitted a more detailed characterization of individual phenotypic defects. All five mutants examined were shown to be entirely or partially defective in both envelope assembly and post-translational cleavage of two prominent polypeptides of the virus core, thereby mimicking the effects produced by the antibiotic rifampicin. Attention was consequently focused on the singularity or plurality of these mutations, which would imply either the existence of a "hot-spot" for mutagenesis in the vaccinia genome, i.e., a region that is unusually sensitive in generating temperature-sensitive mutations, or the participation of a number of individual functions in a coordinated fashion whereby interruption of any one blocks simultaneously virus morphogenesis associated with all. Since post-translational cleavage involves an enzymatic

function, the establishment of a linkage between group E mutations might suggest that phenotypic aberrations arise from the enzyme defect itself or some other regulatory factor common to all five mutants. On the other hand absence of such a linkage would imply that each lesion involves a separate virus product, each involved independently sometimes as a substrate for this protease.

The results of the complementation experiments and the patterns of polypeptides produced at the restrictive temperature supported each other. The mutations were found to occur in distinct loci each affecting a different function as evident from the data on precursor product relationships involving p94 and p65. Thus, although assembly appeared to be a tightly coupled process proteolytic cleavage of one precursor was not necessarily dependent on the prior processing of another precursor polypeptide. Despite the fact that the defect in *ts* 7743 could be mapped as a single mutation, notable lack of complementation between this and the other four mutants, shown in Table 1, implies that *ts* 7743 could be endowed with more than one defect or is a type of regulatory mutant.

The RF values obtained from recombination experiments must be imprecise: (a) judging by the variability of the data (b) in view of the relatively few mutants employed (c) an unexplained leakiness of *ts* mutants after acquisition of the rifampicin resistance or R marker, and (d) the high inoculum multiplicity used. Concerning the last point, infection with 20 PFU/cell of each *ts* partner in recombination analyses could have created massive cytoplasmic factories of predominantly one *ts* mutant type, thereby reducing rather than enhancing the possibility of efficient recombinations between the replicating virus genomes. This question will be investigated in the future to ascertain whether high %RF among poxviruses, reported by others (Chernos *et al.* 1978; Padgett and Tomkins, 1968), reflects the more usual state of affairs.

Since both group E *ts* lesions and rifampicin interfere with envelope formation and

processing of two major precursor polypeptides of the core, the observed partial reversion to wild-type phenotype in terms of plaque morphology and increase in PFUs produced at 40° when the R locus is incorporated into a preexisting *ts* defect, might indicate that presence of R somehow alleviates the *ts* lesion. Such covariance of two markers has been reported in polio virus where *ts* mutations in structural polypeptides alter the sensitivity of the virus to treatment with guanidine carbonate (Cooper *et al.*, 1970). The observed covariance of rifampicin resistance and the partial alleviation of the *ts* mutations support the close involvement of the rifampicin resistance locus with the *ts* mutations. Further analyses on other among our 17 groups of *ts* mutants are currently underway involving a combination of genetic, biochemical, and morphological investigations to elucidate further the complex mechanisms of poxvirus morphogenesis.

ACKNOWLEDGMENTS

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SHORT COMMUNICATIONS

Evidence against Involvement of Host Transcription in the Replication of Vaccinia and Herpes Simplex Viruses¹

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Vaccinia and herpes simplex viruses are unable to multiply in cells treated with the toxin α -amanitin, which specifically blocks host transcription involving RNA polymerase II (pol II). However, these agents are replicated in host cells made nonfunctional by intense γ irradiation when RNA synthesis, including poly(A)-containing RNA and induction of biological functions, exemplified by the antiviral state, are inhibited. From this and previous evidence it is concluded that requirement for pol II or subunit(s) thereof by pox- and herpesviruses is probably related to transcription from the viral, not the host genome.

Involvement of the host nucleus in pox-virus replication is a subject of current interest and controversy. Initially, Pennington and Follett (1) and more recently Hruby *et al.* (29) suggested from their studies of infected cytoplasts derived from BHK21 or BSC cells, that the nucleus may be essential possibly because of some requirement for host-related transcriptional event(s) for completion of vaccinia virus development. Discovery that there exists an obligatory requirement for host transcriptional factors, specifically, for the DNA-dependent RNA polymerase II (pol II), in the vaccinia life cycle (3, 4) has been invoked in support of the hypothesis that transcription from the host genome is involved in the poxvirus replication cycle (4). This notion appears, however, to be inconsistent with the capability of profoundly γ -irradiated cells to fully support production of infectious vaccinia virus (3). As an alternative hypothesis it may be assumed that pol II or some of the enzyme subunits, including the one sensitive to inhibition with α -amanitin, facilitate transcription directly from the viral genome

of some critical virus-specified functions. This view is in line with our findings that *in vitro* transcriptional activity associated with isolated vaccinia virus factories is partially responsive to α -amanitin when such factories originate from infected H9 cells sensitive to this toxin. By contrast, *in vitro* vaccinia-related transcription is unaffected by α -amanitin when tested on factories derived from mutant Ama 102 cells resistant to the drug (G. McFadden, M. Silver, and S. Dales, unpublished observations).

Ability of extensively preirradiated cells to function as a fully permissive host for growth of vaccinia virus, raised the central question concerning the extent of inhibition of host transcription and gene expression by the doses of γ -irradiation applied. This was tested by irradiating uninfected primate and rodent cell lines with varying doses of γ rays from a ⁶⁰Co source, 1 hr prior to labeling the RNA with [³H]uridine. Following synthesis the labeled RNA was isolated and monitored as total and polyadenylated (poly(A)) messenger RNA (mRNA) fractions. The data, summarized in Table 1, revealed, as expected, that the degree of inhibition with BSC-1 cells was directly proportional to the dose applied. Furthermore, synthesis of poly(A)-containing RNA was inhibited to about the

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TABLE 1
EFFECT OF γ IRRADIATION ON SYNTHESIS OF RNA IN PRIMATE AND RODENT CELL LINES

γ Irradiation dose (kilorads)	BSC-1						L_2						L_6H_9	
	Total RNA ^a		Poly(A) RNA ^a		Total RNA ^a		Poly(A) RNA ^a		Total RNA ^a		Poly(A) RNA ^a		Total RNA ^a	Poly(A) RNA ^a
	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%
0	2.99×10^6	100	7.85×10^4	100	3.8×10^6	100	—	2.12×10^4	100	2.03×10^6	100	—	1.64×10^6	100
10	2.20×10^6	73.3	6.50×10^4	82.8	—	—	—	—	—	—	—	—	—	—
20	7.34×10^5	24.5	1.39×10^4	17.7	—	—	—	—	—	—	—	—	—	—
30	3.37×10^5	11.3	8.68×10^3	11.1	—	—	—	—	—	—	—	—	—	—
40	3.19×10^5	10.7	6.83×10^3	8.7	—	—	—	—	—	—	—	—	—	—
70	2.7×10^4	0.9	4.48×10^3	5.7	5.09×10^5	13.2	1.83×10^3	8.6	2.39×10^5	11.8	1.58×10^5	9.6	—	—

Note. The three strains of cells originating from sources identified previously (3, 5) were irradiated as monolayers using a ^{60}Co source (9), returned to incubators at 37° for 1 hr prior to labeling for 2 hr with $2.5 \mu\text{Ci}/\text{ml}$ ^3H -thymidine (New England Nuclear). Following extraction of the RNA, employing the guanidine-HCl procedure (6), aliquots were removed for determining radioactivity of the whole extract and the remaining material was applied to oligo(dT)-cellulose columns for selecting the poly(A)-containing RNA fraction (7, 8). To facilitate comparisons between different samples, aliquots of material labeled with $[\text{U}-^{14}\text{C}]$ thymidine, obtained from unirradiated L_2 cells were mixed with the $[\text{U}-^3\text{H}]$ thymidine-labeled material prior to RNA extraction.

^a Calculated per $\sim 1 \times 10^6$ cells.

same extent as that of the bulk RNA. Of particular interest were data pertaining to the effects produced by 40 and 70 kilorad which inhibited labeling of both the total and poly(A) RNA components by 90-94%. Very similar results were obtained with mouse L₂ fibroblasts and the rat L6H9 myoblast line (Table 1). Incidentally, the latter is the wild type from which the α -amanitin-resistant Ama 102 mutant line, used by us previously (3), had been derived. These data revealed that host-related RNA synthesis and transcription into mRNA are profoundly suppressed following γ irradiation with doses which do not influence appreciably or at all the replication of vaccinia virus (Ref. (3) and Table 2 of this article).

Of course, as demonstrated previously, a dose of 50 kilorad when applied to vaccinia-infected cells can effectively inhibit viral gene expression measured in terms of biological functions such as the activity of the hemagglutinin (3). In order to assess the influence of irradiation on some defined cellular activity we chose to investigate the expression of the interferon-mediated antiviral state. This function was selected because, first of all, it is an inducible one, second, because it is amenable to quantitative assay, and finally, because it manifests or is related to the induction and expression of a multicomponent system involving a number of enzymatic functions (9).

