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ORF VIRUS: ITS POLYPEPTIDES AND CELL CYCLE

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ABSTRACT

To obtain information on the biology of orf virus *in vitro*, a cell culture system was established. Orf virus grew best in primary lung and testis cells of ovine and bovine origin compared with MDBK, MDCK and RK 13 cell lines and the highest yields of virus were obtained when growth medium was supplemented with 0.5% lactalbumin hydrolysate (LAH). Over 95% of the progeny virus remained cell-associated and thus virus yields could be enhanced by freezing and thawing and sonication.

The plaque and quantal assay, as compared with fluorescent focus assay, proved to be the simplest means of assaying viral titres although a period of a week was usually required before the results of the assay could be read.

One-step growth experiments showed that orf virus DNA synthesis in primary bovine testis cells began at 4-8 hr post infection (p.i.), rapidly increased from 12-14 hr and continued for up to 30-35 hr after infection. New infectious particles could be detected at 16-18 hr p.i. and rose to a maximum by 48 hr. In comparison, vaccinia virus DNA synthesis in the same cell system began at 3 hr p.i. and was almost complete by 12 hr. Orf virus DNA synthesis was inhibited by cytosine arabinoside, bromouracyldeoxyribose and hydroxyurea but was not inhibited by adenosine arabinoside.

A comparison of the virion polypeptides of 6 New Zealand orf isolates was undertaken. Sodium dodecyl sulphate-polyacrylamide gel electrophoretic analysis of virion polypeptides revealed similar profiles for 5 isolates while the remaining isolate gave a profile distinct from the others. Treatment of virions with nonidet P-40 and 2-mercaptoethanol solubilised 12 polypeptides which were believed to be surface components of the virion. One polypeptide with a molecular weight of 38.5K was believed to be a major component of the characteristic surface tubule of the virion.

The sequential appearance of virus-induced polypeptides in bovine testis cells was also determined. Bovine testis cells were infected at 15-25 pfu/cell and pulse-labelled with ^{35}S-methionine for 2 hr at various times after infection. Only 1 or 2 labelled polypeptides were detected within 8 hr p.i. Most of the virus-coded polypeptides were detected at 10 hr p.i. and thereafter. Most of the detectable polypeptides were also found in labelled whole virus preparations. A virion polypeptide with a molecular weight of 93K was believed to be a product of post-translational protein cleavage or other modification.

Another aspect of the orf virus/cell cycle investigated was the virus-specific cytopathic effect. Early cell rounding, seen as early as 2 hr p.i. in orf virus-infected bovine testis cells, was inhibited by cycloheximide (300 µg/ml) and actinomycin D (5 µg/ml). It was not inhibited when actinomycin D was added at 2 hrs p.i. Also, inhibition of

early cell rounding was seen when α-amanitine (10 μg/ml) or tunicamycin (2 μg/ml) was used. Ultraviolet irradiation of virus inoculum at a dose of 0.38 x $10^3 J/m^2$ reduced early cell rounding by 50% while 50% viral infectivity was reduced by a dose of 0.39 x $10^2 J/m^2$. A comparison of these results with those obtained by others indicate that early cell rounding is mediated by a gene product expressed early in infection, rather than by some toxic virion component(s).

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CHAPTER 1

GENERAL INTRODUCTION

Orf, or contagious ecthyma, is a common poxvirus disease of sheep and goats. Robinson and Balassu (1981) have reviewed the disease and its epidemiology. Control of the disease in sheep is mainly by vaccination using a live virus. Although vaccination is effective, the scab produced at the site of inoculation can be a source of infection.

There is a need for an alternative vaccine which immunises but does not contaminate the environment. The development of recombinant DNA technology, the use of monoclonal antibodies and more recently transfection, which allows direct transfer of DNA into cells, have augmented approaches for the development of more effective, less innocuous vaccines. However, before these advances in technology can be utilised a better understanding of the molecular biology of the virus is needed.

Studies on the molecular biology of poxviruses have concentrated mainly on vaccinia virus; there is limited information available on other members of the poxvirus family.

This thesis was designed to study the biology of orf virus as a preliminary to vaccine studies. Its objectives included:

1. The establishment of a suitable cell culture system for growing orf virus to increase its yield and to establish a system to assay viral infectivity.

2. To determine the time course of appearance of infectious particles in a one-step growth cycle.

3. To determine the time course of DNA synthesis.

4. To study the sequential appearance of virus-induced polypeptides in bovine testis cells.

5. To investigate the early cell rounding (ecr) of orf virus-infected cells and to determine if this is due to a toxic effect or to the effect of an early gene product.

7. To examine cell surface antigens i.e. proteins derived from, and expressed by, the virus during infection and which may play a role in the cell-mediated immune response to orf virus infection.

CHAPTER 2

A REVIEW OF THE MOLECULAR BIOLOGY OF POXVIRUSES

2.1 INTRODUCTION AND SCOPE OF THE REVIEW

The molecular biology of poxviruses has been reviewed by Fenner et al. (1974), Moss (1974, 1978) and Dales and Pogo (1981). Reviews on poxvirus structures and replication were made by Joklik (1966, 1968), McAuslan (1969), Woodson (1968) and Moss (1974). DNA replication has been reviewed by Esteban et al. (1977a, 1979), Esteban and Holowczak (1977, 1980) and Holowczak (1982).

In this review, the molecular biology of poxviruses is discussed. Emphasis is given to rapidly growing research areas such as the mechanism of DNA replication, gene expression, protein synthesis and gene mapping. The roles of plasma membrane modification by poxviruses and surface tubule antigens in immunity to poxviruses are discussed as is the recent use of poxviruses as cloning vectors and their possible use in vaccines.

2.2 CLASSIFICATION

All the poxviruses are similar in size, morphology, DNA structure, DNA size (140-300 kilobase pairs [kbp]) and in their mode of replication within the cytoplasm of the host

cell. Members are grouped into genera based on antigenic cross-reactivity, host range, and size and structure of the DNA. A summary of the subclassification of the family Poxviridae recently approved by the International Committee on Taxonomy of Viruses (ICTV) is presented in Table I (Matthews, 1982). Relatedness within the group can be demonstrated by immunodiffusion and complement fixation (Esposito et al., 1977) or with the fluorescent antibody technique (Takahashi et al., 1959; Woodroofe and Fenner, 1962).

A more recent method of analysing poxviruses, with a view to classification, is by DNA restriction endonuclease analysis. This has been used in the study of orthopoxviruses (Gangemi and Sharp, 1976; Wittek et al.,1977; McCarron et al., 1978; Mackett and Archard, 1979; Esposito et al., 1978, 1981; DeFelippes, 1982), parapoxviruses (Wittek et al., 1980) and orf viruses (Robinson et al., 1982). These studies have shown that some genera are heterogeneous (parapoxviruses) and some are less so (orthopoxviruses). It has also been shown that a degree of heterogeneity exists between and within members of the same species. Nucleic acid hybridization will perhaps provide a more sensitive way of showing the relatedness among the members for further subclassification. This is illustrated in the studies of 38 orthopoxviruses by Esposito and Knight (1985) and parapoxviruses by Gassmann et al. (1985). Using nucleic acid hybridization, it was found that the middle region of the genome is highly conserved within a genus and that the species or strain-variant specific differences were mainly variations in the terminal regions.

Table I. Classification and Salient Features of the Family Poxviridae

Genera	Members	Main Features
Subfamily Chordopoxvirinae:		
Orthopoxviruses	Vaccinia (type species) Buffalopox (buffaloes) Camelpox (camels) Cowpox (cattle, man) Ectromelia (mice) Monkeypox (monkey, man) Rabbitpox (rabbits) Variola (man)	Linear ds DNA, MW = 160 x 10^6. Infectivity of virion is ether resistant. Exhibits extensive serological cross-reactivity and nucleic acid homology. A lipoprotein haemagglutinin is produced in infected cells and becomes incorporated in modified cell membrane. INV and EEV are antigenically different. Host range is usually limited to a single animal host.
Parapoxviruses (Orf subgroup)	Orf (type species) Bovine Pustular Stomatitis Chamois contagious ecthyma Milker's Node (Pseudocowpox)	Linear ds DNA, MW = 85 x 10^6. Virion is ovoid, 220-300 nm x 140-170 nm. External coat and filaments are thicker than in vaccinia virions and are arranged as a regular spiral coil consisting of a single thread. Members show serologicalcross-reactivity infected cells do not produce haemagglutin. Viruses of ungulates that may infect man.
Fowlpox subgroup	Fowlpox (type species) Canary pox Junco pox Pigeon pox Quail pox Starling pox Turkey pox Sparrow pox	One molecule dsDNA, MW = 200 x10^6. Members show serological cross-reactivity. Infectivity is ether-resistant. Type A inclusion bodies contain much lipid, and infected cells do not produce haemagglutinin. Viruses of birds. Mechanical transmission by anthropods is common.
Sheep pox subgroup	Sheep pox (type species) Goat pox Lumpy skin disease	Virions longer and narrower than vaccinia virions; infectivity is ether-sensitive; members show serological cross-reactivity but produce no haemagglutinins. Viruses of ungulates. Mechanical transmission by arthropods occur.
Myxoma subgroup	Myxoma virus (type species) Hare fibroma Squirrel fibroma	DNA MW = 150 x 10^6. Serological cross-reactivity occurs between members. Infected cells do not produce haemagglutinin. Infectivity ether-sensitive. Viruses of liporids and squirrels. Mechanical transmission by arthropods is common.

Table I. Continuation

Genera	Members	Main Features
Swinepox subgroup	Swinepox virus (type species) no other members	Viruses of swine. Limited host range. Infection marked by several types of cytoplasmic inclusion and vacuolation of nuclei.
Unclassified	Carnivorepox	Related to cowpox
	Elephantpox	Related to cowpox
	Molluscum contagiosum (human)	
	Raccoonpox	Probable orthopox
	Tanapox	}Serologically related
	Yaba monkey tumor pox	}
Subfamily Entomopoxvirinae		
Probable genera: Genus A	Melolontha melolontha Celoptera entomopoxvirus Anomala cuparea Aphodius tasmaniae Demoderma boranensis	One molecule dsDNA, MW = 170-240 x 10^6. Virions ovoid, 450 x 250 nm, with one lateral body and unilateral concave core; surface with globular units 22nm in diameter
Genus B	Amsacta moorei (Lepidoptera entomopoxvirus) Aerobasis zilleri Choristoneura diversuma Clorizagrotis auxillaris Orthoptera	One molecule dsDNA, MW = 132.42 x 10^6. G and C content about 26%. Virions ovoid, 350 x 250 nm, with sleeve-shaped lateral body and cylindrical core; surface with globular unit 40nm in diameter.
Genus C	Chironomus limidus Diptera entomopoxvirus	One molecule dsDNA, MW = 165-250 x 10^6. Virions are brick-shaped 320 nm x 230 nm x 110 nm with two lateral bodies and biconcave core.

Derivation of names:
- pox : from plural of pock (old English poc, pocc), pustule or ulcer
- ortho : from Greek *orthos*, 'straight, correct'
- avi : from Latin *avis*, 'bird'
- capri : from Latin *caper, capri*, 'goat'
- lepori : from Latin *lepus, leporis*, 'hare'
- para : from Latin *para*, 'by the side of'
- entomo : from Greek *entomon*, 'insect'
- sui : from Latin *sus*, 'swine'

Reference: Matthews, R.E.F. 1982

2.3 VIRION STRUCTURE

The orthopoxviruses (Fig. 2-1A), as visualised by electron microscopy (EM), are long, ovoid or brick-shaped particles. Each particle is about 170-260 nm x 300-450 nm in size. Using freeze-etching and negative staining, two forms of virus particles can be recognised. One is the M form (mulberry) with a ridged or beaded surface (Dales, 1962; Westwood et al., 1964; Bergoin and Dales, 1971). The other type is a more dense particle called the C form (capsular or clear) and revealing some internal structures. The C forms are considered to be damaged or denatured particles (Westwood et al., 1964). The surface tubular structures of the M particles measure about 9 nm x 90-100 nm and are believed to be of protein composition (Sarov and Joklik, 1972; Stern and Dales, 1976).

In contrast to the orthopoxviruses the parapoxviruses form a morphologically distinct group within the family Poxviridae. The virions are ovoid rather than brick-shaped and they measure about 220-300 nm x 140-170 nm (Nagington and Horne, 1962). Two forms of the virion (Fig. 2-1B) are recognisable by EM of negatively stained preparations. The surface of the Type $1_{\Lambda}^{(M)}$ particle consists of a single tubular or thread-like structure, 10-20 nm x 800 nm, spirally wound around the virion in a left-hand screw arrangement (Nagington et al., 1964). The superimposed image of the upper and lower surfaces of the particle produce a "criss-crossing" pattern of the tubule giving the "ball of wool" appearance of the virus (Peters et al., 1964; Nagington et al., 1964).

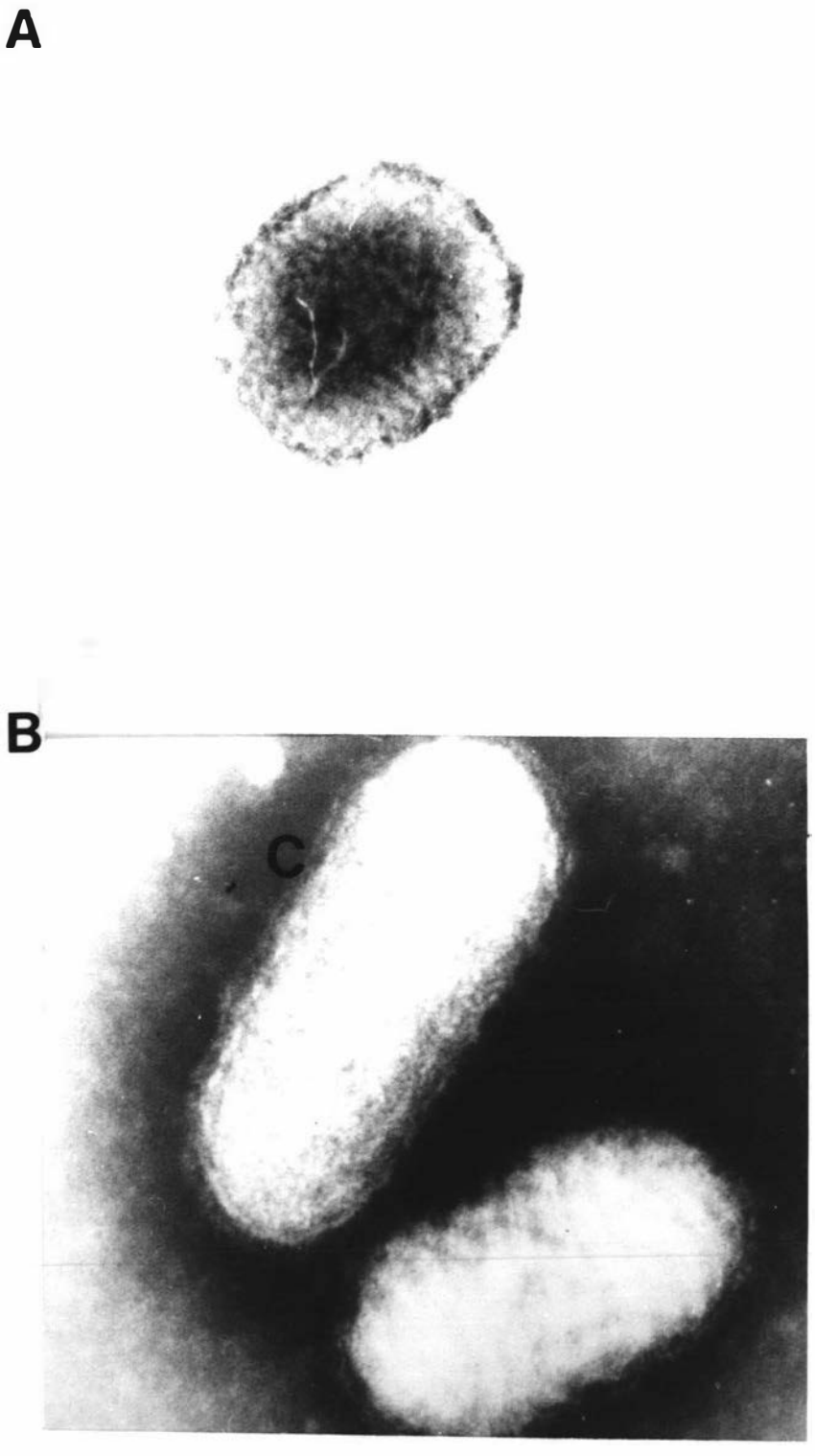


Fig. 2-1. **Electron micrograph of poxvirus particles.** (A) orthopoxvirus; (B) parapoxvirus; (C) Clear form; (M) Mulberry form.

Controlled degradation of purified pox virus particles with non-ionic detergents separates the tubular structures and envelope from the core (Easterbrook, 1966; Sarov and Joklik, 1972; Stern and Dales, 1976; Thomas et al., 1980; Buddle et al., 1984).

2.4 CHEMICAL COMPOSITION

Highly purified particles can be obtained by zonal sedimentation in a sucrose density gradient (Joklik, 1962b; Zwartouw et al., 1962), equilibrium centrifugation in cesium chloride (Planterose et al.,1962), potassium tartrate (Pfau and McCrea, 1963) or sodium diatrizoate (Esposito et al., 1978). Poxvirus particles are composed of approximately 92% protein (14.7% nitrogen), 3.2% DNA, 1.2% cholesterol, 2.1% phospholipids, and 1.7% neutral fat (Zwartouw, 1964). Table II shows the chemical composition of vaccinia virus.

Nucleic Acid

Poxvirus particles contain a single molecule of linear, double-stranded DNA which comprises about 3.5% of the weight of the particle in the case of vaccinia virus (Joklik, 1962a; Zwartouw, 1964). Poxvirus DNAs are the largest nucleic acid molecules among the vertebrate viruses. The sizes of the different poxvirus DNAs are listed in Table III.

Table II. Chemical Composition of Vaccinia Virus as Percentage of Dryweight

Substance	References		
	Smadel & Lavin, 1940 Hoagland *et al*, 1940 Smadel & Hoagland, 1942	Zwartouw, 1964	Joklik, 1966
Physical component			
Nitrogen	15.3	14.7	
Phosphorus	0.57	0.49	
Sulphur	-	0.76	
DNA	5.6	3.2	5.25
Cholesterol	1.4	1.2	
Phospholipid	2.2	2.1	
Neutral fat	2.2	1.7	
Trace material			
Carbohydrate	2.8	0.2	
Copper	0.05	0.02	0.02
Riboflavin	1.3×10^{-3}	0.5×10^{-3}	
Biotin	present	1.3×10^{-5}	
RNA	trace	0.1	0.1

Source: Dales and Pogo, 1981

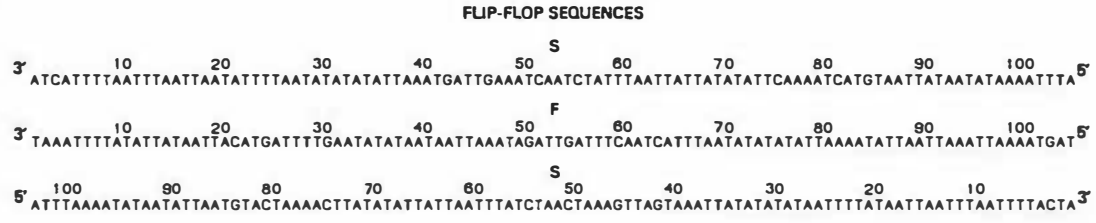
Table III. Sizes of Poxvirus DNA

Virus	Intact full length contour measurement	MW x 10^6	Kilobase pairs	References
Vaccinia (Elstree)		123	193	Geshelin and Berns, 1974 Wittek *et al.*, 1977
Vaccinia (WR)	62.27 μm[a]	120-130[b]	180-193[b]	[a] Esteban *et al.*, 1977 [b] Geshelin and Berns, 1974
Rabbitpox (Utrecht)		118	184	Wittek *et al.*, 1977
Red cowpox (Brighton)		145[a]	222[b]	[a] Archard and Mackett, 1979 [b] Esposito and knight, 1985
White cowpox		128		Archard and Mackett, 1979
Smallpox-like Disease		195		Esposito and Knight, 1985
Fowlpox	100 μm	190-300		Hyde *et al.*, 1967
Shope fibroma	80.3 μm	153		Jacquemont *et al.*, 1972
Molluscum contagiosum	50.02 μm	118		Parr *et al.*, 1977
Milker's node		85-87[a]	141.8-147[b]	[a] Thomas *et al.*, 1980 [b] Gassman *et al.*, 1985
Orf	46.3 μm[a]	88.8[a]	138.7-140[b]	[a] Robinson *et al.*, 1982 [b] Gassman *et al.*, 1985
Stomatitis papulosa		86[a]	129-148[b]	[a] Menna *et al.*, 1979 [b] Gassman *et al.*, 1985
Variola			181-183	Esposito and Knight, 1985
Ectromelia			211	Esposito and Knight, 1985
Monkeypox			192-198	Esposito and knight, 1985
Monkeypox (whitepox)			179-205	Esposito and Knight, 1985
Camelpox			196	Esposito and Knight, 1985
Tatera poxvirus			185	Esposito and Knight, 1985
Racoon			227	Esposito and Knight, 1985

The two strands of the DNA are cross-linked at both ends to form a continuous polynucleotide loop (Baroudy et al., 1982). The cross-links were first observed by Szybalski et al. (1963) who noted that when purified fowlpox DNA was heated and rapidly cooled, a proportion of the DNA would spontaneously reanneal. This observation was also reported by Jungwirth and David (1967) for cowpox and vaccinia virus DNA. Electron microscopic evidence of the presence of cross-links was presented by Geshelin and Berns (1974) in their study of vaccinia DNA and electron microscopy has shown continuous, unbroken circles in denatured molluscum contagiosum DNA suggesting the presence of terminal or near-terminal cross-links (Parr et al., 1977). There are two forms of the loops or "hairpins" at each end of the genome. They are characterised as F (fast) or S (slow) loops when analysed by non-denaturing polyacrylamide gel electrophoresis. Both The F and S loops are 140 nucleotides long, incompletely base-paired and are inverted and complimentary to each other (flip-flopped) as shown in Fig. 2-2 (Baroudy et al., 1982).

Restriction endonuclease analysis (Wittek et al., 1978), cross-hybridization (Wittek et al., 1977; Mackett and Archard, 1979) and electron microscopic studies (Garon et al., 1978) show that the vaccinia virus genome contains an inverted terminal repetition (ITR). This confirms earlier observations of Geshelin and Berns (1974) and Parr et al. (1977) that the two ends have similar base compositions. The sizes of the ITRs of several poxviruses are shown in Table IV. In vaccinia virus these ITRs are about 10 kbp

A.



B.

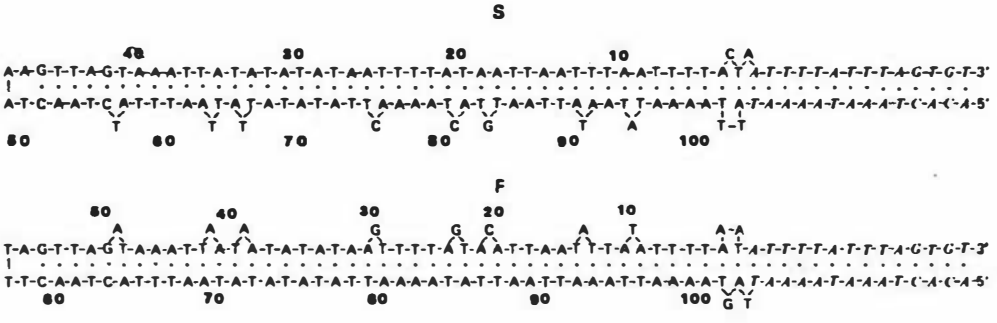


Fig. 2-2. Terminal loop structures of vaccinia virus DNA.
A. Inverted Complementary (Flip-Flop) Sequences. The 104 nucleotide divergent segments of the F and S forms of the terminal loop are shown in 3' to 5' or 5' to 3' direction as indicated.
B. The F and S terminal loop forms are shown with maximum base-pairing. The nucleotides of the divergent 104 nucleotides are numbered. A portion of the contiguous identical sequence is shown in italics on the right (From Baroudy et al., 1982).

(Garon et al., 1978; Wittek et al., 1978) and have been shown to contain tandem repeated sequences (Grady and Paoletti, 1977; Pedrali-Noy and Weissback, 1977; Wittek et al., 1978). Wittek and Moss (1980) found two sets of 70 base pairs (bps) tandem repeats. The first set of 13 repeats is separated from the second set of 17 repeats by a unique 435 bp sequence (Fig. 2-3A). Further sequence studies (Baroudy et al., 1982) (Fig. 2-3B) show the first set of 70 bp repeats to start 87 bps from the proximal end of the loop and that the 70 bps tandem repeats contain a 13 bp internal redundancy. From the same study, an additional repeat was found in the second set and the size of the intervening sequence between the two sets was 325 bp. Some degeneracy in the nucleotide sequences were observed in at least the first 3 repeats of the second set.

The function of the ITR is not known but may be important in the reannealing process of single-stranded DNA during replication (Wittek and Moss, 1980). Electron microscopic studies show that early mRNAs are transcribed from some sequences in the ITR and translation studies show that the RNAs code for polypeptides (Wittek et al., 1980). The function of these polypeptides is unknown.

Restriction endonuclease analysis of poxvirus DNA has shown a high degree of genetic heterogeneity between and within genera (Wittek et al., 1977; McCarron et al., 1978; Robinson et al., 1982). Restriction maps of orthopoxvirus genomes (Fig. 2-4) show that the internal or central part is highly conserved. Differences or variations occur most commonly

Table IV. Sizes of Poxvirus Inverted Terminal Repeats (ITR)

Virus	Size		
	MW x 10^6	Kilobasepairs	References
Vaccinia (WR)	6.8	10.3	Garon *et al*, 1978 Wittek *et al*, 1980
Vaccinia (Elstree)	7.4-8.0		Wittek *et al*, 1978
Rabbitpox (Utrecht)	3.4-3.6		Wittek *et al*, 1978
Red cowpox (Brighton)	6.3-6.6		Archard and Mackett, 1979
Monkeypox (Congo or Denmark España)	5.6-6.1		Mackett and Archard, 1979
Ectromelia (Hamstead and Moscow)	3.1-3.4[a]	6.5[b]	[a] Mackett and Archard, 1979 [b] Esposito and Knight, 1985
Variola		.5	Esposito and Knight, 1985
Tatera		4.5	Esposito and Knight, 1985

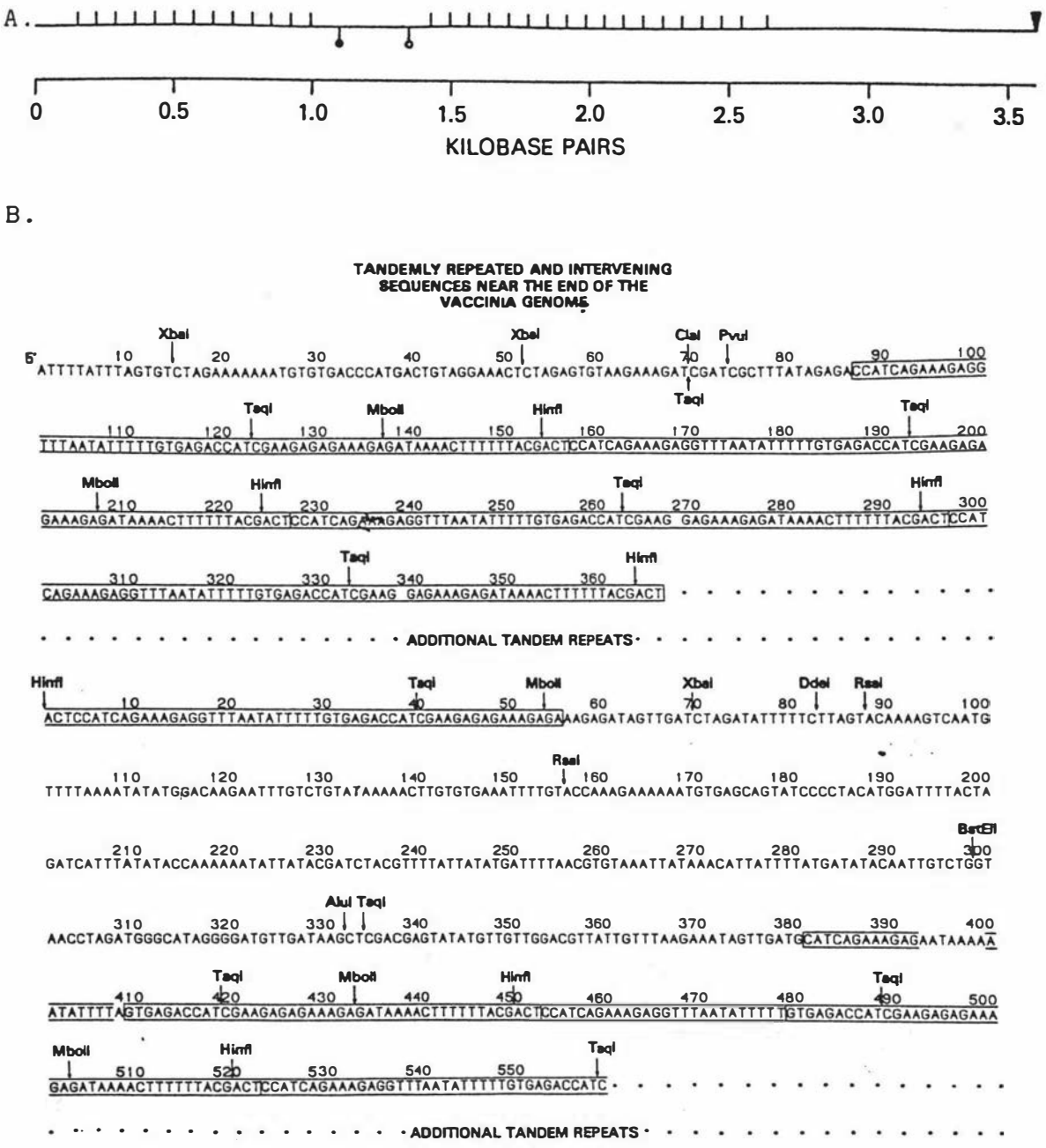
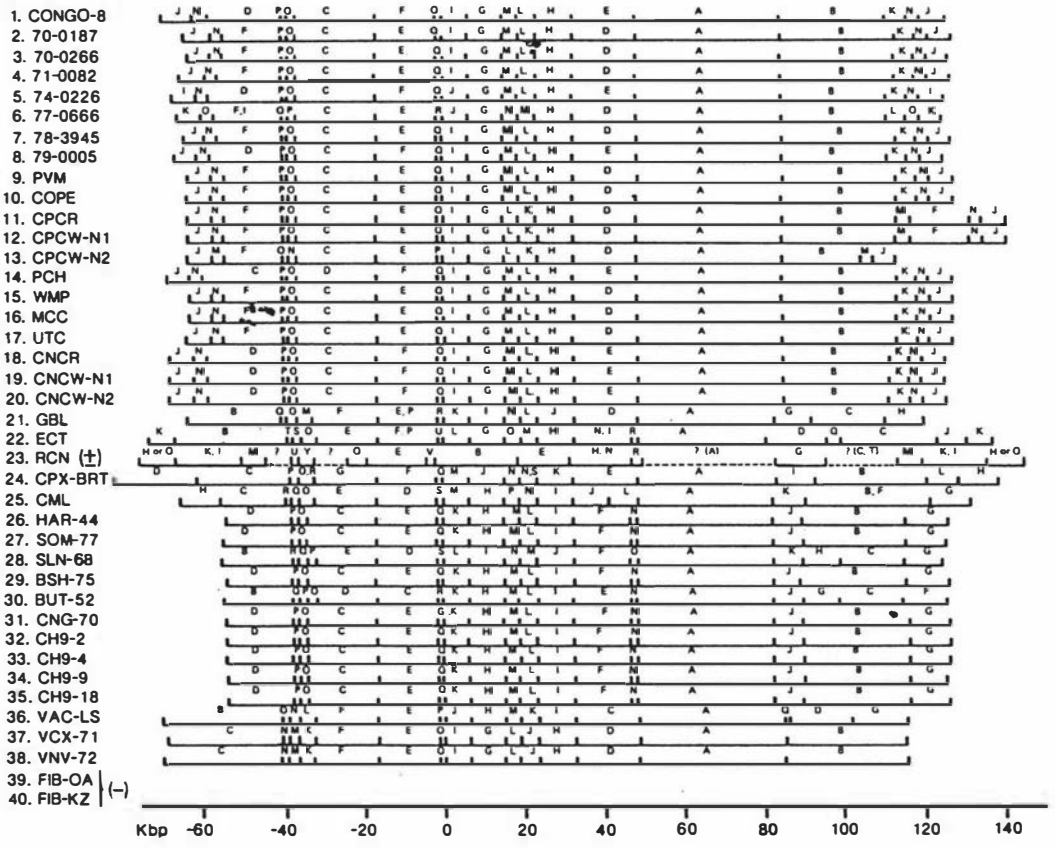


Fig. 2-3. Inverted terminal repitition of the vaccinia virus genome.
A. Location of HinfI, AluI, DdeI and SalI Sites within the terminal region of vaccinia virus DNA. (|) HinfI; (●) DdeI; (○) AluI; (▼) SalI. (From Wittek and Moss, 1980).
B. Nucleotide sequence of cloned terminal tegion of the vaccinia genome. (Top) Terminal sequence and four complete tandem repeats of first set. Tandem 70 bp repeats are boxed. (Bottom) Sequence of the region between the sets of repeats flanked by the last repeat of the first set and the first few repeats of the second set. The first nucleotide is the HinfI site in the last repeat of the first set. (From Baroudy et al., 1982).