The antiviral state was induced in BSC-1 cells following various doses of γ irradiation 1 hr prior to the addition to monolayer cultures, each containing 1.2×10^6 cells, of 100 units/ml of human leukocyte interferon (INF) kindly provided and standardized by Dr. Tan, University of Calgary. After an 8-hr period of INF treatment, the monolayers were inoculated with vesicular stomatitis virus (VSV, Indiana strain) at a multiplicity of infection (m.o.i.) of 0.01 plaque-forming units (PFU)/cell. Following incubation for 10 hrs postinfection (p.i.) the infectious virus formed was assayed as plaque-forming units on monolayers of BSC-1 cells. Cell monolayers untreated with INF, but otherwise irradiated and incubated under

TABLE 2
EFFECTS OF γ IRRADIATION AND α -AMANITIN ON REPLICATION OF VACCINIA AND HERPES SIMPLEX VIRUSES

Sample culture	Titer $\times 10^4$ PFU/plate	
	Vaccinia ^a	HSV ^b
Following adsorption ^c	6	0.5
Following replication—controls ^d	1000	1400
Following replication— γ irradiated ^e	900	650
Following replication—treated with α -amanitin ^f	6	0

^a Monolayers of 10^6 L₆H₉ cells/plate inoculated at m.o.i. = 10.

^b Monolayers of 1.2×10^6 BSC-1 cells/plate infected with HSV type 1 at m.o.i. = 0.2.

^c Sampled 2 hr p.i.

^d Sampled 24 hr p.i. for vaccinia, 16 hr p.i. for HSV-1.

^e L₆H₉ cells γ irradiated with 70 kilorad 1 hr before inoculation; BSC-1 cells irradiated with 40 kilorad 1 hr before inoculation.

^f α -Amanitin, 10 μ g/ml, was present throughout the infection. L₆H₉ cells were pretreated with the drug for 10 hr (3) and BSC-1 cells for 24 hr.

identical circumstances, were used as the controls for replication of VSV. The results, summarized in Fig. 1, indicated that induction of the antiviral state by the INF treatment used, was effective as evident by the reduction in the PFU of about 4 log units. Irradiation of monolayers with 20 kilorad prior to INF treatment appeared to have little or no effect on the induction of the antiviral state. However, at doses of 40-50 kilorad there was over 1 log increase in VSV production in the irradiated and INF-treated cultures. However, since irradiation per se at 30-50 kilorad adversely affected VSV replication in BSC-1 cells, it may be assumed that the actual difference in titer between INF-treated and untreated cells exposed to 30 kilorad was only 3 log units, at 40 kilorad about 2 log units, and at 50 kilorad about 1 log unit. These data are consistent with the view that the biochemical sequence of

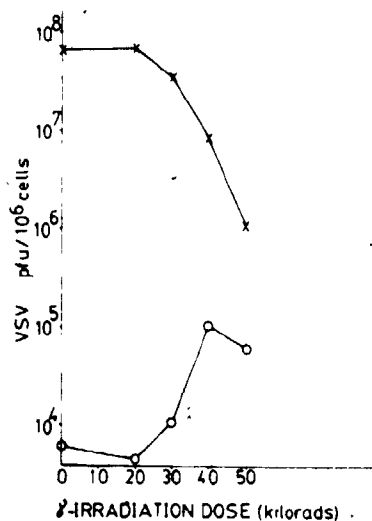


FIG. 1. Effect of γ irradiation on the INF-induced antiviral state. BSC-1 cells were incubated in the absence (X) or in the presence of (O) of human leukocyte INF for 8 hr commencing 1 hr after γ irradiation. Following removal of INF by repeated washings, the culture was inoculated with VSV and incubated for 10 hr until harvested for PFU assays.

events related to induction of the antiviral state by INF was being effectively suppressed by high doses of γ irradiation. Therefore, host-specified functions, expression of which is dependent on ongoing transcription, were effectively suppressed by 40-50 kilorad, doses which do not reduce yields of vaccinia virus (3).

Since intense irradiation, followed by prolonged incubation decreased the capacity of BSC-1 cells to support VSV replication, INF treatment over shorter durations was instituted. Thereby, it was possible to enhance the replicating capability of the irradiated host while allowing sufficient time for induction of the antiviral state. Cells exposed to 40 kilorad were incubated for 1 hr before commencing INF treatment for 4 to 6 hr, as described above. As evident from the data in Fig. 2, INF-untreated cells irradiated 5 hr prior to inoculation produced about half a log less virus (1.5×10^7 PFU) than the unirradiated and untreated cells (5×10^7 PFU), hereafter referred to as controls. By contrast, the unirradiated cells

treated with INF for 4 hr yielded >2.5 log units less (1×10^5 PFU) of VSV than the controls. Following γ irradiation and INF treatment for 4 hr, the yield of VSV (4.7×10^6 PFU) was only 1 log less than obtained with the controls. With the more prolonged treatment cultures irradiated 7 hr prior to infection replicated approximately a half log unit less VSV (1.1×10^7 PFU) than the controls. A 6-hr period of INF treatment of unirradiated cells caused an approximate 3.5 log unit reduction in titer (Fig. 2), but preirradiation enabled cells treated with INF to produce 1.3×10^6 PFU of VSV, i.e., a reduction in yield of only 1.6 log units compared with the controls. Therefore, γ irradiation enhanced VSV replication in INF-treated cells by over 2 log units.

These combined data in Figs. 1 and 2 indicated that preirradiation with 40 or more kilorad can effectively inhibit induction of the antiviral state and must,

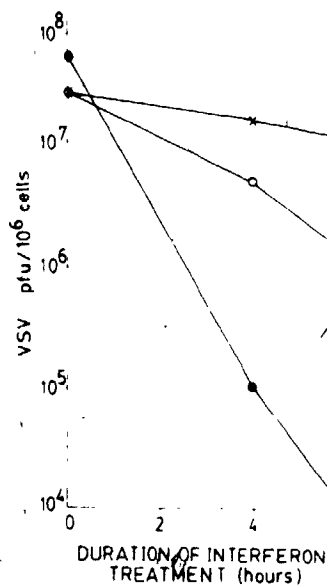


FIG. 2. Effect of γ irradiation and duration of INF treatment on the antiviral state. BSC-1 cells were γ irradiated (40 kilorad) 1 hr prior to addition of INF, as described in Fig. 1. After incubation for the periods indicated, the monolayers were washed, inoculated with VSV, and harvested 10 hr p.i. for PFU assays. γ Irradiated (X); INF treated (●); γ irradiated, then INF treated (O).

therefore, cause suppression of gene expression manifested both as mRNA synthesis and specific biological functions.

In agreement with previous and current results which showed that L₂ cells irradiated with 40-70 kilorad are a fully competent host for vaccinia virus (3), although their own transcription is profoundly reduced (Table 1), present experiments revealed that preirradiation with 40 kilorad did not affect the ability of BSC-1 cells to replicate vaccinia virus (data not shown). Likewise in the case of L6H9 rat myoblasts, preirradiation with 70 kilorad had little effect on vaccinia virus production (Table 2). By contrast treatment of these cells, containing pol II sensitive to α -amanitin, with the drug completely abolished vaccinia virus replication (Table 2), confirming our previous observations. Since the present data imply that ongoing host-related transcription is not required, the pol II or subunit(s) thereof might be involved in transcription from the vaccinia genome.

It seemed appropriate to examine, as a control, replication of another DNA agent, herpes simplex virus (HSV), known to pass through a replication phase during which transcription into early rather than late functions, as with vaccinia (2, 3), is blocked by α -amanitin. In agreement with previous findings of others (10), exposure of BSC-1 cells to the drug profoundly inhibited HSV replication implying that pol II is intimately involved in the life cycle of this virus (Table 2). Infection of preirradiated BSC-1 cells, shown to be effectively suppressed in their own transcription and gene expression, affected only partially production of HSV. These observations imply that, as in the case of vaccinia virus, pol II may be involved in HSV transcription (11), but host-specified transcription is probably not involved in this process.

To summarize, it was shown in a variety of cell types that high dose γ irradiation is an effective method for severely inhibiting cellular synthesis of bulk and the poly(A) RNA, as well as host gene expression exemplified by induction of the INF-mediated antiviral state. Such irradiation does not affect appreciably the replication of vaccinia virus or HSV-1, two agents with an obligatory requirement for pol II activity. Taken together, these observations favor the idea that pol II, or a subunit thereof, participates directly in transcription from the viral genome. In the case of vaccinia, such transcription is most likely into late function(s) following DNA replication. Concentration of pol II at its normal site of activity in the host nucleus could, therefore, explain why cytoplasts are unable to or have a very low efficiency for supporting vaccinia virus replication (1, 2).