1 - 20	Monkey pox	23	Racoon	26 - 35	Variola
21	Tatera	24	Cowpox	36 - 38	Vaccinia
22	Ectromelia	25	Camelpox	39 - 40	Leporepox

Fig. 2-4. Orthopoxvirus DNA HindIII maps: Physical arrangement of HindIII genome DNA of 38 orthopoxviruses. The consecutive order of the digest fragments was determined by cross-hybridization with cloned DNA fragments of orthopoxviruses, the HindIII DNA maps of which already were established. (From Esposito and Knight, 1985).

near or within the ITRs (Wittek et al., 1977; Muller et al., 1977; Esposito et al., 1978, 1981; Mackett and Archard, 1979; Dumbell and Archard, 1980; Moyer et al., 1980; Schumperli et al., 1980; Esposito and Knight,1985). However, type-specific differences in the central region (Mackett and Archard, 1979; Schumperli et al., 1980) and similarities in the heterologous regions also occur between rabbitpox and vaccinia virus (Schumperli et al., 1980).

Restriction endonuclease maps of the parapoxviruses are shown in Fig. 2-5. The fragment patterns vary considerably confirming the presence of genetic heterogeneity among the parapoxviruses. Although the viruses studied could fall into groups of isolates with similar fragment patterns, isolates of such different groups showed little or no similarities. Thus classification of a new isolate into such group on the basis of restriction endonuclease patterns alone would be difficult.

Proteins

Pox virions contain large numbers of polypeptides and several enzymes. The use of strong denaturing and reducing agents, such as guanidine HCl and sodium dodecyl sulphate (SDS) and 2-mercaptoethanol to break the disulfide bonds, enables a study to be made of structural and non-structural polypeptides coded for by the virus.

Early studies of vaccinia virus (genus Orthopoxvirus) by Holowczak and Joklik (1967) showed 17 isotope-labelled

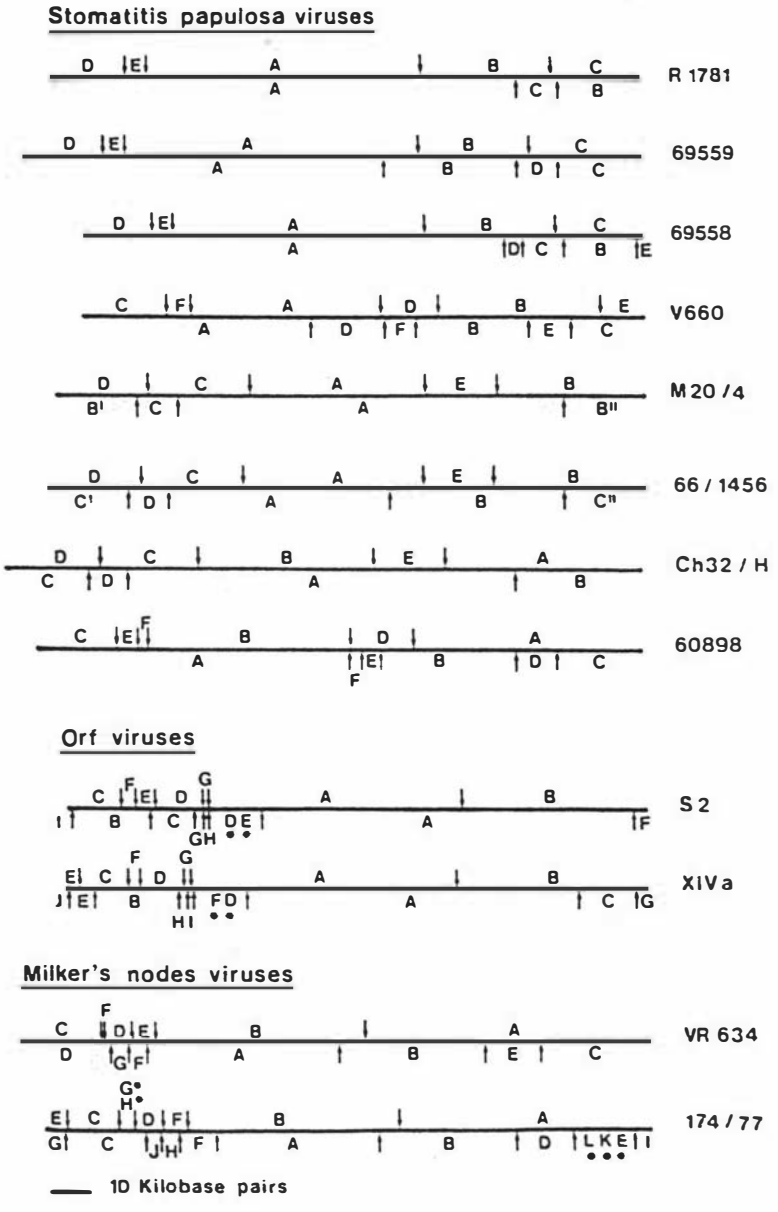


Fig. 2-5. Restriction maps of 12 parapoxvirus isolates. The position of EcoRI (↓) and HindIII (↑) restriction sites are indicated. * shows the the position of fragments for which the precise position has not been determined. (From Gassmann et al., 1985).

polypeptides as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Using longer, cylindrical gels, Sarov and Joklik (1972) detected 30 stained or isotope-labelled polypeptides. Similar complexity was observed in fowlpox virus (genus Avipoxvirus) by Obijeski et al. (1973) and in Yaba monkey tumour viruses (Fenger and Rouhandeh, 1976). Improvements in the technique such as the discontinuous buffer system of Laemmli (1970) have increased the number of polypeptides detected. McCrea and Szilagyi (1975) reported 40 major polypeptides while Stern and Dales (1976) detected 55-60 ^{35}S-methionine-labelled polypeptides (Fig. 2-6A). In 1979 Essani and Dales using 2-dimensional SDS-PAGE resolved about 110 polypeptides (Fig. 2-6B). This was still an underestimation since polypeptides with molecular weights (MWs) higher than 100k and lower than 9k that were resolved in a single dimension gel were not resolved in the two-dimensional gel.

The MWs of these polypeptides range from 200,000 to 10,000. Detergent treatment showed that at least 5 of these polypeptides are located on or near the surface of the intact virion and 17 are associated with the core (Fig. 2-7A). Two of the polypeptides are glycopolypeptides containing glucosamine and 6 are phosphoproteins. One phosphoprotein, P11 (MW = 11K), is associated with the core (Sarov and Joklik, 1972; Pogo et al., 1975). Polypeptide P58 (Stern and Dales, 1976; Sarov and Joklik,1972), with a MW of 58,000, is believed to be a major component of the surface tubules (Fig. 2-7B). Some extracellular virus polypeptides, believed to be glycoproteins, were not present in the intracellular

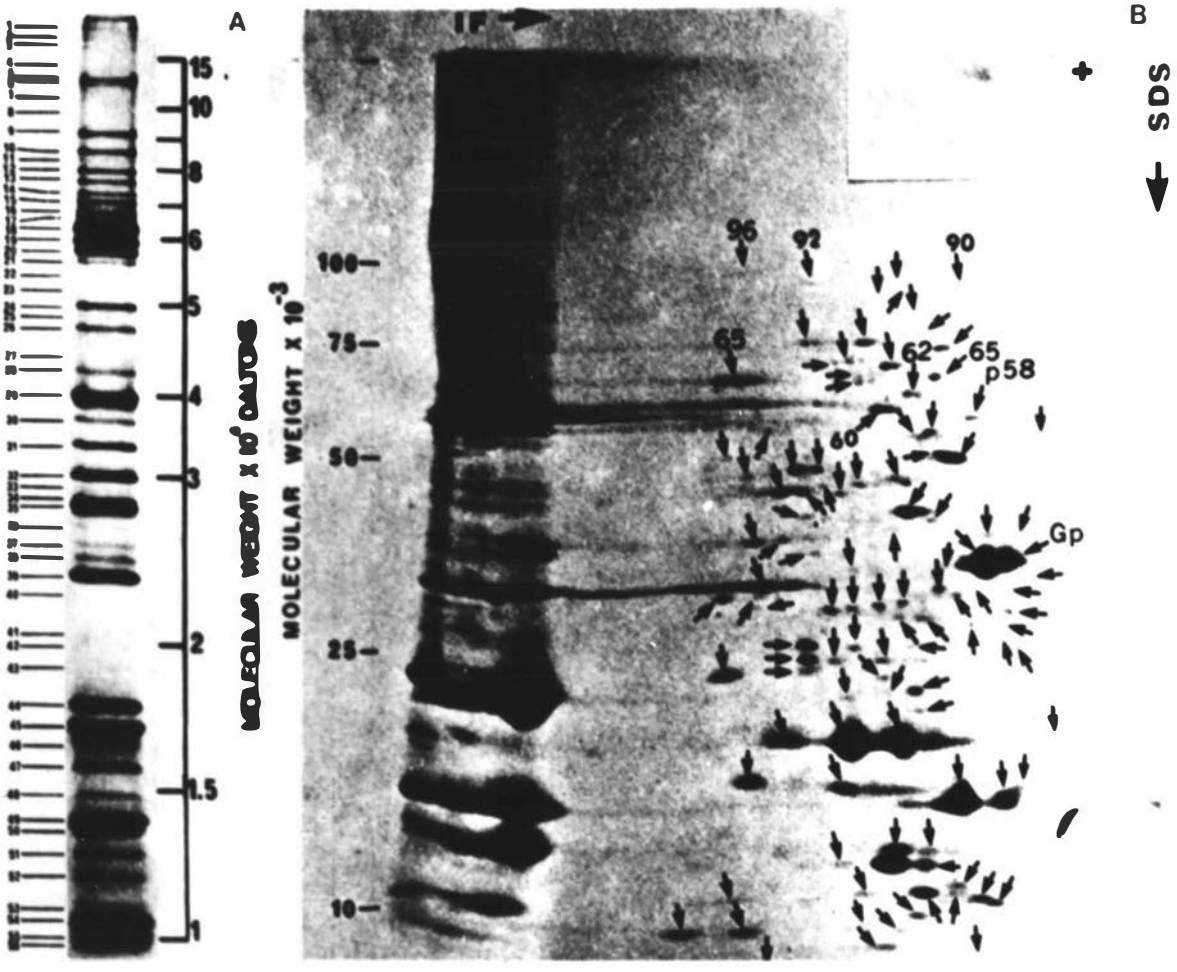


Fig. 2-6 *A* Autoradiogram of one-dimensional SDS-PAGE of [^{35}S]methionine-labeled pure vaccinia virus. All identifiable bands are numbered on the left and the molecular weight scale is shown on the right. About 20 μg of virus protein containing 20,000 cpm was applied to 11% acrylamide slab gels. The numbers on the left indicate the numerical order of the bands

B Autoradiogram of a two-dimensional separation of vaccinia virion polypeptides. The large panel is a preparation of whole virions, and in the inset, lower right, a sample of purified 58 K surface tubular elements (STE). Pure [^{35}S]methionine-labeled virus was dissociated and aliquots, each containing 15 μg of protein and 170,000 cpm, were introduced into isoelectrofocusing (IF) gels at the cathode. Electrophoresis was conducted for 6000 V · hr, following warming to 37° for 60 min to equilibrate the system. The IF gels were then placed onto slab gels and electrophoresis in the second dimension was carried out for 5 hours at 25 A. Arrows indicate individual polypeptides (from Essani and Dales, 1979)

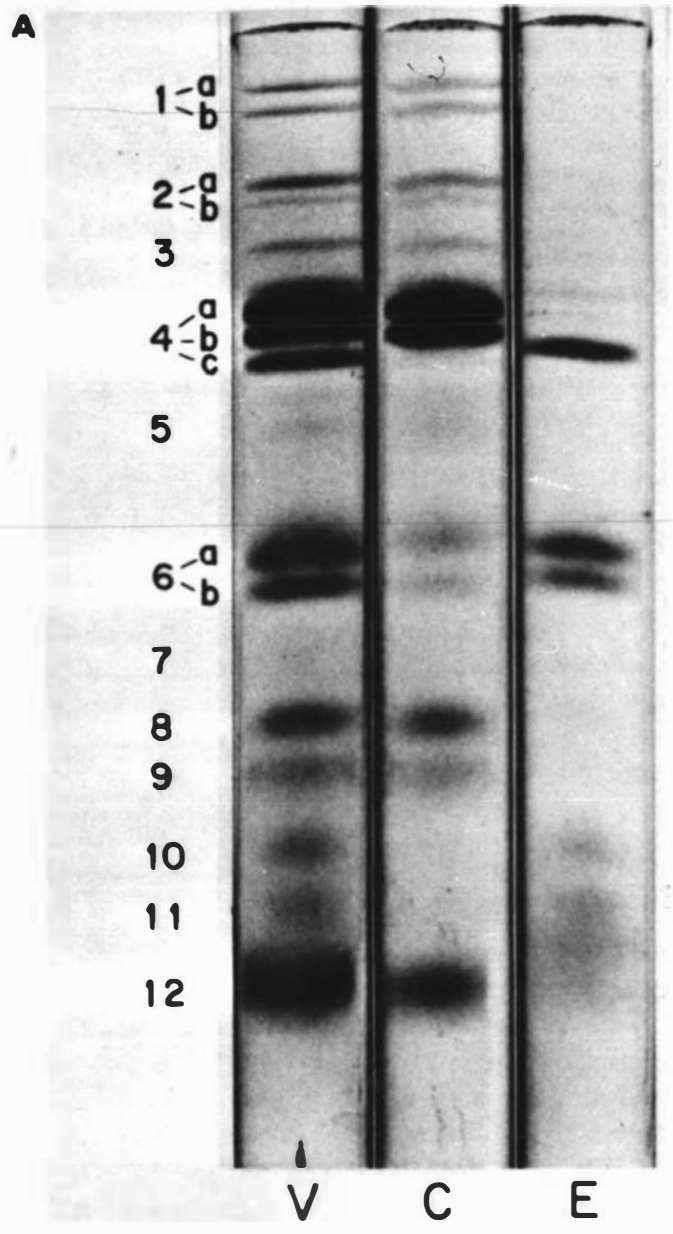


Fig. 2-7 Comparison of polypeptides from whole vaccinia virions and fractions obtained by detergent and mercaptoethanol treatment. Polypeptides were separated by polyacrylamide gel electrophoresis in sodium dodecylsulfate; V, virions; C, cores; E, envelope.

(From Moss et al., 1973).

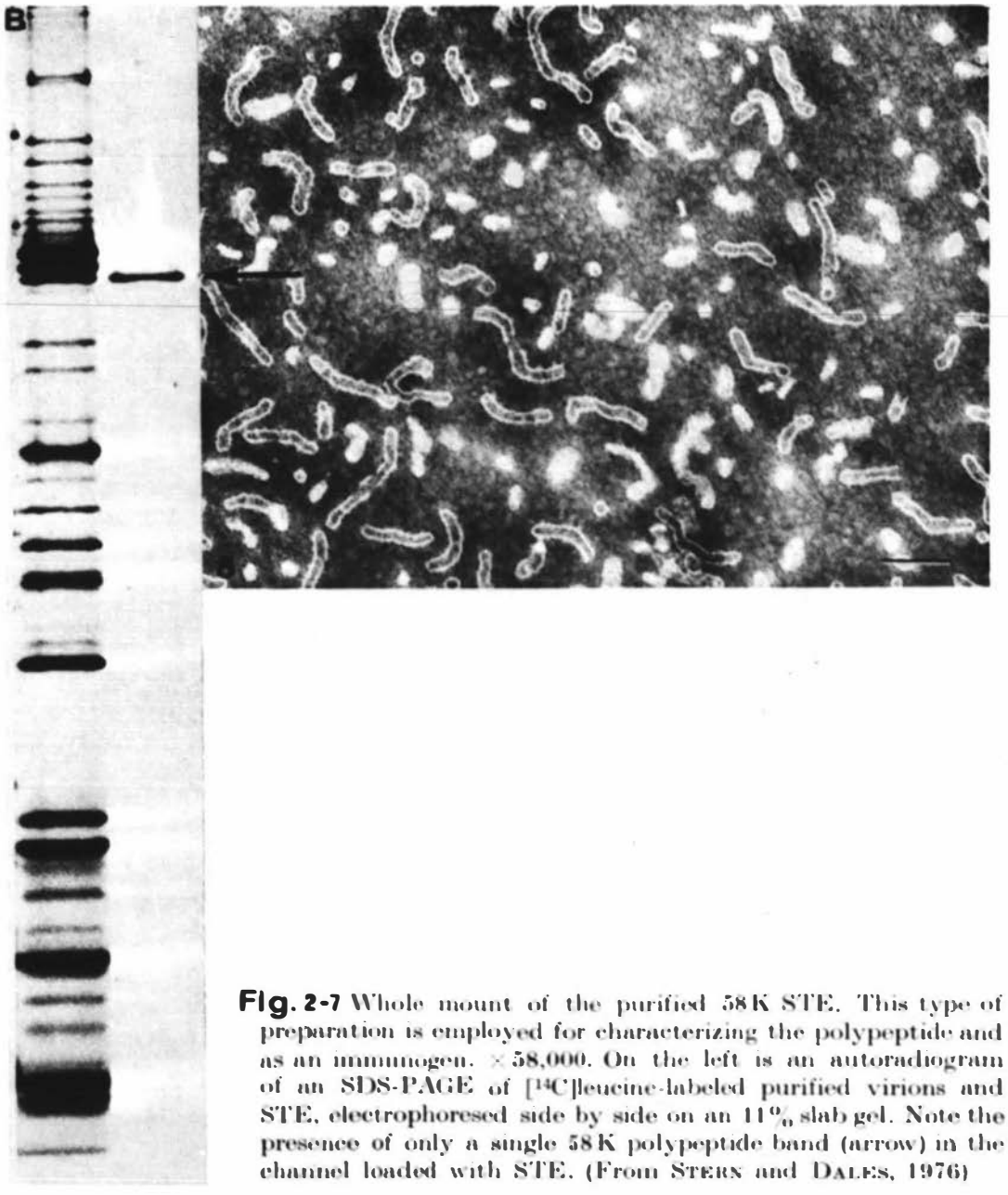


Fig. 2-7 Whole mount of the purified 58K STE. This type of preparation is employed for characterizing the polypeptide and as an immunogen. ×58,000. On the left is an autoradiogram of an SDS-PAGE of [^{14}C]leucine-labeled purified virions and STE, electrophoresed side by side on an 11% slab gel. Note the presence of only a single 58K polypeptide band (arrow) in the channel loaded with STE. (From Stern and Dales, 1976)

virus (Payne, 1978; Hiller et al., 1981; Mallon and Holowczak, 1985). Table V shows the location of the polypeptides in the vaccinia virion and their functions where these are known.