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Biogenesis of Vaccinia: Interrelationship between Post-translational Cleavage, Virus Assembly, and Maturation¹

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Previously published investigations with inhibitors and temperature-sensitive (*ts*) mutants revealed that post-translational cleavage (PTC) of virion core polypeptides is a necessary step for development of infectious, mature vaccinia virus. Present studies focused on the nature of the protease factor(s) required for vaccinia-biogenesis. To ascertain whether the proteolytic factor(s) can move freely through the cytoplasm, PTC occurring during complementation between cleavage defective and DNA-*ts* mutants was compared with that evident following induced syncytiogenesis, involving cells singly inoculated with wild-type and cleavage-defective *ts* 1085 virus. Since PTC can occur during coinfection but not after cell-cell fusion, the protease factor is presumed to be nondiffusible. This notion is supported by the incapacity of extracts from infected cells to bring about *in vitro* PTC. Data from temperature shift experiments with *ts* 1085 indicate that the factor(s) for proteolysis is probably a short-lived activity and affinity labeling suggests that it may be a virus-induced, nonvirion polypeptide p 12.5 in molecular weight, possessing the specificity of chymotrypsin for protease inhibitors TPCK and ZPCK. Evidence indicating that the factor has a brief half-life implies that it must be synthesized on a continuous basis to effect viral maturation. A model of vaccinia self-assembly, which takes into account previous observations and current data, is proposed according to which induction of core enzymatic activities, internal differentiation, and acquisition of infectiousness are temporally coordinated, closely coupled phenomena requiring PTC.

INTRODUCTION

Our laboratory has been investigating the assembly process of vaccinia virus for a number of years using inhibitors and more recently temperature-sensitive (*ts*) mutants. One of our mutants, *ts* 1085, which under nonpermissive conditions mimics the effects of the antibiotic rifampicin on envelope and virus formation (Nagayama *et al.*, 1970; Katz and Moss, 1970) has been particularly useful. With *ts* 1085 early functions and DNA synthesis are normal but membrane assembly is aberrant. Furthermore, post-translational cleavage (PTC) of specific core polypeptides and induction of certain core en-

zymes, both regulated as late functions, are inhibited. While all of these defects are reversible upon "shift-down" to the permissive temperature, only assembly of immature virus envelopes can occur in the absence of continuing protein synthesis (Stern *et al.*, 1977). Combined evidence from several lines of investigation indicates that PTC of core precursor polypeptides as well as the induction of core enzymes, virus maturation, and infectiousness must be temporally coordinated and somehow interconnected events. Data from this and previous studies suggest that proteins related to core enzymes and precursor polypeptides must be packaged within immature particles before PTC and differentiation into the core and lateral bodies, connected with virion maturation, can occur.

In this article, examination in consid-

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erable detail of the nature and role of the envelopes and PTC led us to propose a revised model of vaccinia virus biogenesis.

MATERIALS AND METHODS

Virus and cells. The syncytiogenic IHD-W parental strain of vaccinia virus (Hanafasa, 1950), designated as wild-type (WT) and two conditional-lethal, temperature-sensitive (*ts*) mutants 1085, 7743, defective in maturation and other *ts* 6389 defective in DNA synthesis, have been described previously (Dales *et al.*, 1978; Lake *et al.*, 1979). Monolayers of L₂ mouse fibroblasts (Rothfels *et al.*, 1959), used for virus propagation and assays of plaque-forming units (PFU), were maintained in nutrient medium (NM) consisting of Eagle's minimal essential medium (MEM) (Eagle, 1959) supplemented with 10% fetal calf serum (FCS). For experiments employing *ts* mutants, the permissive temperature was ~33°, and restrictive temperature 39–40°.

Reagents. The *Conavalia ensiformis* lectin (concanavalin A type IV) (Con A) and protease inhibitors phenylmethylsulfonyl fluoride (PMSF), L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK), *N*- α -*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and *N*-carbobenzoxyl-L-phenylamine chloromethyl ketone (ZPCK), were purchased from Sigma Chemical Co. Actinomycin D was obtained from Schwarz/Mann, rifampicin from CIBA Pharmaceutical Co., and polyethylene glycol 6000 (PEG) from BDH Chemical Co. Streptoviticin A (str A) was a gift from Upjohn Co.

The [³H]ZPCK (specific activity 330 mCi/mmol) was prepared by Amersham Corp. employing random labeling of ZPCK by means of the catalytic exchange method in tritiated aqueous medium (code TR.1). L-[³⁵S]methionine (1010.6 Ci/mmol) and L-[³H]amino acid mixture (NET-250) were purchased from New England Nuclear.

Complementation analysis. Complementation between *ts* 1085 and *ts* 6389 was tested using 35-mm monolayer cultures (1 × 10⁶ cells/plates) singly or doubly infected with each mutant at a multiplicity of infection (m.o.i.) of 5 PFU/cell to mea-

sure rescue in terms of infectivity and at m.o.i. of 10 for gel analysis of virus polypeptides. After adsorption at 39° for 1 hr, the unadsorbed inoculum was removed by repeated washing of the monolayers with MEM prewarmed to ~39°. Uneclipsed virus was neutralized with NM containing antivaccinia serum during 1 hr incubation at ~39°. The antiserum was removed by washing with MEM and cultures were either harvested forthwith or after a further incubation for 22 hr at ~33 or ~39°. The phenotypes of progeny derived after complementation from the mixed infection at ~39° were checked by plating diluted lysates, growing up virus stocks from individually-picked plaques (Dales *et al.*, 1978), and analyzing polypeptide patterns employing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE of Laemmli, 1970, as applied to vaccinia virus by Stern and Dales, 1976b).

Particle counting by quantitative electron microscopy (Dales, 1963) was made on singly or doubly infected 60-mm monolayer cultures (3 × 10⁶ cells/plate) inoculated with the individual mutants at m.o.i. of 2.

Cell-cell fusion. Fusion between infected cells was initiated by a modified method of Mercer and Schlegel (1979). Briefly, semiconfluent (1.5 × 10⁶ cells/plate) 60-mm monolayer cultures were inoculated with *ts* 1085 at a m.o.i. of 10. Following 19 hr incubation at the nonpermissive temperature, the monolayers were washed with MEM and placed for 1 hr at ~39° in NM containing 10 µg/ml of Con A to enhance cell-cell contact with the 3 × 10⁶ cells/dish added from suspension. The latter had, prior to plating, been infected with either WT or *ts* 1085 at an m.o.i. of 10 and incubated at ~39° for 9 hr. The mixed adherent and added cells were incubated further for 1 hr at ~39° to establish more stable contact. The Con A-containing medium was then removed and replaced for 1 min by carefully added 1 ml of MEM containing 44% polyethylene glycol (PEG) and 10% dimethyl sulfoxide (DMSO), prepared as described by Davidson and Gerald (1976). Upon removal of the PEG-containing medium, by washing three times

with prewarmed MEM and 10% DMSO, each plate received 5 ml of NM and incubation continued at ~ 33 or $\sim 39^\circ$ for 8 hr prior to harvesting cells for electron microscopy, SDS-PAGE analysis, or enumeration of polykaryocytes.

Efficiency of fusion was ascertained after fixation *in situ* with 2% glutaraldehyde and staining with Giemsa. For enumerating nuclei following fusion, cells were released from monolayers by trypsinization, disrupted in lysis buffer (1% Nonidet P.40 (NP₄₀), 10 mM Tris [tris (hydroxymethyl)aminoethane]-HCl, pH 7.2, and 1 mM MgCl₂, then the free nuclei centrifuged into pellets at 800 *g* for 5 min. The pellets were washed twice with phosphate-buffered saline (PBS), resuspended in 10 ml PBS, and the nuclei enumerated using a hemacytometer. Cultures prepared as described above with the omission of PEG were used as controls.

Isotopic labeling and PAGE analysis. Cytoplasmic extracts and purified viroplasmic matrices or "factories" isolated from labeled cells were analyzed by SDS-PAGE using 11% polyacrylamide slab gels, as described by Lake *et al.* (1979), except in the case of [³H]ZPCK labeling experiments for which 10-20% gradient gels were used. Unless otherwise stated, labeling with [³⁵S]methionine (10 μ Ci/ml) or [³H]amino acids (10 μ Ci/ml) occurred from 9 to 10 hr postinfection (pi). In experiments involving cell-cell fusion, the adherent *ts* 1085-infected cultures were labeled prior to initiation of the fusion process. The chase medium used after [³⁵S]methionine labeling contained five times the normal concentration of methionine.

Inhibitors. Synthesis of RNA was suppressed by adding 4 μ g/ml of actinomycin D to the NM and of protein by including str A in varying concentrations. The protease inhibitors PMSF, TLCK, TPCK, ZPCK, and [³H]ZPCK were dissolved in DMSO and used in MEM containing 1% DMSO.

The effect of protease inhibitors on rates of protein synthesis was determined as follows: suspensions of 2×10^7 cells in 1 ml NM were inoculated for 1 hr at 4°

with WT vaccina at an m.o.i. of 10, then were washed twice with MEM to remove unadsorbed virus, resuspended in 50 ml of NM containing 0.1% methylcellulose, and incubated at 37° for 9.5 hr with agitation. The cells were collected into pellets by centrifugation at 800 *g* for 10 min, washed once with MEM, and resuspended in 50 ml MEM, 1% DMSO, 0.1% methylcellulose. Str A or one of the protease inhibitors was added to duplicate 1-ml aliquots, and the samples incubated with agitation at 37° for 1 hr in a water bath. [³⁵S]Methionine was added at a final concentration of 0.25 μ Ci/ml, incubation was continued at 37° for 1 hr, whereupon the samples were cooled and kept on ice during addition, with simultaneous mixing on a Vortex mixer, of SDS at a final concentration of 1% and bovine serum albumin at a final concentration of 0.1 mg/ml. Protein was precipitated with a 10% final concentration of trichloroacetic acid (TCA), the precipitate was trapped on Whatmann 3 MM paper discs, washed sequentially with 10% TCA and 95% ethanol, and placed in vials containing toluene-based scintillation fluid for determining cpm precipitable counts in a Beckman LS-350 scintillation counter.

Labeling of polypeptides with [³H]ZPCK. Four 100-mm monolayer cultures were infected with WT or *ts* 1085 at an m.o.i. of 25. At 10 hr pi, the NM was removed, the monolayers washed once with MEM + 1% DMSO, and MEM-containing 1% DMSO and [³H]ZPCK at a final concentration of 10^{-4} M was added to the cells. After 3 hr incubation at ~ 33 , 37 , or $\sim 39^\circ$, cells were harvested by scraping, washed twice with MEM + 1% DMSO, and disrupted in lysis buffer as described above. The cytoplasmic extracts were layered on a 1% deoxycholate, 10 mM Tris-HCl (pH 7.4) cushion and centrifuged at 10,000 *g* for 20 min to concentrate into pellets the virus "factories" (Pogo and Dales, 1969) which were then used for SDS-PAGE analysis.

Electron microscopy. Methods for collecting and preparing cell samples, thin sectioning, and examination by transmission electron microscopy were the same as those described in a previous study (Dales and Mosbach, 1968).

γ -Irradiation. Monolayers of cells were placed on ice and irradiated using a ^{60}Co source (Gamma cell 220, Atomic Energy Canada, Ltd., calibrated to deliver 110 rad/sec; 1 rad = 10^{-2} J/kg). The NM was then replaced and cultures returned to incubators and kept at 37° for 1 hr before use for experimentation.

RESULTS

Description of Assembly and Maturation Studied Previously and Presently by Mutants Defective in Processing of Core Proteins

The advantages of employing conditional-lethal mutants of vaccinia virus for analysis of the assembly sequence were established previously (Dales *et al.*, 1978; Lake *et al.*, 1979). Two among these mutants, *ts* 1085 and 7743 were especially useful in elucidating the requirement for processing, also termed post-translational cleavage (PTC), of certain core polypeptides and involvement of the lipoprotein envelope during morphopoeisis and maturation. These two group E mutants, described by Dales *et al.* (1978), are blocked at a stage of assembly which mimics closely the effects on morphology of the antibiotic rifampicin (Nagayama *et al.*, 1970; Katz and Moss, 1970). At the restrictive temperature the normally spherical envelopes covered by an external layer of spicules, evident on immature particles, failed to develop. Instead, the factories were surrounded by flexible envelopes devoid of spicules. Following a temperature "shift-down," from 39° to the permissive 33°, normal development of *ts* 1085 and 7743 was resumed and mature virions were formed. However, when temperature shift-down was carried out in the presence of str A, a rapidly acting inhibitor of protein synthesis, the normal spherical membranes associated with immature virus were assembled, but further development was arrested. Thus, defects in envelope formation related to *ts* mutations were readily reversible but subsequent development culminating in mature virions was contingent on continuation of protein synthesis.

The morphological defects associated with *ts* 1085 and 7743, described above, were closely linked with defective PTC of certain core polypeptides, as revealed by PAGE analysis. Incidentally, assignment of molecular weight of individual polypeptides conformed to the system adopted by this laboratory (Stern and Dales, 1976a). To exemplify, a protein migrating to a molecular weight position of 100,000 in the Laemmli (1970) PAGE system was designated p100. During infection with *ts* 1085 and pulse labeling with [^{35}S]methionine at the restrictive temperature, core precursor polypeptides p94 and 65 accumulated without PTC. After shifting the labeled cultures to permissive conditions, the precursor proteins were processed into p62, 60, 23, 18.5, and 18.0 polypeptides, confirming the previous findings of Stern *et al.* (1977). With *ts* 7743, PTC of p94 to 62 was defective and appearance of p23, 18.5, and 18.0 absent or reduced, with the p65 core precursor polypeptide was cleaved normally to its p60 product. Once again, after shift-down to 33°, normal PTC was resumed. However, in the case of wild-type virus (WT), as well as with both mutants, exposure to inhibitors of protein synthesis such as str A, at the time of reversal to permissive conditions, prevented all PTC (Stern *et al.*, 1977, and Fig. 7a of this article), despite conversion from aberrant to spherical envelopes, described above. Therefore, PTC is intimately connected with differentiation from immature into mature virions.

Effect of Complementation on Assembly of Infectious Progeny

Another approach toward elucidation of the role of envelopes and PTC in vaccinia virus assembly involved coinfection with *ts* 1085 and *ts* 6389, a tight mutant defective in DNA synthesis (McFadden and Dales, 1980). From data on yields of progeny, expressed as formation of PFUs, it was evident that the two mutants complemented each other efficiently, the index of complementation, calculated according to Lake *et al.* (1979), being ~30 to 70. Any possibility that increase in PFU after dual

infection resulted from appearance of revertants with WT phenotype was eliminated by analysis of plaque-purified progeny, as described below. Complementation data on PFU were corroborated by particle counting in the electron microscope. Various stages of virus development, evident in thinly sectioned cells, were enumerated by according to the procedure of Dales (1963). It is clear from the data in Table 1 that dual infection with *ts* 1085 and 6389, under nonpermissive conditions, produced about five times more mature virions than did the sum of infections with individual mutants. By contrast, there was no increase, even perhaps a reduction, in the number of incomplete and complete immature particles following dual infection (Table 1). Defectiveness of *ts* 6389 in DNA synthesis was reflected in the number of early factories, i.e., those which did not contain any recognizable virion structures. Rescue of one *ts* mutant by the other became also evident from enhanced PTC of all precursor polypeptides, as demonstrated by means of SDS-PAGE and autoradiography. Gels such as that shown in Fig. 1 revealed, first of all, that in cells infected with WT vaccinia virus and har-

vested immediately after the pulse-labeling with [³⁵S]methionine, the cleavage products p62, 60, 23, 18.5, and 18.0 were present in low amounts but increased in quantity after an 8 hr chase at 39°. With *ts* 1085, also evident in Fig. 1, PTC was blocked effectively during the chase at restrictive temperature. In the case of *ts* 6389, the DNA mutant, the precursor polypeptides being late functions (Stern *et al.*, 1977) did not appear during pulse labeling and, therefore, failed to generate cleavage products during the chase at 39°. Following dual infection with *ts* 1085 and 6389, p94 and 65 precursors were synthesized, then underwent normal processing as evident from the presence in Fig. 1 of five cleavage product bands following the 8 hr chase under nonpermissive conditions.

To establish that enhanced PTC and mature virus production were, indeed, the consequence of complementation, not reversion of mutated virus to the WT phenotype, progeny from dual *ts* 1085 + *ts* 6389 infection at 39° were inoculated after suitable dilution onto cell monolayers and allowed to form plaques under methylcellulose. Twenty individual plaques were picked, most were grown into virus stocks,

TABLE 1
STAGES IN VIRUS ASSEMBLY DURING SINGLE AND DUAL INFECTION WITH *ts* MUTANTS^a

	Temperature	Virus titer ^b (PFU/ml × 10 ⁻⁵)	Electron microscopy ^c			
			Foci of viroplasm (factories)		Immature particles ^e	Mature particles
			Early ^d	Late		
<i>ts</i> 1085	33°	650	0	65	1091	2667
	39°	1.3	8	51	360	42
<i>ts</i> 6389	33°	120	20	38	516	1154
	39°	0.4	59	7	159	48
<i>ts</i> 1085 + <i>ts</i> 6389	39°	33	14	35	211	477

^a Confluent 60-mm culture plates were infected at m.o.i. of 2 with *ts* 1085 or *ts* 6389 or 2 of each of the two mutants, incubated at 33 or 39° for 24 hr and sampled for virus titer and electron microscopy.

^b Virus titers were determined by plaque assay on L cells at 33°.

^c The electron microscopy data were obtained by examining 100 thinly sectioned cell profiles per sample.

^d Early factories were those from which recognizable virion structures were absent.