Milker's node virus (Parapoxvirus genus) has about 40 ^{35}S-methionine-labelled polypeptides with MWs ranging from 200,000 to 10,000. Nine of these are presumed to be surface envelope polypeptides as they are released when virions are treated with non-ionic detergents and reducing agents. A polypeptide with a MW of 45,000 was found to be a component of the surface tubule (Thomas et al., 1980).

Buddle and associates (1984) have detected at least 31 polypeptides in orf virus. Fourteen of these were released by detergent treatment. Three polypeptides with MWs of 37,000, 39,000 and 45,000, either singly or in pairs, were components of the surface tubule. It was suggested by Buddle et al. (1984) that the polypeptide components of the surface tubule might be useful in classifying viruses within the poxvirus family.

Post-translational modification of proteins has been detected in vaccinia virus. Two amino acid modifications can occur in some structural proteins during maturation and/or assembly of virus. Both glycosylation and phosphorylation have been recorded. At least one (Moss et al., 1971; Holowczak, 1970; Garon and Moss, 1971) or possibly 2 proteins (Sarov and Joklik, 1972) contain a sugar side chain which consists mainly of glucosamine (Garon and Moss, 1971). One major and

Table V Structural and Functional Identity of Some Vaccinia Virion Polypeptides

No	Molecular weight x 10^3	Position in Virion Core	Inter-mediate	Surface	Associated biological structure or function
1	~250	+			
2	~200	+			
3	158				
4	145				
5	138	+			RNA polymerase subunit
6	131	+			RNA polymerase subunit
7	120				
8	101				
9	97	+			guanyl methyl transferase subunit
10	89	+			polynucleotide 5' phosphatase subunit
11	86				
12	84.5	+			
13	79	+			RNA polymerase subunit
14	76				
15	75			+	
16	72.5				
17	70	+			Nucleotide phosphohydrolase I & II
18	63.5	+			Protein kinase
19	60	+			Nucleotide phosphohydrolase I & II guanylyl transferase with 7-methyl-transferase
20	58			+	surface tubular element
21	57	+			poly A polymerase subunit
22	55				
23	53.5				
24	51	+			poly A polymerase subunit(?)
25	50	+			SS endo- and SS exonuclease
26	48				
27	45				
28	43				
29	41.5		+		glycopeptide
30	38		+		glycopeptide

Table V (Continuation)

No	Molecular weight x 10^3	Position in Virion Core	Inter-mediate	Surface	Associated biological structure or function
31	36.5	+			Poly A polymerase subunit
		+			RNA methylase
32	35	+		+	DNA nicking-closing enzyme Poly A polymerase subunit(?) RNA polymerase subunit
33	31	+			guanine methyl transferase subunit
34	29.5				
35	28.5			+	
36	27.5	+			
37	26.5	+			polynucleotide 5'-phosphatse subunit
38	25.5				
39	24.5				
40	23				DNA nicking-closing enzyme component
41	21.5				
42	20				
43	19.5				
44	~18	+			
45	~17	+			
46	~17	+			
47	16				
48	15				
49	~14			+	
50	~14				RNA polymerase subunit
51	13				
52	12				
53	~11	+			Phosphoprotein
54	~10.5	+			
55	~10	+			
56	~10				

SOURCE: Dales and Pogo, 1981.

a few minor structural polypeptides can be labelled with ^{32}P (Sarov and Joklik, 1972). Polypeptide P11 (MW = 11,000) is a major core polypeptide and is associated with the DNA (Pogo et al., 1975). These polypeptides contain phosphoserine but not phosphothreonine.

Virion-Associated Enzymes

Several enzymatic activities have been found associated with the virus core. The first of these enzymes to be discovered was the DNA-dependent RNA polymerase (Kates and McAuslan, 1967b; Munyon et al., 1967). To date 12 to 13 enzymatic activities have been detected and at least 5 are involved in the transcription and modification of mRNAs. These enzymes are classified as either "early" or "late" depending on whether activities are detected before or after the onset of DNA synthesis (see Table VI). A more detailed discussion appears below (Section 2.5.).

Lipids

The envelope of vaccinia virus consists mainly of phospholipids derived from the host cell (Stern and Dales, 1974; 1976). The lipid accounts for 2.1% of the total dry weight and 1.7% of this is neutral fat and 1.2% cholesterol (Zwartouw, 1964). The phospholipid is mainly lecithin (Dales and Mossbach, 1968). The lipid composition is similar to that of the host cell membrane except for a lower amount of

Table VI. Enzymes Associated With the Core of Vaccinia

Activity	Class	MW	References
A. Related to transcription and modification of RNA			
1. DNA-dependent RNA polymerase	Late	(140k; 130k; 37k; 35k; 31k; 22k; 17k)	Kates and McAuslan, 1967; Munyon *et al.*, 1967; Baroudy and Moss, 1980.
2. Endoribonuclease			Kates, 1970; Paoletti and Lipinskas, 1978.
3. Polyadenelate polymerase (terminal riboadenylate transferase)	Early	80k (51k; 35k)	Kates and Beeson, 1970; Moss *et al.*, 1973; Moss *et al.*, 1975.
4. Polynucleotide 5'-triphosphate		113k (90k; 26k)	Tutas and Paoletti, 1978.
5. mRNA guanylyltransferase	Early	127k	Martin and Moss, 1975; Moss *et al.*, 1976.
6. mRNA(guanine-7) methyltransferase	Early	(95k; 31k)	Martin and Moss, 1975; Moss *et al.*, 1976; Monroy, 1978.
7. mRNA methylase	Early		Wei and Moss, 1974; Barbosa and Moss, 1978.
B. Related to DNA and its functions			
1. SS DNAse, exonuclease pH 4.5	Late	100k (50k)	Pogo and Dales, 1969; Pogo and O'Shea, 1977.
2. SS DNAse, endonuclease	Late	100k	Pogo and Dales, 1969; Pogo and Dales, 1977.
3. DNA nicking-closing enzyme	Early	(35k; 24k)	Bauer, 1977; Lakritz, 1985.
C. Neucloside triphosphate phosphohydrolase I		61k	Wei and Moss, 1974
II		68k	Paoletti and Moss, 1974
D. Kinase			
1. Protein kinase		62k	Kleiman and Moss, 1973.
2. Phosphate polyribonucleotide kinase			Spencer *et al.*, 1978.
E. Alkaline protease			Arzoglou *et al.*, 1979.

( ) = subunits

phosphatidylethanolamine (Stern and Dales, 1974). In fowlpox, lipids account for 34% of the total dry weight (Randall et al., 1964) and contains squalene (White et al., 1968).

Trace Substances Associated with the Virion

Trace amounts of RNA can be demonstrated in the virion (Zwartouw, 1964; Joklik, 1966). The source of this RNA is unknown but it is either incidentally incorporated during assembly or may be virus-related transcripts that have not become dissociated from the DNA before packaging in the virus core (Roening and Holowczak, 1974). Copper ions were also found on the surface of highly purified virions (Smadel and Hoagland, 1942).

2.5 GROWTH CYCLE

During a natural infection, poxviruses produce either a generalised, often fatal disease or a mild surface infection. For example, smallpox, mousepox, rabbit myxomatosis, and sheep pox viruses produce a generalised infection of the host while Shope fibroma, Yaba tumour, milker's node and orf viruses produce a localised skin infection (Fenner et al., 1974). What determines whether a particular virus becomes generalised or not remains unknown. Poxviruses also vary considerably in their replication times in cell culture. For instance, vaccinia produces newly replicated infectious virus

within 6 hr of infection while other poxviruses are much slower. There appears to be no correlation, however, between generalised versus surface infection and speed of replication.

The growth cycle of vaccinia in cell cultures is a very rapid event. At a very high multiplicity of infection (50-100 pfu/cell), DNA synthesis begins 1.5-2 hr after infection and is nearly complete by 5 hr, at which time new infectious particles can be detected (Joklik and Becker, 1964; Esteban and Holowczak, 1977b; 1977c). Fig. 2-8 shows the time course of vaccinia viral DNA synthesis and the appearance of new infectious particles.

The synthesis of milker's node virus (MNV) DNA starts between 4 and 8 hr after infection of primary bovine kidney cells. This continues for up to 30-35 hr throughout the growth cycle. Infectious particles are detected 20 hr p.i. (Thomas et al., 1980). Hessami et al. (1979) detected new infectious particles in orf virus-infected bovine spleen cells at 8 hr p.i. and a maximum titre at 36 p.i. Slow rates of DNA synthesis have also been reported in other poxviruses: fowlpox, starts at 12 hr p.i. with a rapid increase up to 24 hr (Gafford et al., 1972); Shope fibroma, starts at 6 hr, peaks at 12 hr and continues to 25 hr (Ewton and Hodes, 1967). In Yaba monkey tumour virus, DNA synthesis starts at 3 hr, reaches a peak at 20-30 hr p.i. and infectious particles are first detected at 15-20 hr and reach a maximum at 70-80 hr (Yohn et al., 1970). Despite these differrent rates of growth in cell culture the overall pattern of

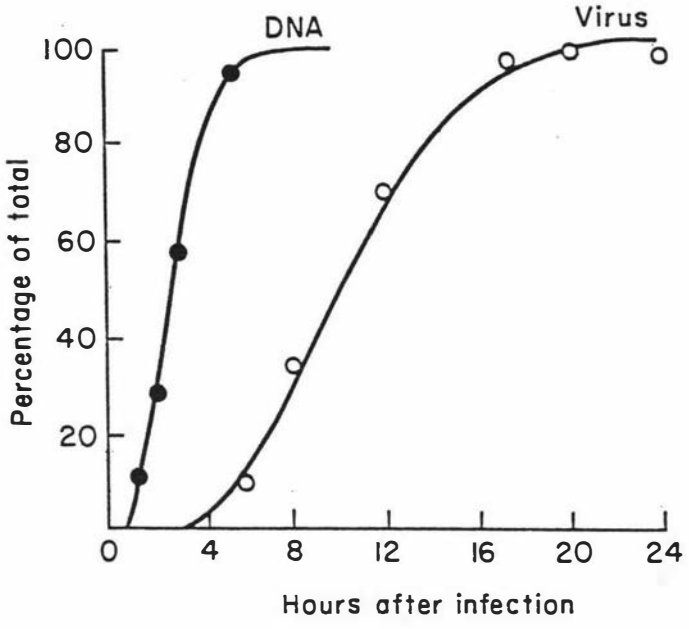


Fig. 2-8. Biogenesis of vaccinia virus: Time course of DNA synthesis and appearance of infectious vaccinia virus in infected Hela spinner cells. (From Fenner et al., 1974).

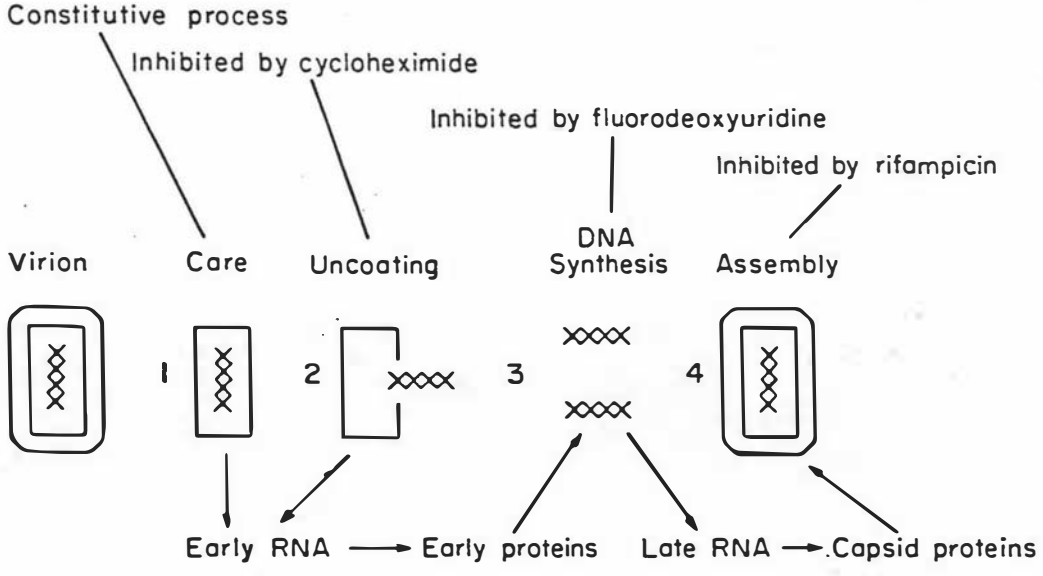


Fig. 2-9. Pattern of multiplication of poxviruses. (From Fenner et al., 1974).

multiplicaton is the same for all poxviruses examined, as shown in Fig. 2-9.

Adsorption, Penetration and Uncoating

Attachment of vaccinia virus to host cells does not appear to involve specific cellular receptors. However, it was shown in the recent studies of Eppstein et al. (1985) that occupancy of the epidermal growth factor (EGF) receptor inhibits vaccinia virus infection. Twardzik et al. (1985) isolated a protein with a MW of 25K from the supernatant of vaccinia virus-infected cells which was shown to bind to the EGF receptor and to have a mitogenic activity. However, they found no serological similarity between it and EGF and no sequence study to show its homology to EGF was performed. Stroobant et al. (1985) reported the isolation and identification of a vaccinia virus growth factor (VVGF) from infected cells; VVGF was also secreted into the medium. Two polypeptides were identified from infected cells with MWs of 28K and 23K; the secreted polypeptide had a MW of 23K. This VVGF was found to be serologically related to, and to bear some homologous sequences to, mouse EGF. It also binds to EGF receptors and stimulates its autophosphorylation. Amino acid sequences deduced from the vaccinia DNA sequence showed that the 23K polypeptide is a processed form of the predicted vaccinia virus polypeptide encoded in the ITR region of the genome. However, unlike the EGF and transforming growth factor-1 (TGF-1) it is glycosylated which may account for the presence of the 28K dalton entity in infected cells.

Earlier studies by Blomquist et al. (1984), Brown et al. (1985) and Reisner (1985) revealed a 19K protein which, from sequencing studies by computer search, was deduced to bear homology to TGF-1 and EGF (Fig. 2-10). The function of the 19K protein is unknown but it was suggested by Reisner (1985) that it has an EGF-like function. This polypeptide is translated early during viral infection and the gene is located at the hypervariable or terminal region of the vaccinia virus genome (Venkatesan et al., 1982). If this polypeptide is located on the surface of the virion it might mediate binding of the virus to the cell expressing the EGF receptor. If, on the other hand, a soluble EGF-like peptide is released from an infected cell, neighbouring cells might be stimulated to proliferate (Brown et al., 1985). This latter speculation has been confirmed by the studies of Stroobant et al. (1985). Eppstein et al. (1985) did not obtain complete inhibition of virus infection by EGF or a homologous peptide. This suggests that EGF receptor binding is not the only pathway by which vaccinia virus might infect a cell. Furthermore, Stroobant et al. (1985) reported that vaccinia virus can infect mouse NR-6 cells which lack detectable EGF receptors.