^e In this category were included complete spherical particles and immature forms with incomplete membranes

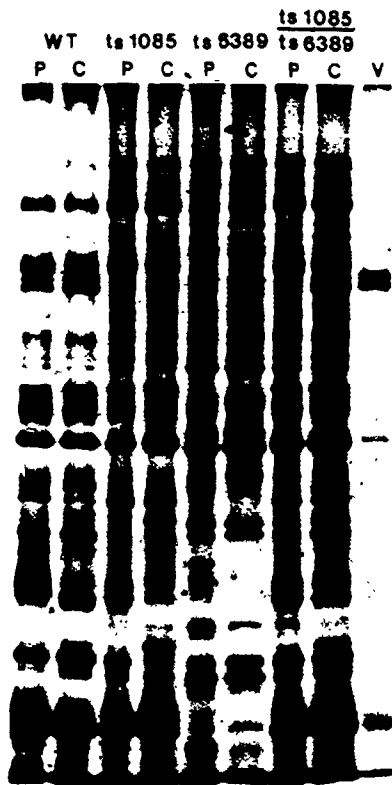


FIG. 1. Rescue of the defect in post-translational cleavage by complementation during dual infection. Autoradiogram was prepared from cytoplasmic extracts following single or double infection with *ts* 1085 and *ts* 6389. Cultures maintained throughout at 39° were pulse-labeled with [³⁵S]methionine 9 to 10 hr pi and either harvested immediately (P) or 8 hr later (C), after incubation in chase medium. Arrowheads indicate positions of p94, 95, 62, 60, 23, 18.5, and 18, respectively. Channel V pure virion polypeptides.

and subjected to SDS-PAGE analysis. Among progeny from randomly isolated plaques, seven displayed a protein banding profile identical with that of *ts* 6389 and four with that of *ts* 1085 (data not shown), confirming the evidence for bonafide complementation.

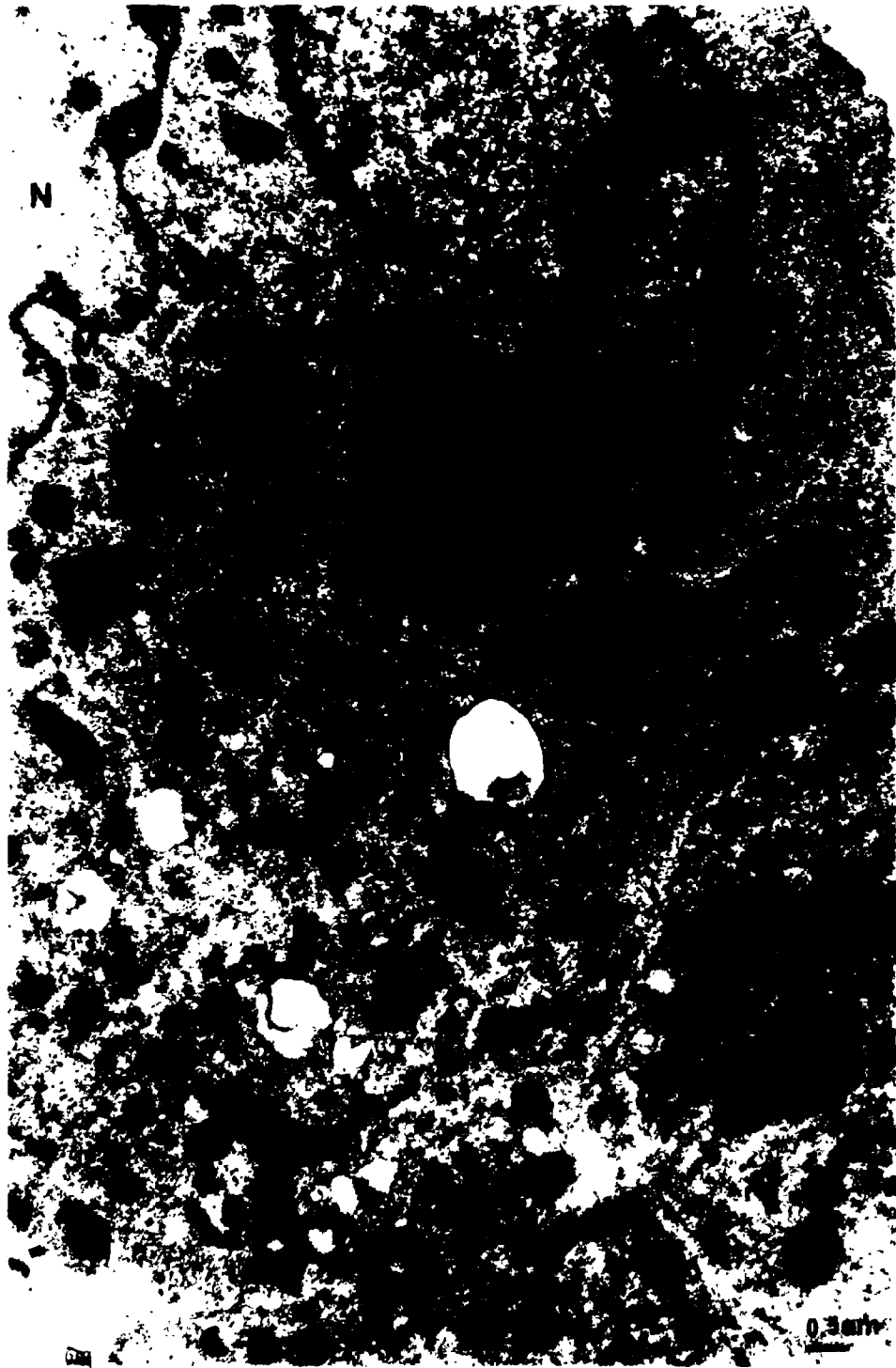
Taken together, the data from complementation experiments provide evidence for close linkage between development of mature, infectious virions, and normal PTC.

Attempt to Transfer Capacity for PTC by Cell-Cell Fusion

The above results showing that different *ts* mutants can act in a complementary manner to facilitate PTC, raised questions concerning the mobility and intracellular site where factor(s) required for processing may be active. Experiments designed to answer these questions involved coalescence of cytoplasmic compartments from cells singly infected with cleavage defective *ts* 1085 and WT vaccinia virus. Cultures inoculated with *ts* 1085 and maintained at the restrictive temperature were pulse-labeled with [³⁵S]methionine for 1 hr commencing at 9 hr postinfection (pi), while companion cultures, infected independently with WT vaccinia virus, were incubated for 10 hr without protein labeling. Then cells, in the process of infection by the WT and mutant viruses, were induced to fuse under the influence of PEG, as described under Materials and Methods, and incubation was resumed at 33 or 39°.

An electron microscopic image of a typical "hybrid" polykaryocyte from a culture maintained at the nonpermissive temperature, is shown in Fig. 2. The cytoplasm of such fusion-induced polykaryocytes contained viroplasmic foci characteristic of both WT and *ts* 1085 infections and numerous mature particles. Approximately one-third of all cells present on the monolayer were bi- or multinucleated. Fusion also produced a 48% increase in the total number of nuclei in the monolayer cultures as compared to monolayers not subjected to cell-cell fusion, but otherwise

FIG. 2. Electron micrograph of a selected area of a thinly sectioned polykaryocyte. Cell-cell fusion mediated by PEG involved cultures independently infected at 39° with *ts* 1085 or WT vaccinia virus. Following coalescence of cytoplasm from the 2 infections, both aberrant factories (F), typical of *ts* 1085 development, and normal immature (I) and mature (M) virions, associated with WT infection, were evident. Nucleus (N); mitochondrion (m). ×14,250.



treated similarly. The morphological observations indicated that extensive fusion between cells, independently infected with *ts* 1085 or WT vaccinia virus, must have occurred.

To ascertain whether it is possible to transfer a factor(s) from WT-infected cells which elicits PTC of *ts* 1085 proteins at 39° by means of cell-cell fusion, extracts from labeled cells were analyzed by PAGE. It is necessary to point out that the bulk of virus polypeptides resides within the isolable virus factories (Stern *et al.*, 1977). It is evident from the band pattern in Fig. 3 that, regardless of induced fusion, polypeptides synthesized after infection with *ts* 1085 underwent PTC at 33° but failed to do so at 39°. This result indicated that any factor(s) involved in proteolysis of vaccinia virus core polypeptides which were active during infection with the WT could not effect PTC of *ts* 1085 precursor proteins concentrated in viroplasmic foci, following cytoplasmic mixing.

Another approach toward characterization of the factor(s) functioning in PTC was to isolate cytoplasmic components containing the factories for investigation as follows: the entire cytoplasmic material, or concentrated factories, from unlabeled cells infected for 10 hr with WT vaccinia virus were mixed with comparable material from cells infected with *ts* 1085 for 10 hr at 39° and labeled with [³⁵S]methionine 9-10 hr pi. Once again, in line with data revealed by PAGE analysis following cell-cell fusion experiments, the autoradiograms (results not shown) indicated that cytoplasmic components isolated from WT vaccinia-infected cells did not induce processing of core protein precursors specified by *ts* 1085. From these combined *in vivo* and *in vitro* experiments it may be deduced that any late protein factor(s) required for PTC of core polypeptides was not a readily diffusible cytoplasmic component which could be moved between the cytoplasmic factories. Conversely, such factor(s) may be able to elicit proteolysis only when present in close proximity to the site of synthesis or in-

teraction with the core precursor polypeptides.

Instability of Factor(s) Required for PTC

Since the defect in processing associated with the *ts* 1085 and 7743 mutations is readily reversible after temperature shift-down, the question arose whether PTC, once it had been initiated for brief periods under permissive conditions, continued after return to the restrictive environment. The answer was sought by means of a series of studies employing pulse-labeling with [³⁵S]methionine and pulse-chases during infection with *ts* 1085 at 33° or 39°. Following a 1-hr labeling interval at 39°, commencing 9 hour pi, individual cultures were placed in chase medium and either incubated at 33° for 15-360 min before harvesting or at 39° for 15-360 min, then shifted to 39° and incubated until 16 hr pi had elapsed. The results, illustrated in the autoradiogram (Fig. 4), suggested, first of all, that the quantity of material migrating as the p62 and p60 bands was directly proportional to the duration cells were held at the permissive temperature before sampling (compare channels 6 with 10 and 11 of Fig. 4). Second, they revealed that during prolonged incubation under restrictive conditions, after shift-up from varying periods at 33°, no further processing of p94 and 65 into the products p62 and 60 had occurred (channels 6-10 of Fig. 4). Therefore, the extent of PTC was directly related to duration of incubation at 33°, implying that any induced protease factor(s) involved were either thermolabile or active for only brief periods.

Because in the case of *ts* 1085, coincident with activation of PTC, the assembly-arrest phenotype can be reversed, experiments were conducted to examine effects of temperature shifts, like those described in Fig. 4, on virus morphopoeisis. The virus-related structures, evident by electron microscopic examination of thinly sectioned cells, were grouped and enumerated as aberrant factories, incomplete normal envelopes, complete immature forms, and mature virions. A summary of these data

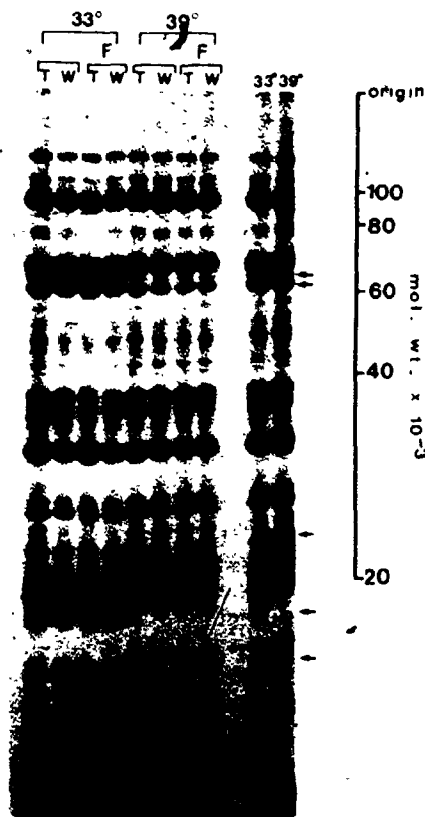


FIG. 3. Failure to rescue the *ts* 1085 defect in PTC by cell-cell fusion induced with PEG. Autoradiogram was prepared from extracts of cells inoculated with *ts* 1085, which were incubated, pulse-labeled with [³⁵S]methionine, and then fused (F) with either WT-infected (W), unlabeled cells or with *ts* 1085 infected (T), unlabeled cells at 39°, as described under Materials and Methods. After formation of the polykaryocytes, incubation was continued at 33 or 39°. Two channels at the extreme right represent material from cells infected with *ts* 1085 which had not been subjected to fusion with PEG. Arrows are at position of p62, 60, 23, 18.5, and 18.

is presented in Fig. 5. Examination of panel A of this figure reveals that the number of aberrant envelopes decreased in direct proportion to the duration of incubation at 33°. Coincidentally, the number of incomplete (panel B) or complete immature progeny virions with spherical envelopes of normal appearance (panel C) increased in relation to the duration of the shift-down period. Likewise, as shown in

panel D of Fig. 5, there was a gradual increase in the number of mature virions as the duration of incubation at 33° was extended. In cultures kept for 60-120 min at 33° then returned to 39° for another 4-5 hr, the number of incomplete particles decreased (Fig. 5B), while the number of complete, immature particles increased by about 50% (Fig. 5C). There was an approximately threefold elevation in the number of mature virions during resumed incubation at 39°, compared with number observed in cells preserved at the end of incubation at 33° (Fig. 5D). Regardless of the duration of the shift-down period or extent of incubation at the nonpermissive

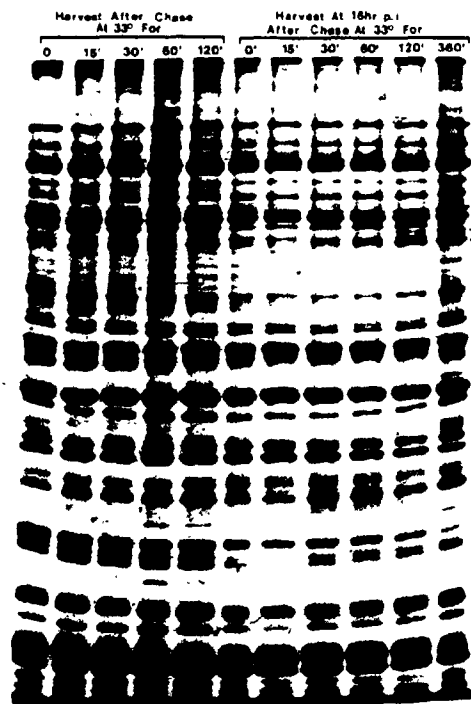


FIG. 4. Autoradiogram showing effect of incubating *ts* 1085 infected cells for varying periods at the permissive temperature. Cultures, maintained at 39° were pulse-labeled with [³⁵S]methionine 9-10 hr pi, placed in chase medium, and shifted to 33° for the duration indicated. Samples were then either collected forthwith (5 left-hand channels) or following shift-up to 39° and further incubation for a total of 16 hr pi (6 right-hand channels). Arrows indicate positions of p62 and 60.

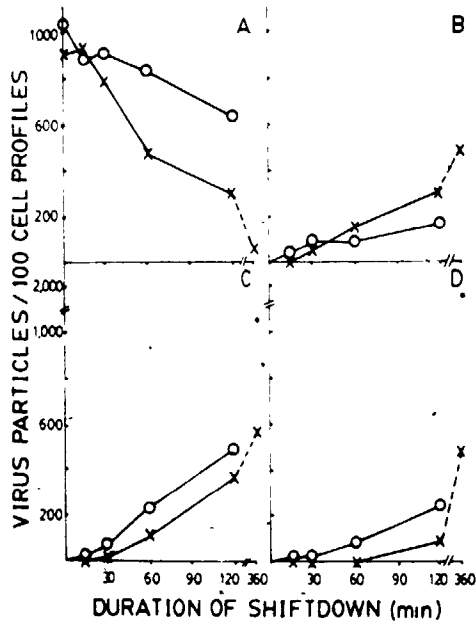


FIG. 5. Enumeration by quantitative electron microscopy of stages in *ts* 1085 development following incubation for various periods under the permissive temperature. Temperature shifts, commencing at 10 hr pi, were carried out according to the schedule outlined in the legend to Fig. 4. Each point is a cumulative count derived from 100 thinly sectioned cell profiles. Samples were taken either when incubation at 33° was ended (x) or after an additional incubation at 39° for a total of 16 hr from the time of inoculation (o). (A) Aberrant factories; (B) incomplete normal envelopes; (C) complete spherical immature particles; (D) mature virions. The number of progeny virions present in equivalent samples of cultures incubated throughout at the permissive temperature is indicated by an asterisk.

temperature, the number of immature and mature virions observed was far lower than that seen with the *ts* 1085 infection maintained throughout the 16-hr period at the permissive temperature (asterisk in Figs. 5C and D). The results revealed that conversion from aberrant to normal envelopes is connected directly to the *ts* 1085 morphological defect. In agreement with the data on PTC presented in Fig. 4, particle counting revealed that extend of normal assembly of incomplete, immature particles and their envelopes during brief intervals at 33° was proportional to duration of the *ts* 1085 infection under per-

missive conditions (Fig. 5B). On the other hand, conversion of some incomplete, immature particles into the complete, spherical immature form could occur at 39°, as evident by the decrease of the former (Fig. 5B) and increase of the latter (Fig. 5C), following temperature shift-up and further incubation. These data indicate that both the extent of PTC and assembly of normal, immature virions including envelopes may be subject to factor(s) which function briefly and/or have a brief half-life at 33°.

Attempts to Identify the Factor(s) Involved in PTC of Core Precursor Polypeptides

Current experiments, described above, indicate that the factor(s) which are required for protein processing and vaccinia virus maturation probably function at the site of virus assembly within factories, may act for short periods or become rapidly turned over and remain nonfunctional in several *ts* mutants, manifesting the phenotype with mimics inhibition by rifampicin. The active site-specificity of the proteolytic factor(s) was tested with several protease inhibitors possessing known specificity for their native substrates.