Under optimal conditions, adsorption of orthopoxvirus occurs very rapidly and is temperature independent. About half of the inoculum attaches in 15 min (Joklik, 1964) and more than 90% in 60 min (Dales, 1963). The efficiency of adsorption may be increased by increasing the virus concentration or by centrifugal force (Sharp and Smith, 1960). There was no difference in the efficiency of attachment between

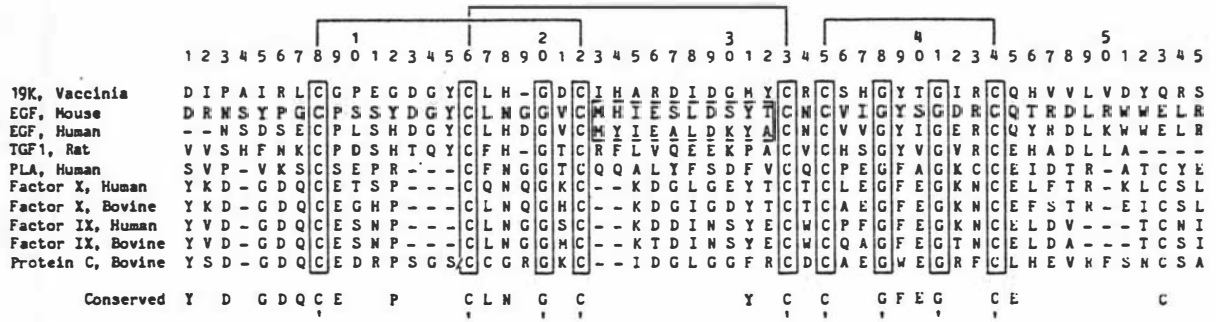


Fig. 2-10. Alignment of vaccinia virus 19K protein with EGF, TGF1, and the first EGF-like domain from the light chains of four blood clotting factors. Boxes enclose the 9 invariant residues. (From Blomquist et al., 1985)

intracellularly-derived and extracellularly-derived virus when inoculated. The host-derived additional membrane of the extracellular enveloped virus (EEV) did not seem to interfere with the attachment, and penetration appears to be more efficient than in the intracellular virus (Payne and Norrby, 1978).

Penetration is a temperature dependent event. It is accompanied by either viropexis (Dales and Siminovitch, 1961; Dales, 1963; Dales and Kajioka, 1964; Shand et al., 1976) and/or fusion with the cell membrane (Granados, 1973; Chang and Metz, 1976; Armstrong et al., 1973). It was shown by EM that viropexis is the more common route of entry. Penetration can be enhanced by proteolytic treatment of the virus (Ichihashi and Oie, 1980; Ichihashi et al., 1982). Inactivation by heating or U.V. irradiation does not prevent penetration (Dales and Kajioka, 1964) whereas high concentrations of specific antibody are effective in this respect (Stern and Dales, 1976). Protease inhibitors e.g. phenylmethylsulphonylfluoride (PMSF) also have an inhibitory effect on penetration (Ichihashi and Oie, 1982).

An efficient adsorption and penetration of orf virus to bovine testis cells was also reported by Traykova and Argirova (1985). The adsorption rate as determined by attachment of ^{3}H-thymidine-labelled virus to calf testis cell monolayers was calculated to be 5×10^{-7} $cm^{3} \times min^{-1} \times cell^{-1}$. Absorption was almost complete within 30 min.

Following the fusion of the viral envelope with the plasma membrane the core is released into the cytoplasmic matrix (Dales, 1973; Armstrong et al., 1973). The envelope is then degraded by cellular enzymes (Dales, 1965). At the time of penetration, antigenic components of the envelope are rapidly and widely dispersed within the plane of the cell membrane as revealed by immunoferritin tagging (Chang and Metz, 1976). Some of these virion-derived antigens have now been identified (see Section 2.5.3). Present evidence shows that these antigens, together with viral antigens expressed early on the cell surface after infection, may participate in the formation of the "target-antigen complexes". These complexes render the vaccinia virus-infected cells susceptible to recognition and killing by syngeneic, vaccinia virus-specific cytotoxic T lymphocytes (VV-CTLS) (Hapel et al., 1978; Mallon and Holowczak, 1985; Mallon et al., 1985)

The uncoating process has been analysed by E.M. and autoradiography of infected cells (Dales and Kajioka, 1964; Dales, 1962, 1963, 1965). Joklik (1964) initially conceptualised that mRNA production and protein synthesis are necessary for uncoating the DNA from its core and that this is mediated by the host genome. The role of RNA and proteins in the release of DNA was confirmed by Woodson (1967) but he discounted the concept of host genome involvement. The discovery of the DNA-dependent RNA polymerase in the core of vaccinia virus provided evidence that viral DNA is capable of directing early functions e.g. mRNAs specifying uncoating proteins, while still in the core (Kates and McAuslan, 1967a; Munyon et al. 1967). Work by Prescott et al. (1971) and

Pennington and Follet (1974) also provided evidence that the nucleus of the host cell is not required for poxvirus uncoating. The DNA passes through a break in the proteinaceous coat of the core (Dales, 1965) leaving behind an empty core or shell. The DNA is then conserved and replicated within cytoplasmic "factories" throughout the cell cycle.

Transcription and Translation

Transcription and translation can be divided into pre-replicative, or early, and post-replicative, or late phases in relation to DNA replication. They can be further subdivided into immediate early, which includes functions related to transcription from the core, early, which includes functions expressed after uncoating, late, covering functions involved in virion assembly and maturation and late-late which covers functions after maturation, e.g. haemagglutinin production.

Transcription

Poxviruses carry their own transcriptase. This enables the virus to synthesise mRNA immediately after infection, before DNA is released from the core (Munyon and Kit, 1966; Woodson, 1967). This presence of a DNA-dependent RNA polymerase in virions was confirmed by Kates and McAuslan (1967b) and Munyon et al. (1967). Other factors may be involved in the

initiation of RNA synthesis besides the viral RNA polymerase (Golini and Kates, 1984).

The rate of early and late RNA synthesis depends on the multiplicity of infection (Woodson, 1967; Oda and Joklik, 1967). There is an initial burst of viral RNA synthesis during the first hour of infection as shown by pulse-labelling (Becker and Joklik, 1964). These species of immediate early RNA are virion-specific, cistron-sized RNAs transcribed from the core. They have sedimentation coefficient of 10-12S (Kates and McAuslan, 1967a; Oda and Joklik, 1967). Higher molecular weight RNAs, of about 20-30S, have been identified and are believed to be precursors of 8-12S mRNAs as shown by hybridisation-competition experiments (Paoletti, 1977a; 1977b). The 8-12S RNAs are probably equivalent to the 10-12S RNAs described by the earlier investigators. The precursor molecules are methylated but not polyadenylated. It was believed that during immediate early transcription, polycistronic RNA molecules are produced which become rapidly cleaved, polyadenylated and extruded from the core as functional monocistronic RNAs (Paoletti and Lipinskas, 1978). However, Bossart *et al.* (1978a) showed that mRNAs coding for the individual viral translation products in a cell-free translation system are transcribed from individual promoter sites rather than from polycistronic mRNA precusor molecules. Enzymatic activities involving polyadenylation and capping have been identified in the cores of vaccinia virus. Poly A is added to the 3' end of the RNA chain (Sheldon *et al.*, 1972; Nevins and Joklik, 1975) and the cap or 7-methyl-

guanosine (m^7G) to the 5' terminus. The m^7G is connected from the 5' position by a triphosphate to the 5' position of an adjacent 2'-O-methyladenosine (A^m) or 2-O-methylguanosine (G^m) residues (Wei and Moss, 1974; Moss et al., 1976; Boone and Moss, 1977; Bossart et al., 1978a; 1978b). The modified terminus of the vaccinia mRNA bears the structure $m^7G(5')pppN_1{}^m\text{-}N_2{}^m$ where N_1 is A^m or G^m. This structure is depicted in Fig. 2-11. The adenosine residue in the penultimate position is often double-methylated to form N6,2-O-dimethyladenosine ($m6A^m$) and N_2 is methylated to form the structure $m^7G(5')pppN_1{}^m\text{-}N_2{}^m$. This modification makes the RNA resistant to the combined action of 5'-exonucleases and alkaline phosphatase (Ensinger et al., 1975) and is presumably required for efficient translation of mRNA in vitro (Both et al., 1975).

Other early RNA species are made after the initiation of protein synthesis when uncoating is completed (Kates and McAuslan, 1967a). Some experiments suggest that the rate of RNA synthesis is controlled by the uncoating process. If the second stage of uncoating is blocked by an inhibitor of protein synthesis, synthesis of immediate early RNA is enhanced for an extended period of time (Munyon and Kit, 1966, Kates and McAuslan, 1967a). Interferon (Joklik and Merrigan, 1966) and U.V. irradiation of viruses (Kates and McAuslan, 1967a) produce the same effect. On the other hand, uncoating of the virus and an associated burst of early RNA synthesis, which rapidly declines, occurs in the presence of inhibitors of DNA synthesis. Early mRNA synthesis continues throughout the multiplication cycle. This may be due to the

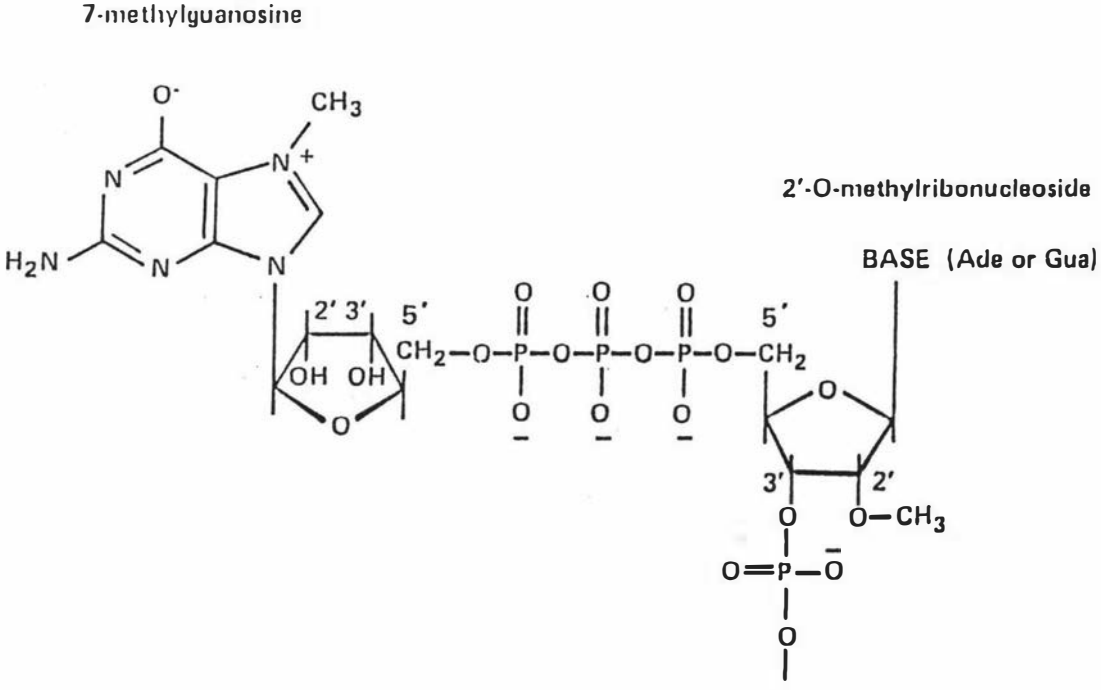


Fig. 2-11 Modified 5′-terminal structures of vaccinia virus mRNA synthesized in vitro in the presence of S-adenosylmethionine.

portion of the virus inoculum that remains incompletely uncoated, since only 50% of virus input becomes completely uncoated (Joklik, 1964). Sequences coding for some early mRNAs have been located within the ITR (Wittek et al., 1980).

Late RNAs are made after the onset of DNA replication. Synthesis of this species of RNA is prevented by inhibitors of DNA synthesis. Like the early mRNAs they also contain poly A and are capped and methylated (Kates, 1970; Sheldon et al., 1972; Nevins and Joklik, 1975). Early and late mRNAs can be distinguished from one another by hybridization-competition and by differences in their sedimentation rates. Early vaccinia virus RNA competes with only half of the RNA made at late times whereas the late RNA competes with all the early RNA (Oda and Joklik, 1967). The sedimentation rates of the late RNAs range from 16-23S compared with the 10-14S (Oda and Joklik, 1967; Salzman et al., 1964) or 10-12S (Kates and McAuslan, 1967) of the earlier species. Caps in the early mRNAs also contain more G^m than A^m and $m6A^m$ at the N_1 position. The early ones are also methylated more extensively at N_2 than the late ones.

In addition to DNA synthesis, it is also believed that arginine is required for late mRNA synthesis. It was shown that arginine deprivation allows early mRNA to be transcribed and DNA synthesis to proceed but prevents the transcription of late mRNAs (Obert, et al., 1971).

The development of a cell-free transcription system has enabled the transcription of cloned fragments of the genome. Transcription maps of these genes (see Fig. 2-12) have given information as to the initiation and termination sites as well as to the regulatory mechanisms involved (Cooper et al., 1981; Puckett and Moss, 1983; Mahr and Roberts, 1984a; 1984b; Wittek, et al., 1984; Weir and Moss, 1984; Golini and Kates, 1984; Jones and Moss, 1985; Cochran et al., 1985). The relative complexity of the sequences represented among these RNAs suggests the presence of a large number of efficient promoters throughout the genome (Paoletti et al., 1980). These studies also show that transcription can either go leftward or rightward. Clustering or overlapping of vaccinia virus genes was also observed (Golini and Kates, 1984; Jones and Moss, 1985).

The metabolic stability of the different classes of RNAs has been studied. The mRNAs for thymidine kinase (McAuslan, 1963a; 1963b) and for induced DNA polymerase (Jungwirth and Joklik, 1965) have half-lifes of 6 hr. Other early mRNAs are less stable (Kates and McAuslan, 1967a). Experiments by Sebring and Salzman (1967) showed half-lifes of 13 min for late mRNAs and 120 min for early mRNAs. However, Oda and Joklik (1967) claimed that late messengers were as stable as early messengers.

Translation

To initiate translation, vaccinia mRNA engages the smaller subunit of the ribosomes, similar to the cellular message

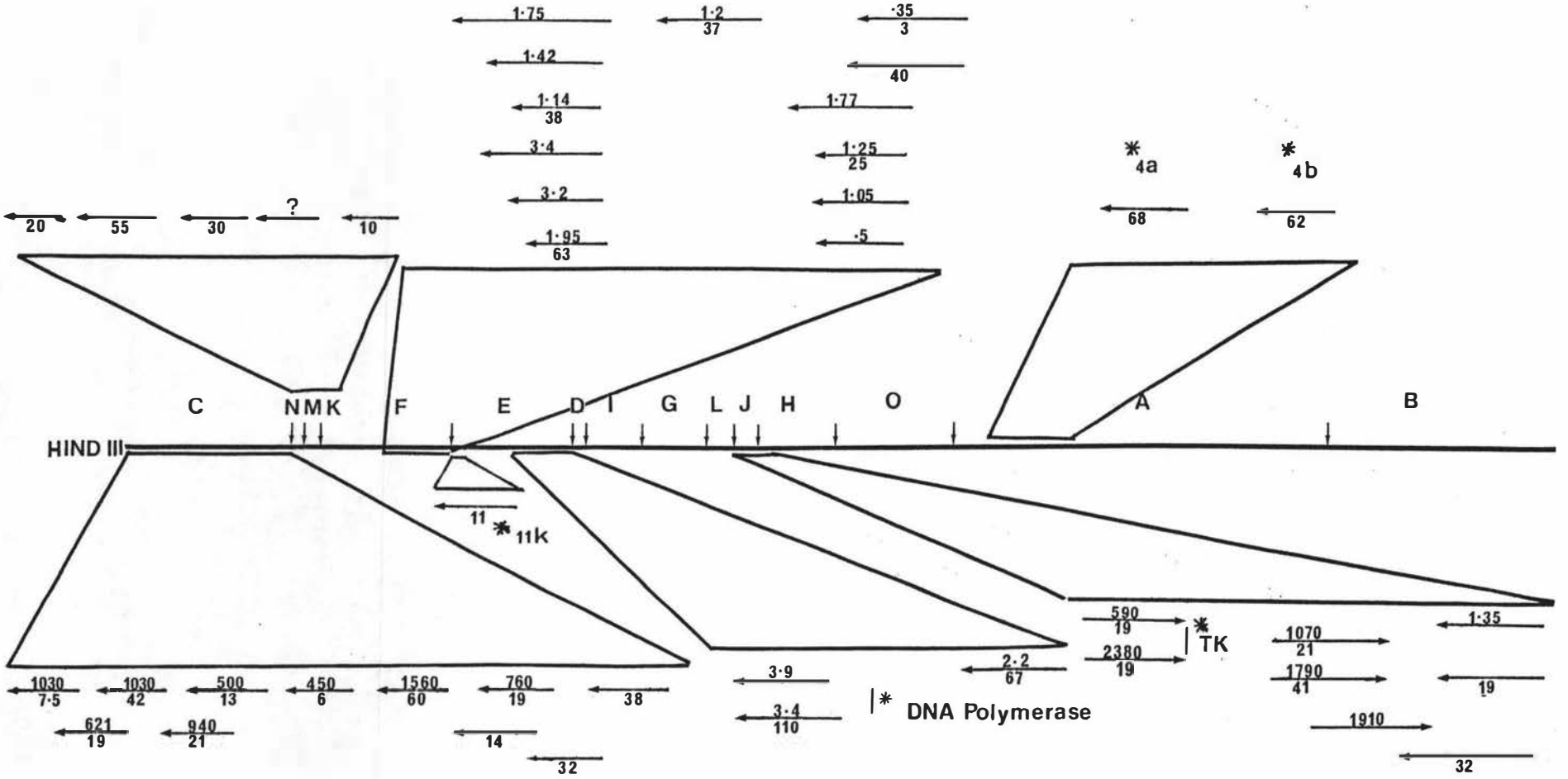


Fig. 2-12. Transcription/translation map of the vaccinia virus genome. Arrows indicate the direction of transcription. Numbers above the arrows represent the size of the mRNAs in base pairs; numbers below represent the size of translational products in kilodaltons. Genes that have been mapped are also indicated by asterisks. (Data from Wittek *et al.*, 1981; Cooper *et al.*, 1981; Moss *et al.*, 1981; Venkatesan and Moss, 1981; Bajszar *et al.*, 1983; Mahr and Roberts, 1984; Jones and Moss, 1984; Morgan and Roberts, 1984; Wittek *et al.*, 1984).