Compounds such as TLCK and TPCK have affinities for and are recognized as substrates by trypsin-like and chymotrypsin-like proteases, respectively, which they covalently bind and inhibit at the active site (reviewed by Shaw, 1975). However, since these inhibitors also suppress protein synthesis at higher concentration (Pong *et al.*, 1975) it was necessary to determine the molarity at which these compounds acted specifically as protease, rather than translation, inhibitors. Str A was used to check the effects of a specific inhibitor of translation. The results, summarized in Fig. 6, revealed that TLCK at 10^{-4} M inhibited protein synthesis by about 25% without affecting detectably PTC, as illustrated in the autoradiogram of Fig. 7a. At 10^{-3} M, TLCK, which inhibited protein synthesis by over 80%, did not interfere with conversion of p94 and 65 to p62 and 60, but reduced the amount of p23, 18.5, and 18.0 product polypeptides. These

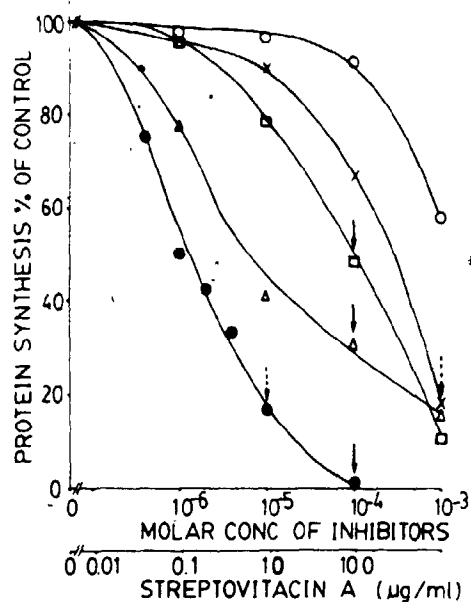


FIG. 6. Relationship between concentration of str A or protease inhibitors and inhibition of protein synthesis. At 9 hr pi WT infected cultures were treated for 1 hr with varying concentration of one of the following: str A (●); PMSF (○); TLCK, (×); TPCK (Δ) or ZPCK (□), then were pulse-labeled for 1 hr at 37° with [³⁵S]methionine, prior to sampling for determination of CPM in protein. Each point represents an average value obtained from duplicate cultures. Broken arrow indicates the concentration of the inhibitors which suppressed PTC partially; solid arrow the concentration which blocked PTC entirely, as judged from the data in Fig. 7a and b.

findings imply that the proteolytic factor involved in cleaving core polypeptides does not have the specificity of trypsin. Application of TPCK, at 10⁻⁴ M, which reduced protein synthesis by about 65% (Fig. 6), completely blocked processing of core polypeptides (Figs. 7a and b) indicating that a protease with specificity of chymotrypsin was involved. This result was substantiated using ZPCK, another compound with affinity for chymotrypsin-like proteases, which at 10⁻⁴ M reduced translation by only 50% (Fig. 6) but PTC completely (Fig. 7b). To prove that reduced protein synthesis in the presence of TPCK and ZPCK was not per se responsible for suppression of processing, str A was added in varying concentrations to vaccina in-

fectured cultures during the chase period, following pulse-labeling with [³⁵S]methionine. It is evident that at 0.1 µg/ml of this drug, which reduced protein synthesis by about half (Fig. 6), PTC occurred normally (Fig. 7a) and at 1.0 µg/ml, which lowered the protein synthesis rate by 82%, appreciable cleavage was evident in the autoradiogram (Fig. 7a). Another protease inhibitor, PMSF, was less potent in affecting translation and even at 10⁻³ M had no influence on PTC. Taken together these data revealed that proteolytic factor(s) which may bring about processing of the viral core precursor proteins possess the specificity of a chymotrypsin-like protease.

[³H]ZPCK Labeling of the Protease Involved in PTC

Evidence that protease inhibitors such as ZPCK have affinity for and form covalent bonds with chymotrypsin-like protease(s) involved in vaccina virus development, suggested that such protease(s) might be identified by means of ZPCK carrying a suitable radiolabel. To this end, cultures infected with WT vaccina or mock infected cultures were incubated in the presence of [³H]ZPCK for 3 hr commencing at 10 hr postinfection. As a further test of the origin of the protease(s), cells intensively γ-irradiated with 70 krad from a ⁶⁰C source prior to infection, were analyzed for PTC because such irradiation was shown to suppress profoundly or abolish transcription of host cell genes (Silver and Dales, *Can. J. Biochem.* 1982, in press). Therefore, any polypeptide induced after infection binding ZPCK would most likely be a virus-specified function. When extracts of [³H]ZPCK-labeled cells were subjected to SDS-PAGE analysis, the autoradiograms revealed presence, exclusively in material from infected cells, of a prominent band migrating with an apparent molecular weight of 12,500 (Fig. 8, channels 6 and 7). This band was entirely absent from uninfected, γ-irradiated or unirradiated cells (Fig. 8, channels 8 and 9). Several identical minor polypeptides were also evident in PAGE of extracts from

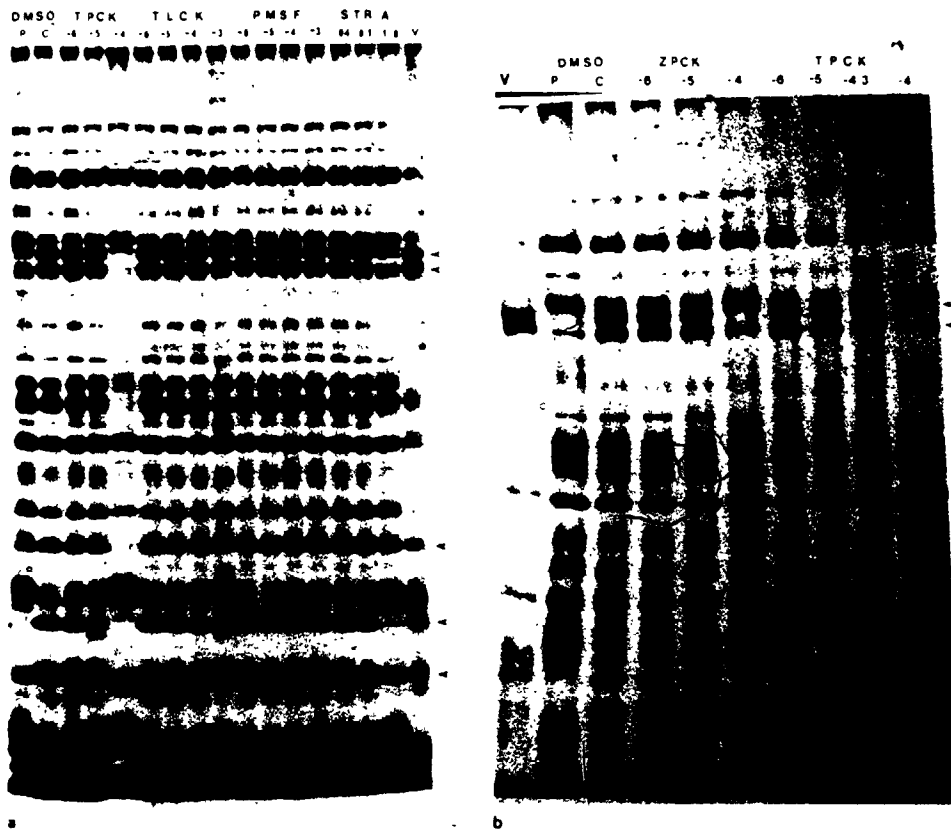


FIG. 7. (a, b) Effect of varying concentrations of protease inhibitors and str A on PTC. The autoradiogram was prepared from cytoplasmic extracts of WT vaccinia virus-infected cells. Cultures were treated as indicated above each channel, following pulse-labeling with [35 S]methionine for 1 hr commencing 9 hr pi (P), and incubation in chase medium in presence of inhibitor for a further 8-hr period (C). Molar concentration of protease inhibitors which were 10^{-6} , 10^{-5} , 5×10^{-5} , 10^{-4} , and 10^{-3} are labeled respectively as -6, -5, -4.3, -4, and -3. Str A concentrations in $\mu\text{g}/\text{ml}$ were 0.04, 0.1, and 1.0. Arrowheads indicate positions of p62, 60, 23, 185, and 18, respectively. Channel V, pure virion polypeptides.

both virus-infected and uninfected cells. None of these corresponded to the prominent polypeptide presumed to have been labeled by [^3H]ZPCK. The major, virus-specified polypeptide, marked by addition of [^3H]ZPCK, did not match in MW any of the [^{35}S]methionine-labeled proteins from purified virus, also displayed in channel V of Fig. 8. These results suggested that a virus-specified p12.5 protein, which has affinity for ZPCK, was induced during vaccinia virus infection, but was absent from mature virions. It is hypothesized that this polypeptide is related to the protease factor(s) involved in PTC.

To ascertain whether the same polypeptide was present or absent during *ts* 1085 infection, which is blocked in processing at the restrictive temperature, infected cultures were labeled as above with [^3H]ZPCK. It is evident from the autoradiogram (Fig. 8, channels 1-5) that regardless of the conditions prevailing during infection, i.e., whether labeling occurred at 33 or 39°, or whether following temperature shift-down transcription was inhibited with actinomycin D or translation with str A at concentrations which block PTC (Nagayama *et al.*, 1970), the p12.5 polypeptide was induced. Therefore,

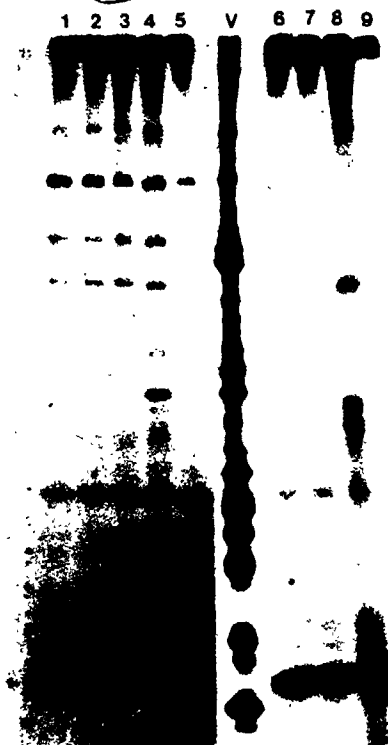


FIG. 8. Identification of a polypeptide with presumed binding affinity for ZPCK. The autoradiogram prepared from cytoplasmic fractions isolated from cells infected with WT or *ts* 1085 vaccinia virus and incubated in the presence of 10^{-4} M [3 H]ZPCK for 3 hr commencing at 10 hr pi. Channels (1-5) *ts* 1085 infected at 33° (1) and at 39° (2); shifted after 10 hr at 39 to 33° during exposure to ZPCK (3); as in (3) but with str A 10 µg/ml added (4); as in (3) but with actinomycin D 4 µg/ml added (5). Channels (6 and 7) infected with WT virus; (8 and 9) mock-infected; γ -irradiated 1 hr prior to infection (7) and (9). Channel V: [35 S]methionine-labeled pure virion polypeptides.

synthesis of the ZPCK-binding protein presumably occurred under circumstances which were nonpermissive for processing and maturation of vaccinia virus. This finding was supported by results obtained during infection with WT vaccinia virus in the presence of 100 µg/ml of rifampicin, an antibiotic which simultaneously blocks morphoiesis and PTC in a manner closely mimicked by *ts* 1085 and other mutants of this phenotype. When assayed during rifampicin treatment or during arrest of

transcription and translation, following removal of rifampicin, there was synthesis of the polypeptide marked by [3 H]ZPCK (data not shown).

Possible identity of the p12.5 protein with any labeled by [3 H]amino acids or [35 S]methionine was examined in gels of cytoplasmic extracts. A band appeared after labeling with [3 H]amino acids or [35 S]methionine corresponding in MW to the p12.5 band evident after addition of [3 H]ZPCK to infected cells (data not shown).

Therefore, during vaccinia virus infection, when host-related protein synthesis is inhibited (Moss and Salzman, 1968), and induction of host-related functions blocked by γ -irradiation, a p12.5 polypeptide, with presumed affinity for chymotrypsin protease inhibitors, may be induced under circumstances either permissive or restrictive for PTC and envelope assembly and maturation. This polypeptide, if it was present in isolated factories originating from cells infected with WT virus, was not labeled *in vitro* by [3 H]ZPCK. The lack of *in vitro* affinity labeling could explain the absence of PTC during *in vitro* incubation on the supposition that the protease factor(s) was lost or became inactive during isolation.

DISCUSSION

In the current investigation, focusing on the role of PTC and envelopes in vaccinia virus biogenesis, use of conditional lethal, *ts* mutants made possible an incisive analysis of the complex sequence of virus development. In particular, mutants such as *ts* 1085 and 7743, defective in both PTC of core polypeptides and normal envelope formation (Dales *et al.*, 1978; Stern *et al.*, 1977) were amenable to the multidisciplinary analysis described here.

The finding that different mutants like *ts* 1085 and 7743, although of the same phenotype as far as envelope formation is concerned, have individual defects involving processing of different polypeptides (Lake *et al.*, 1979) indicates, first of all, that PTC of one core polypeptide may occur independently of any other and second,

that assembly of immature virion envelopes with normal morphology is not contingent upon PTC, in support of previous data of Stern and Dales (1976b) and Stern *et al.* (1977).

In an endeavor to elucidate further the connection between processing and virus assembly, experiments were formulated to identify protease factor(s) which might be involved. As one approach, complementary infections were initiated from which it became evident the dual infection at 39° with *ts* 1085 and 6389, a DNA-mutant, resulted in enhanced production of infectious, mature virus as well as rescue from the processing defect inherent in *ts* 1085. This experiment demonstrated that an active proteolytic factor, once induced, could bring about cleavage of core polypeptide precursor specified by a cleavage-deficient mutant. Therefore, the defect in *ts* 1085 probably involves proteolysis itself, not the protein substrates being cleaved. By contrast, with mutants like *ts* 7743, in which some precursor core proteins are processed, these proteins may themselves be mutated, as demonstrated by McFadden *et al.* (1980) for a p37 core polypeptide of *ts* 9251. Such proteins might, therefore, not be amenable to PTC. Data from complementation experiments also ruled out the possibility that an inhibitor of PTC was acting during *ts* 1085 infection. In contrast to the above data on complementation by dual infection, results from cell-cell fusion experiments designed to assess the mobility of protease factor(s), showed that PTC was not rescued after coalescence of cytoplasm of cells infected by WT virus permissive for PTC with cells infected by cleavage-defective *ts* 1085. This result indicates that the proteolytic factor(s) involved may not be readily diffusible between factories within a continuous cytoplasmic compartment, perhaps because the site of synthesis and activity in PTC must be somehow spatially coordinated.

A series of experiments on cells infected with *ts* 1085, in which cultures were shifted for short periods to the permissive temperature then returned to the restrictive one, provided results consistent with the view that PTC, once initiated under per-

missive conditions, does not continue to function after "shift-up" to the restrictive temperature. This evidence is consistent with the finding that continuous transcription and translation are necessary to maintain PTC, even in the case of WT vaccinia infection (present data and Stern *et al.*, 1977) and implies that the proteolytic activity required to process vaccinia core polypeptides and facilitate the maturation process can be active for only short periods. Data from quantitative electron microscopic analysis are entirely consistent with the idea that control of PTC determines also the assembly and maturation process. With *ts* 1085 this is evident from the direct relationship between time spent at 33° and the number of immature and mature progeny formed (see Fig. 5). Continuation of virus development following shift-up to 39°, albeit limited in extent, presumably involves those immature virions which can utilize the preformed protease factor(s) available to them. This suggestion is in agreement with the finding by Dales and Mosbach (1968), that after rapid suppression of translation with str A, during infection with WT vaccinia, assembly of small quantities of mature, infectious progeny may continue.

Present studies, employing specific protease inhibitors, were useful in characterizing the vaccinia virus protease factor(s). Specific affinity for TPCK and ZPCK implies a chymotrypsin-like active site on the protease. Binding studies employing isotopically labeled ZPCK, although not definitive because dissociation of ³H from the inhibitor molecule was not ruled out, suggest that this protease is a p12.5 virus-specified polypeptide, which is absent from the mature virion itself. In terms of molecular weight, this protein is substantially smaller than p24 MW of chymotrypsin, but approximates the p15 size of the protease identified within avian sarcoma viruses, which is involved in PTC of the virion p76 precursor polyprotein and maturation (von der Helm, 1977).

Synthesis of a viral protein with affinity for ZPCK, under a variety of circumstances permissive or restrictive for PTC and maturation, suggests that it is not the inhibited synthesis of the factor per se

which is connected with arrest in proteolysis. Rather, the basis for the defects may result from (1) unavailability at the time and place required, of nascent protease, as evident with WT infection, or (2) protease interactions with abnormal products and or aberrantly constructed immature particles. Thus, in the case of mutants like *ts* 1085 and 7743, conformational state of each polypeptide precursor within the assembling immature virion and spatial relationship to the envelope could conceivably determine whether PTC can occur. With WT vaccinia infection, it might be assumed that the protease factor has to be in the appropriate place and active state within assembling immature forms to permit PTC to occur.

Combined data from current and previous investigation lead us to the following model of vaccinia virus biogenesis: the envelopes, assembled *de novo* and coated externally by spicules which determine the spherical form of the immature particles (Dales and Mosbach, 1968), are formed around quanta of DNA and polypeptides, among them proteins destined as enzymatic or structural components of the core (Pogo and Dales, 1971; Nagayama *et al.*, 1970; Stern and Dales, 1976b; Stern *et al.*, 1977). The internal components then acquire precise conformation with respect to the envelope, an event which becomes critical for subsequent PTC controlled by the virus-specific protease. The protease or a factor required for activity, with a rapid turnover rate or short half-life, has to be incorporated as a nascent polypeptide into immature particles before the envelope has been completed or sealed. Once PTC is initiated all precursors are processed, although cleavage of one is not contingent on that of another. PTC is obligatory for progression from immature into the mature form. As a consequence PTC, induction of core enzymes, internal differentiation into the core, and lateral bodies and acquisition of infectiousness are temporally coordinated, tightly coupled events.

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