(Metz et al., 1975; Joklik and Becker, 1965). The presence of poly As at the 3' end, or their absence, does not seem to affect the rate of translation (Nevins and Joklik, 1975).

The early proteins translated can be classified into 3 classes: Class A are those that are synthesised immediately and rapidly after infection, continue for a period then switch off after 60 min e.g. P60 and P27; Class B are those that are synthesised more slowly and reach maximum levels between 1-2 hr e.g. P125, 103, P51 while Class C are those which are synthesised at a constant rate during the first 2 hr of infection e.g. P55, P40, P31 (Esteban and Metz. 1973). Two classes of late proteins have been described. One type is synthesised at the peak of DNA synthesis and thereafter declines and the other appears slightly later and continues throughout the virus growth cycle.

Possible control mechanisms of vaccinia virus protein synthesis are transcriptional or translational, or a combination of both. The differential regulation of transcription could account for the appearance of class A proteins before class B proteins i.e. messengers for class A would be transcribed before that of class B. The switch-off of synthesis of class A protein requires either that the mRNAs for class A proteins have short lives or that there is competition between the different virus messenger species for rate-limiting components in translation. The regulation of translation may involve the action of newly synthesised virus protein e.g. class A protein switching on the transcription of mRNA for class B proteins. Alternatively, it may involve

a programmed virus particle in which RNA polymerase trancribes, without external control, first one set of genes and then another. Regulation of the overall rate of transcription by the core is well established (Kates and McAuslan, 1967a; Woodson, 1967) and is probably effected by a newly synthesised virus protein.

The differential regulation of translation could account for the changing patterns of early polypeptide synthesis in the absence of control of transcription. Thus a class A protein could switch on the synthesis of the class B protein and class B protein could switch off the synthesis of the class A protein. Evidence for this type of regulation is found with the synthesis of thymidine kinase (McAuslan, 1963a; 1963b; 1969). Moss and Filler (1970) also reported that virus replication is irreversibly inhibited if protein synthesis in vaccinia virus-infected HeLa cells is blocked between 30 and 60 min after infection, but not at earlier times. These authors postulate that early synthesis of virus-specific translation initiation factors may be necessary for the subsequent regulation of virus protein synthesis.

Cell-free translation experiments indicate that early RNA directs the synthesis of authentic early polypeptides and the late RNA directs the synthesis of authentic late polypeptides (Cooper and Moss, 1979).

The rapid switch-off of host cellular protein synthesis by vaccinia infection makes possible the identification of virus-coded proteins synthesised immediately after infection.

Within 15-60 min, virus-induced protein can be detected and can be related to the prior appearance of new polyribosomes which contain viral mRNA (Becker and Joklik, 1964).

The vacinia virus genome is capable of coding for 200 polypeptides. About 75 early and 40 late polypeptides have been mapped by cell-free translation of mRNAs selected by hybridization to purified (Cooper and Moss, 1979) or cloned fragments (Belle Isle et al., 1981). Transcription/ translation maps of the fragments of the genome analysed to date are shown in Fig. 2-12.

The use of SDS-PAGE enabled the sequential appearance of the virus-induced polypeptides to be studied. Pennington (1974) detected about 80 polypeptides, 30 of which appeared before and 50 after the onset of DNA synthesis (Fig. 2-13). Virus-coded protein synthesis starts as early as 30 min after infection using a high multiplicity of infection (50 pfu/cell).

Some early as well as some late proteins are expressed on the plasma membrane of infected cells. The sequential appearance of these virus-specific antigens on the cell surface was seen in mouse L cells infected with WR, IHD-J and CV-1 strains of vaccinia virus (Mallon et al., 1985; Mallon and Holowczak, 1985). At 1 hr p.i., polypeptides with MWs of 48-50K and 36-37K were detected on cell surfaces and by 2 hr p.i. polypeptides with MWs of 48K-50K, 42K-44K, 36K-37K, 29K-30K and 16K-17K were detected. When protein synthesis was allowed to proceed for 2.5 hr p.i. and then stopped,

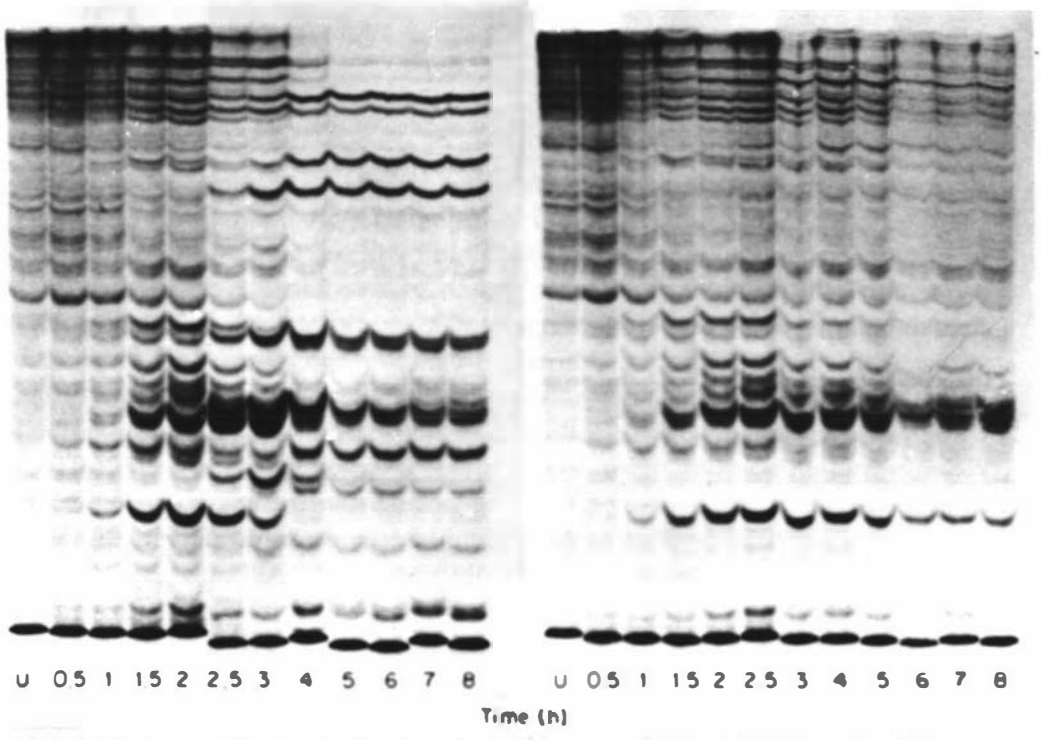


Fig. 2-13 Autoradiograms showing time of course of polypeptide synthesis in vaccinia virus-infected cells in the absence (left) and presence (right) of cytosine arabinoside, an inhibitor of DNA synthesis. U, uninfected cells.
(From Pennington, 1974).

polypeptides with MWs of 58K, 48K-50K, 42K, 36K-37K, 32K-33K, 27K-29K and 16K-17K were expressed on the plasma membranes. By 3 hr p.i., polypeptides with MWs of 78K-82K, 65K, 50K, 42K-45K, 35K-37K, 32Kk-34K, 30K, 20K and 17K-18K had appeared on the plasma membranes and by 4 hr, only the 32Kd-34Kd polypeptide was detected. Polypeptides of 32K and 37K were virion-derived antigens (Mallon and Holowczak, 1985; Mallon et al., 1985). The role of these antigens in relation to recognition and lysis of infected cells by cytotoxic T cells has been studied (Jackson et al., 1976; Oie and Ichihashi, 1981; Mallon and Holowczak, 1985; Mallon et al., 1985). An example of a late surface antigen is the haemagglutinin expressed on cells infected with orthopoxviruses of the HA(+) phenotype (Weintraub and Dales, 1974), but this antigen is clearly not essential for T cell recognition of infected cells.

Regulation of vaccinia virus transcription and translation appear to be virus-specified. However, the presence of nuclear associated vaccinia virus-specified mRNA suggests that the nucleus may be involved in virus-specified mRNA synthesis (Bolden et al., 1979). Similar observations were reported in fowlpox virus-infected cells (Gafford and Randall, 1976) and cells infected with Yaba viruses (Taylor et al., 1977). However, a difference exists in the nuclear and cytoplasmic virus-specified mRNAs. It was postulated by Bolden et al. (1979) that the possible role of the nucleus in vaccinia infection is to process the vaccinia mRNA made in the cytoplasm to the appropriate form as needed. Processing and splicing of mRNA, both viral and cellular may be a

necessary step to produce a functional message. It was also observed that virus assembly is inefficient in enucleated cells (Pennington and Follet, 1974) even though early functions and DNA synthesis occur. A specific role for host nuclei in vaccinia virus has been demonstrated by the work of Silver, et al. (1979) using α-amanitine, a specific inhibitor of RNA polymerase II. RNA polymerase II is responsible for nuclear mRNA synthesis. It is suggested that the host nucleus participates in virus assembly. Silver and colleagues (1979) showed that α-amanitine did not effect vaccinia virus replication in mutant cells which possessed a polymerase II resistant to the toxin.

In contrast γ-irradiation in doses sufficient to make the host nucleus dysfunctional does not affect virus replication. Also, EM studies of enucleated primary monkey kidney cells infected with the WR strain of vaccinia showed all the usual stages of virus replication (Klitsunova et al., 1983). Recently, α-amanitine-resistant vaccinia virus mutants have been isolated. One mutant, α-27, is capable of replicating in enucleated BSC-40 cells (Villareal et al., 1984). It is able to catalyse proteolytic processing of two major capsid proteins, VP62 and VP60, which wild type virus failed to do in the presence of α-amanitine. Comparison of restriction enzyme analyses revealed that an XhoI cleavage site was altered in the α-27 DNA molecule.

Structural Proteins

The use of quantitative immunoprecipitation and autoradiography of gel diffusion plates on protein extracts of vaccinia virus-infected cells shows that at least 5 viral structural proteins are synthesised before the onset of viral DNA synthesis (Salzman and Sebring, 1967). Most of the late polypeptides are virion structural entities and are synthesised late in infection. Two of the core polypeptides and one of the surface polypeptides are made early after infection (Holowczak and Joklik, 1967). A third core polypeptide and most of the high molecular weight species appear late. One or 2 polypeptides seem to be made throughout the multiplication cycle (Moss and Salzman, 1968, Holowczak and Joklik, 1967). About 3 polypeptides are formed from higher molecular weight precursors which are made after the commencement of DNA synthesis (Katz and Moss, 1970a; 1970b; Moss and Rosenblum, 1973; Pennington, 1973).

Post-Translational Modification

There are two types of post-translational changes involved in the formation of vaccinia virus polypeptides, namely, peptide cleavage and amino acid modification. Three major structural polypeptides, 4a (MW=68K) 4b (MW=62K) and P9 were found to come from higher molecular weight precursors, namely, P4a (MW=105K), P4b (MW=74K) (Moss and Rosenblum, 1973) and Py (Pennington, 1973), respectively. The precursor/product relationships have been demonstrated by 'pulse-chase' type

experiments (Katz and Moss, 1970a, 1970b; Pennington, 1973) and peptide mapping (Moss and Rosenblum, 1973) (Fig. 2-14). The precursors P4a and P4b have half-lives of 1 - 2 hr and their cleavage appears to be coupled to the assembly of virus cores as shown by inhibition with rifampicin (Katz and Moss, 1970a; 1970b).

Proteins are also modified by glycosylation and phosphorylation. Glycoproteins associated with the cell membrane have been detected by labelling infected cells with radioactive glucosamine, fructose and galactose (Moss et al., 1971; Weintraub and Dales, 1974). Glycosylation is possibly an early function (Jackson, et al., 1974). At least 1 (Moss et al., 1973; Holowczak, 1970; Garon and Moss, 1971; Esposito et al., 1977) and possibly 2 (Sarov and Joklik, 1972) virion proteins are glycosylated with glucosamine as the only sugar component (Garon and Moss, 1971). They have been identified as polypeptides with MWs of 38-39K and 41K. When vaccinia virus is grown in the presence of ^{32}P, several structural polypeptides are labelled (Sarov and Joklik, 1972). About 60% of the radioactive material incorporated was detected in a polypeptide with a MW of 11K-12K. This polypeptide is histone-like in composition and is a major component of the core. It is found to be associated with the DNA (Pogo et al., 1975). Minor phosphoproteins include the 24K, 34K and 58K proteins.

Virus-Induced Enzymes

Several virus-induced enzymatic activities are detected in vaccinia virus-infected cells and some of these are core-associated (see Table VI).

Thymidine kinase (TK) is induced 1.5 - 2 hr after infection and its production continues for about 4-6 hr (Magee, 1962; Kit *et al.*, 1963; McAuslan, 1963 a & b). The induction of this enzyme in TK^+ cells as well as in TK^- cells and the isolation of TK^- mutant, are proof that it is virus-coded (Kit *et al.*, 1963; Dubbs and Kit, 1964). The control of TK expression is mediated by an early or delayed early viral gene (Hruby and Ball, 1981). This gene has now been mapped to the *Hin*dIII J fragment of the genome (Hruby and Ball, 1982; Bajszar *et al.*, 1983). The MW of the native TK is about 80K and the active TK is believed to be a tetramer of 19K subunits (Hruby and Ball, 1982).

The level of a DNA polymerase activity (DNA nucleotidyl transferase) also increases during the early phase of vaccinia virus infection (Jungwirth and Joklik, 1965). The enzyme has a MW of about 110K (Challberg and Englund, 1979). The gene coding for the enzyme is located in a 2,000 bp segment (Jones and Moss, 1985) within the 15 kbp *Hin*dIII E fragment (Jones and Moss, 1984; Traktman *et al.*, 1984). Although it can bind adventitiously to vaccinia virus particles it is not an integral virion component (Tan and McAuslan, 1972). Evidence that DNA polymerase activity is virus-coded came from studies with phosphonoacetate (PAA).

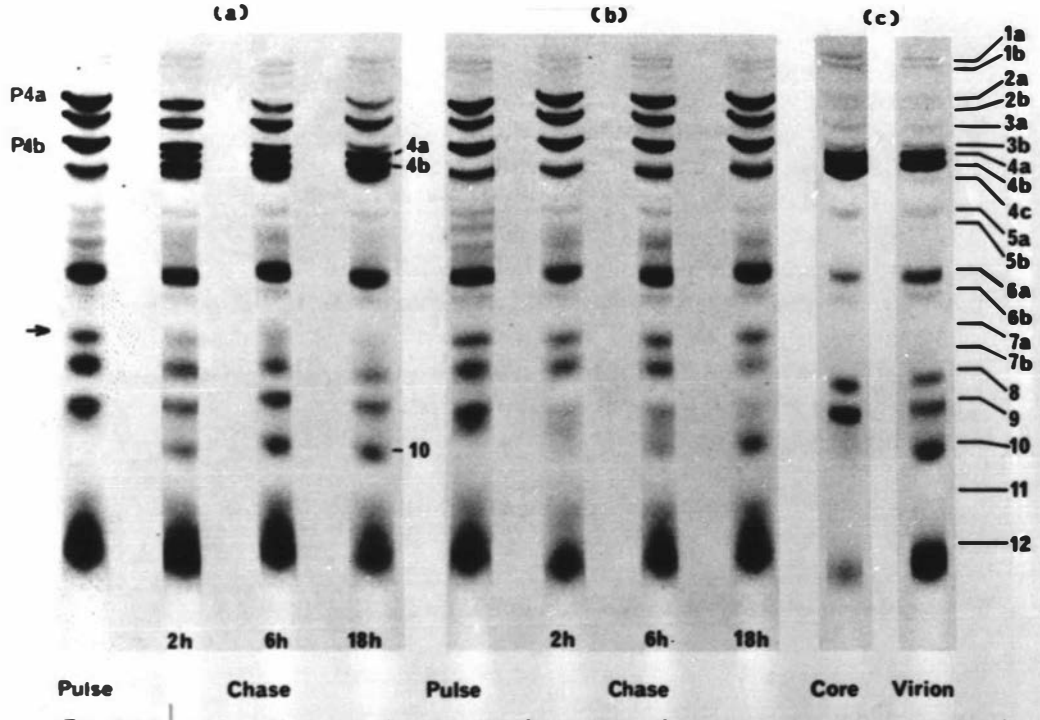


Fig. 2-14 Autoradiograms showing the formation of some vaccinia virus structural proteins from higher-molecular weight precursors. HeLa cells were infected in the absence (left) or presence (center) of rifampicin and incubated with [^{35}S]methionine for 20 min at 6 hr after infection. Cytoplasmic proteins obtained at the end of the labeling period and at intervals thereafter were analyzed by polyacrylamide gel electrophoresis. (From Moss and Rosenblum, 1973).

Citarella et al. (1972) found that the vaccinia DNA polymerase was inhibited by PAA. Subsequently, Condit and Motyczka (1981) isolated a PAA-resistant (PAA^r) mutant later confirmed by Moss and Cooper (1982).

Another early virus-induced enzyme is the polynucleotide ligase which seals single-stranded breaks in duplex DNA and is essential for DNA replication in prokaryotes. It has the same ATP cofactor requirement as cellular ligase. The absence of enzyme induction in the presence of puromycin suggests that this enzyme is virus-induced (Sambrook and Shatkin, 1969).

Infection of monkey kidney (BSC-40) cells with vaccinia virus strain WR resulted in marked increase of ribonucleotide reductase activity as measured by cytidine diphosphate (CDP) reduction in cell-free extracts. Hydroxyurea, EDTA, dATP, and dTTP inhibited CDP, setting this reductase apart from T4 reductase which is not inhibited by dATP, and from herpes virus reductase which requires no activation and is insensitive to deoxyribonucleotide triphosphate inhibition (Slabaugh et al., 1984).

Several enzymatic activities have been isolated from the virus core and some are involved in the transcription and modification of mRNA. The first of these enzymatic activities identified was the DNA-dependent RNA polymerase activity in vaccinia (Kates and McAuslan, 1967b; Munyon et al., 1967). This activity has been detected in other poxviruses including those from rabbitpox (Kates and

McAuslan, 1967b) and Yaba viruses (Schwartz and Dales, 1971). Analysis of the enzyme from infected HeLa cells by SDS-PAGE revealed the presence of 7 polypeptides with molecular weights of 135k, 130k, 77k, 34k, 19.5k, 16.5k and 13.5k (Nevins and Joklik, 1977). The enzyme has been partially purified from the vaccinia virion by Spencer et al. (1980) and Baroudy and Moss (1980). The latter investigators calculated the MW of the native enzyme to be 500K and the MWs of the putative subunits to be 140K, 137K, 37K, 35K, 31K, 22K and 17K.

The poly A polymerase (polynucleotide adenyl transferase or terminal adenylate transferase) is responsible for attaching approximately 100 adenylate residues to the 3' terminus of the virion-associated mRNA (Moss and Rosenblum, 1974). In native form it has a MW of 80K consisting of 2 polypeptide subunits of 51K and 35K as detected by SDS-PAGE (Moss et al., 1975).

Three enzymes responsible for "capping" and methylation at the 5' terminus of mRNA have been isolated from the vaccinia core (Ensinger et al., 1975). These are guanylyltransferase, which transfers a GMP residue from GTP to the 5' terminus of RNA containing a 5' terminal di- or triphosphate, a methyltransferase which transfers a methyl group from S-adenosyl methionine to the 7 position of the added guanine and a second methyltransferase which methylates the 2'-position of the ribose of the second nucleoside. The reactions involved are:

1. $GTP + ppN_1pN_2p \rightarrow G(5')ppp(5')N_1pN_2p + pp_i$

2. $G(5')ppp(5')N_1pN_2p^-$ + SAdoMet >

$m^7G(5')ppp(5')N_1pN_2-$ + SAdoHcy

3. $m^7G(5')pppN_1pN_2p^-$ + SAdoMet >

$m^7G(5')ppp(5')N_1{}^mpN_2-$ + SAdoHcy

The first two activities appear to be associated with a single enzyme with a MW of 127k. The purified enzyme contains 2 polypeptides with MWs of 95K and 34.4K (Martin et al., 1975; Martin and Moss, 1975; Moss et al., 1976; Boone et al., 1977). Both the guanylyltransferase and methyltransferase activities are apparently synthesised as early functions in the cytoplasm of infected HeLa cells (Boone et al., 1977). The enzyme which catalyses methylation has a MW of 36k (Barbosa and Moss, 1978).

Two enzymatic activities are related to the modification of DNA of the virion. Both the exonuclease and endonuclease have similar MWs of about 50K but exist as heterodimers of 100k in the active state (Pogo and Dales, 1969; Schwartz and Dales, 1971; Pogo et al., 1971). Their role is not certain but, by analogy with eukaryotic system, they may act to introduce nicks in the DNA template (Geshelin and Berns, 1974; Pogo, 1977, 1980b). They are distinguished from each other by their pH optima, Km values, sites of nucleolytic attack of their substrates and their activities in the presence of various anions, cations, and transfer RNA. The exonuclease acts optimally at pH 5.0, the endonuclease at pH 7.8. Addition of phosphates to the reaction inhibited DNAse activity at pH 5.0 more than at pH 7.8 while Mg^{++}, Ca^{++}, Na^+, K^+ and NH_4 were relatively more inhibitory at pH 7.8. At pH 5.0, tRNA proved to be a more effective

competitive inhibitor of the reaction than at pH 7.8 (Pogo and Dales, 1969). A third DNAase optimally active at alkaline pH, produced after rabbitpox infection of HeLa cells has been reported (McAuslan and Kates, 1966) but has not been detected in vaccinia-infected cells (Pogo and Dales, 1969).

Modification of the poxvirus genome in prepararion for transcription or replication may involve another enzyme, the nicking and closing enzyme. First identified by Bauer et al. (1977), it has a MW of 70K with 2 polypeptide subunits with MWs of 35K and 24K. It is suggested that this enzyme may have a role in the transcription of the core. Its role in DNA replication has been illustrated by the studies of Lakritz et al. (1985). It was shown that it is capable of relaxing both left- and right-handed superhelical DNA. This relaxation was monitored by the extent of conversion of supercoiled closed duplex DNA (DNA I) to nicked circular DNA (DNA II). When left longer in the enzyme preparations the DNA II would subsequently convert to duplex linear DNA (DNA III).

Other enzymes detected in the virus core are nucleotide triphosphate phosphohydrolases. Two independent ATPases have been noted, ATPase I and II with MWs of 61K and 68K, respectively (Wei and Moss, 1974; Paoletti and Moss, 1974). Functions such as extrusion of nascent mRNA chains from the cores (Kates and Beeson, 1970a) have been attributed to these enzymes. Other biological funtions may be DNA strand separation, initiation of DNA replication, transcription, DNA packing, assembly and the nicking and closing activity.

Kinases have also been detected in virus-infected cells. A vaccinia protein kinase of MW 63K has been solubilised and shown to contain heat-labile and heat-stable components (Kleiman and Moss, 1973). The core-associated 5'-phosphate-polyribonucleotide kinase associated with RNA capping has also been characterised (Spencer et al., 1978).

Alkaline proteases have also been detected but their functions remain unknown (Arsoglou et al., 1979).

DNA Replication

Poxvirus DNA synthesis is initiated in the cell cytoplasm in so called "factories" or viroplasms (Cairns, 1960). Replication appears to be carried out largely by virally coded enzymes. However, reports have shown that fowlpox DNA replication can occur in the nuclei of infected cells (Gafford and Randall, 1976; Hardy et al., 1976). As mentioned above (see Sec. 2.5) the rates of DNA synthesis differ between different pox viruses.

Blocking of protein synthesis with cycloheximide inhibits DNA replication (Kates and McAuslan 1967c) and some of the viral proteins required are discussed below. Core enzymatic acitivites associated with DNA synthesis are shown in Table VI. The DNA polymerase required for DNA replication (Jungwirth and Joklik, 1965) has been purified from infected HeLa cells and found to be a single, large polypeptide with a MW of 110K. It was associated with an exonuclease activity even in the purest form. The polymerase activity requires a

divalent cation and is maximally active in 5mM $MgCl_2$ and in phosphate buffer at pH 8-9. The enzyme has maximal activity on activated ("nicked") DNA. It will add a maximum of one or 2 nucleotides on each nick introduced into ØX174 RF DNA. The exonuclease activity also requires a divalent cation and the optimum pH for activity is similar to that of the polymerase activity. The exonuclease is more active on single-stranded than on duplex DNA (Challberg and Englund, 1979).

The manner in which viral DNA is in poxvirus-infected cells formed conforms in most respects with the semi-conservative, symmetrical replication seen in eukaryotic cells. However, poxvirus genomes have some unique structural features which are probably involved in DNA replication (Moyer and Graves, 1981b; Baroudy et al., 1982). First, the complementary strands of DNA are terminally cross-linked (Geshelin and Berns, 1974) and, secondly, a stretch of about 10,000 nucleotides at one end of the DNA is complementary to the other end, forming an ITR (Garon et al., 1978; Wittek et al., 1978).

Early models of DNA replication suggested that parental genomes bearing one (Esteban and Holowczak, 1977c) or 2 (Pogo, 1977, 1980b) nicks at the terminal cross-links served as templates for DNA replication. Recombination events which occur between parental and/or progeny DNA molecules (Moyer et al., 1980) also suggested that replication is accompanied by nicking. Both ends of the genome serve as the initiation and termination site of DNA replication (Pogo et al., 1981).

Recent experiments (Pogo et al., 1984) show the initiation site to be within the terminal 150 bp. Cross-links are re-inserted into progeny genomes during the terminal stages of genome formation (Holowczak and Diamond, 1976; Pogo and O'Shea, 1978). The "cross-linking" activity is catalysed by an enzyme complex containing exonuclease, DNA polymerase and DNA ligase activites, (Pogo, 1978, 1980a) and a "nicking-closing" enzyme which has been solubilised and identified (Bauer et al., 1977; Lakritz et al., 1985). It was shown by Lakritz et al., (1985) that the "cross-links" are sensivite to nuclease S1 but not to proteinase K which shows that it is a hairpin not maintained by protein links.

The presence of ITRs in the vaccinia genome led some investigators (Garon et al., 1978; Moyer and Graves, 1981a) to believe that the poxvirus genome circularises at some point during replication. This concept is supported by the results of experiments of McFadden and Dales (1979) that mirror-image deletions at the terminal end of vaccinia DNA can be produced only if DNA circularises during replication or that linear head-to-tail concatameric molecules are produced to serve as intermediates in the overall process of replication. Evidence of circularisation was suggested by Archard (1979) as an explanation for fast sedimenting forms of replicating DNA found in alkaline sucrose gradients, which were able to be blocked by prior ethidium bromide treatment of infected cells. Observations by Holowczak and colleagues (cited in Holowczak, 1982) show that if circularisation occurs it must be a transient stage in the viral DNA

replication process since the circular molecules are not present in the "virosomes" of infected cells as judged by EM.

There is evidence against any obligatory head-to-tail DNA end interactions during replication. A deletion mutant of rabbit pox virus exists where a portion of only the left but not the right ITR is deleted (Moyer and Rothe, 1980). It could be argued, however, that this assymmetrical deletion occured after replication.

Wittek and Moss (1980) proposed a model (Fig. 2-15) in which the nucleation event consists of the annealing of any of the nucleotide repeats at the opposite ends of single strands displaced during replication. The next step consists of the annealing of adjacent repeats and the remaining 7,000 nucleotide portion of the ITR. The ITR holds the panhandle structure together while tandem repeats are realligned to maximise base pairing. This realignment step might be favoured by the presence of the short, unique region between the two blocks of tandem repeats. The weakness of this model is that the authors do not describe how the panhandle structure is converted into a duplex DNA molecule, how the 5' ends are completed nor how the hairpins are restored.

A more convincing model whereby a concatameric DNA intermediate is generated consisting of genomes arranged head-to-head or tail-to-tail rather than head-to-tail is presented by Moyer and Graves (1981) for rabbitpox virus DNA as shown in Fig. 2-16A. In this model, replication is initiated within the double-stranded region near one end of

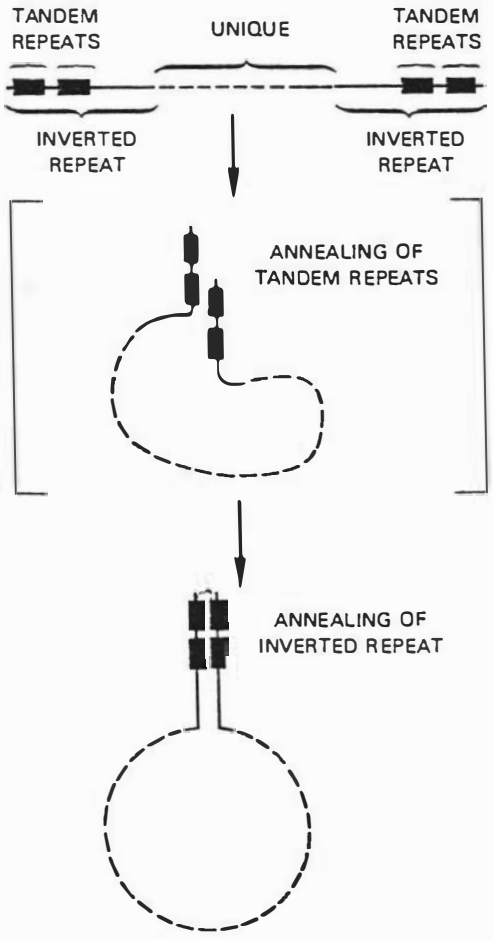


Fig. 2-15. Model showing the cyclization of single strands of vaccinia virus DNA that are displaced during replication. The nucleation event consists of the annealing of 70bp repeats. This is followed by hybridization of the remaining 7,000 nucleotide portion of the inverted terminal repetition and final rearrangement to maximize base pairing. (From Wittek and Moss, 1980)

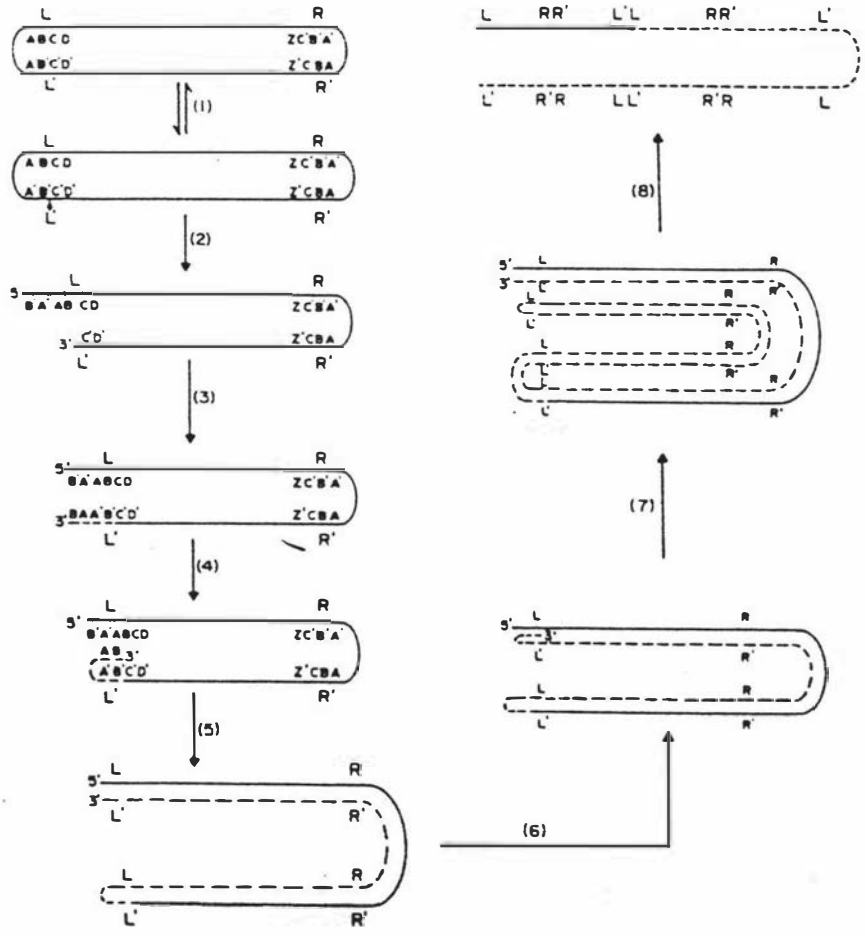


Fig. 2-16A. Model for DNA replication. Replication is initiated by the introduction of a nick into 3' strand at the B'C' sequence within the duplex region near the left end of the DNA. Parental DNA strands are indicated by solid (——) lines and newly synthesised progeny DNA strands with dashed (-----) lines. See text for further details of the model. (From Moyer and Graves, 1981).

the terminal ends of the DNA by a specific single-stranded nick within the 3' strand between the sequences B'C' (step 1 & 2). If the poxvirus contains an ITR, nicking could occur at either or both ends. The nicked end could then be extended using the complementary strands as templates (step 3). This would allow the copying of the sequences constituting the cross-links and then create an inverted repeat that could subsequently generate a priming "hairpin" structure to allow for the replication of the remaining DNA (step 4). Strand extension would yield a complete copy of the genome and result in a dimeric molecule joined through two right (RR) ends in a tail-to-tail position (step 5). The process can be repeated (step 6 & 7) to form tetrameric concatamers where the molecules are linked through two right (RR), then two left (LL) and finally two right (RR) ends. A mechanism is also presented for the excision of the genome-length molecules from head-to-head and tail-to-tail concatameric arrays during immature particle formation (Fig. 2-16B). Scheme A represents a concatamer generated by orthopoxviruses lacking inverted terminal repeats. The repeat distance of the sequence B'C' determines the size of the DNA to be packaged (step 1). The DNA is then excised, annealed (step 2 and 3) and ligated to form a cross-link mature DNA. Scheme B shows a proposed maturation scheme for a poxvirus containing inverted terminal repeats. Packaging is dictated by the spacing of the B'C' nicking site (arrows, step 1). Mature viral DNA forms after self-annealing (step 2) and ligation.

Now that the structure and sequence of the vaccinia virus DNA termini have been worked out, any DNA replication model must

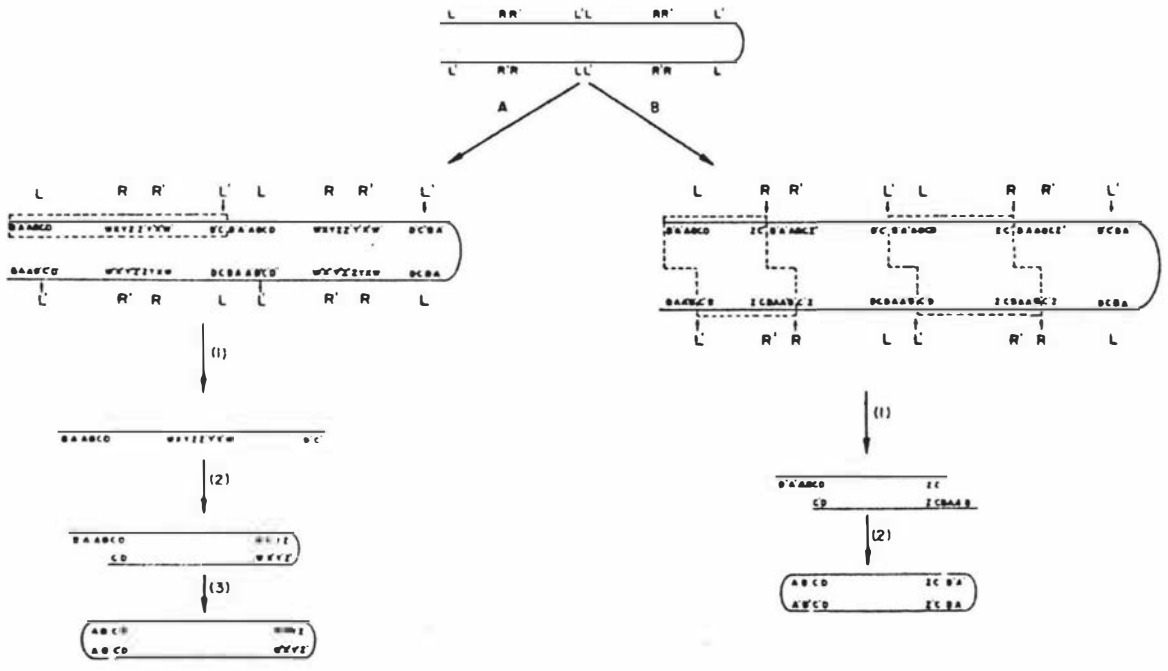


Fig. 2-16B. Excision of the monomeric molecules from virosomal DNA concatemers for packaging. A mechanism is presented for the excision of unit length molecules from concatemers where complexity is determined by a recognition sequence B'C'. Poxviruses are considered that either lack inverted terminal repeats (scheme A) or contain inverted repeats (scheme B). (From Moyer and Graves, 1981)

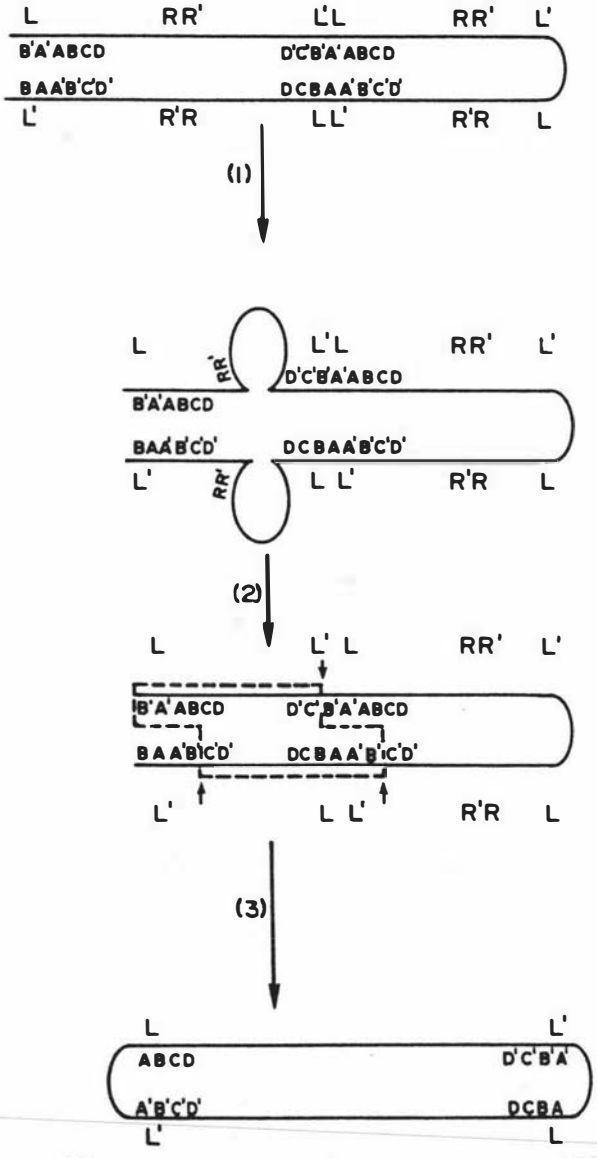


Fig. 2-16C. Generation of RPV gene duplications and inverted repeats. Following deletion (step 1), molecular excision governed by the spacing of the (B'C') recognition sequence occurs and, in the example illustrated, leads to a duplication of the left-hand sequences and the creation of an expanded inverted terminal repeat. (From Moyer and Graves, 1981).

account for the ITRs, the hairpins and the alternating "flip-flop" structure of the 104 imperfectly annealed bases. Some models of DNA replication that incorporate these features have been outlined by Baroudy et al. (1982) are shown in Fig. 2-17 A-C. These include a **self-priming** model, a **de novo** start model and a model which involves circularisation of the DNA.

The **self-priming** DNA replication model involves a site-specific nick resulting in a free 3`OH end that then serves as a primer for DNA replication. Strand displacement with unfolding of the "hairpin" loops occurs. The extended palindrome reforms the "hairpin" loop and the DNA replication continues by a strand displacement mechanism. In this model, replication is initiated at both ends and since the ends are replicated as extended palindromes, this explains the incomplete base-pairing in the loop. Other variations, e.g. a nick at one end, might occur. If this was the case, replication around the opposite un-nicked loop would lead to a concatamer. This model is essentially the model proposed by Moyer and Graves (1981), except that it incorporates the new information on the structure of the "flip-flop" termini.

In the **de novo** start model, DNA replication is assumed to initiate with RNA primers as seen in other eukaryotic replication system. Nicking and rearrangement separate the daughter DNA molecules from the dimer circle generated from one round of replication. Again, important elements of the Moyer and Graves model are incorporated into this model.

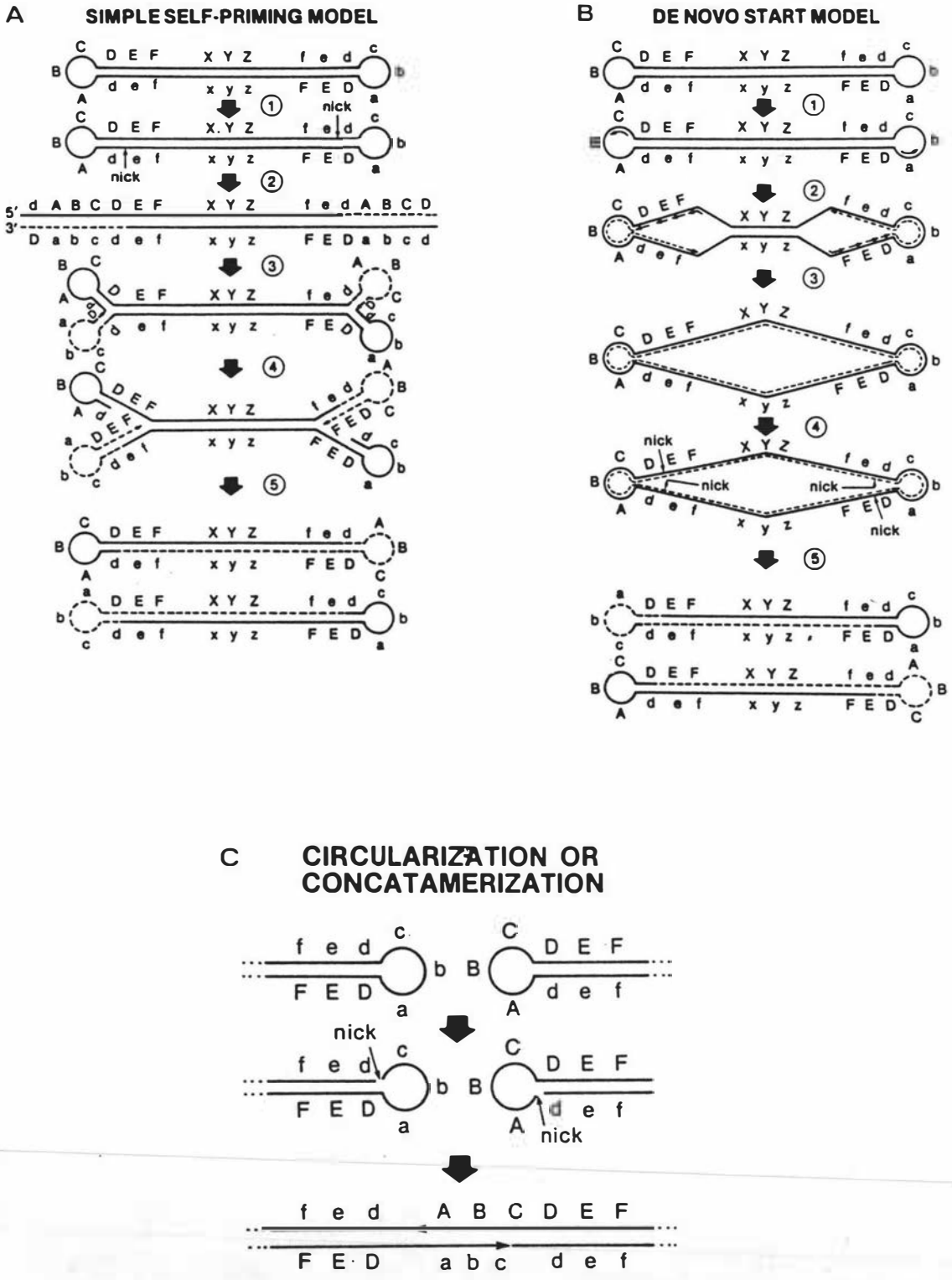


Fig. 2-17. DNA replication models which accounts for the flip-flop terminal loops. See text for details. (From Baroudy et al., 1982)

A third model of DNA replication in which the first step is a site-specific nick to form sticky ends and the second is circularisation or concatemerization is also proposed. Following replication by a mechanism similar to that used in prokaryotic system, site-specific nicking, refolding and ligation would occur. Baroudy et al. (1982) argued that the occurrence of a flip-flop sequence inversion demands that there be site-specific nicks, and religation, and the nick must occur proximal to the last mismatched base.

These models can also explain the generation of mirror image gene duplications or deletions and variably sized ITRs. One explanation proposed by Moyer and Graves (1981b) is the occurrence of gene duplication within the head-to-head and tail-to-tail concatamers prior to packaging (Fig. 2-16C). Following deletion (step 1), packaging is initiated by nicking and excision at the B'C' recognition site (step 2). Upon reannealing and ligation (step 3) a viral variant is generated which contains duplications of the left hand genes as mirror-immage inverted repeats. Mirror-immage deletions can also arise in the same mechanism.

Assembly and Maturation of Virions

The initial stages of virion formation are first detected as circumscribed, granular and electron-dense areas in the cytoplasm. Morphogenesis is similar in all poxviruses studied as revealed by EM studies of thin sections of cells infected with vaccinia (Dales and Siminovitch, 1961; Dales, 1963),

variola (Avakyan and Byckovsky, 1965), fowlpox (Morgan et al., 1954), Yaba tumor (Tsuruhara, 1971), milker's node (Cohen et al., 1964, Thomas et al., 1980) and insect pox (Granados and Roberts, 1970; Bergoin et al., 1969) viruses.

Development of virus particles may be divided into 6 stages. Type I particles are short cresent or "capsule-shaped" segments of membrane material. Type 2 are spheroidal, Type 3 have electron dense granular material or a distinct nucleoid within the cavity. These electron dense nucleoids are probably DNA (Rosenkranz et al., 1966). Type 4 have an elongated shape and an internal membrane structure. Type 5 particles have recognizable cores and lateral bodies while Type 6 are the mature virions (Thomas et al., 1980).

During maturation, precursor polypeptides are processed (Stern et al., 1977), i.e. the 105K precursor becomes 68K, the 74K precursor becomes a 62K product and Py becomes P9. These cleaved polypeptides are major components of the core (Moss and Rosenblum, 1973). Virus assembly is inhibited by beta-isatin thiosemicarbazone (Easterbrook, 1962) and rifampicin (Pennington, 1973; Katz and Moss, 1970a; 1970b). The latter drug prevents attachment of spicules to the bilayer membrane of the envelope (Nagayama et al., 1970; Pennington et al., 1970; Grimley et al., 1970), maturation (Nagayama et al., 1970; Grimley et al., 1970) and induction of core-associated late enzymatic activities (Nagayama et al., 1970).

Release and Dissemination

Virions of the WR strain of vaccinia remain highly cell-associated, only about 10% become extracellular (Easterbrook, 1962). The main mode of spread is by cell-to-cell transfer (Joklik, 1966; Payne, 1980) which can be demonstrated by the formation of plaques in cell cultures containing a high dose of antiserum in the medium (Kaku and Kahamora, 1964; Ichihashi et al., 1971; Appleyard, 1973). The intracellular virions can be released from cells by mechanical means, e.g. freezing-thawing and sonication, and are presumably also released after cell disintegration during natural infection. The intracellular virions have been referred to as naked virions to distinguish them from budded "extracellular" virions (Appleyard, 1971).

The intra- and extracellular virions are both infectious but differ in the nature of their surface antigens, probably due to the additional coat material from cell membranes derived during release from cells (Boulter et al., 1972).

The IHD-J strain of vaccinia, on the other hand, has a propensity to form extracellular virions after infection of certain hosts; about 60% become extracellular when grown in RK 13 and about 30% in L-cells (Mallon et al., 1985). Release of extracellular virus follows a more complex pattern. Electron micrographs of vaccinia-infected cells show 2 mechanisms of release, budding and reverse phagocytosis (exocystosis) of double-membraned virions (Tsutsui, 1983). In the budding process, virions migrate through the cytoplasmic

matrix towards the cell periphery. At the cell surface individual particles become positioned at the tip of microvilli-like extensions. Breakdown of the tips cause the release of the particles (Dales and Siminovitch, 1961; Stokes, 1976).

In the second mechanism, mature virions travel to the Golgi apparatus and become wrapped in double-membrane cisternae. The wrapped particles then migrate to the surface of the cell where the outer walls of the cisternae fuse with the plasma membrane. The combined particle/inner cisternal membrane package may remain intact or become ruptured after release (Morgan, 1976; Ichihashi et al., 1971).

One-step growth cycle experiments, using the IHD-W strain in FL cells (Tsutsui, 1983), demonstrated, by EM, brick-shaped virions on the surface of infected cells within 8 hr of infection. The budding of virions from the cell surface was detected 12 hr p.i. and became more common at later stages of infection (Tsutsui, 1983).

2.6 EFFECTS OF VIRUS INFECTION ON HOST CELLS

Gross Microscopic Changes

The cytopathogenic effect (cpe) that results from poxvirus infection has been simply referred to as cell rounding (Appleyard et al., 1962; Bablanian et al., 1978). In vaccinia it appears within 30 to 60 min following cell infection. This

is followed by the appearance of cytoplasmic inclusions, granularity, loss of cell membrane integrity and, finally, cell death. Depending on the type of virus, cell death occurs as rapidly as 12 hr after infection with vaccinia, to about 1-3 days with avian pox, Yaba and rabbit fibroma viruses (Cho and Wenner, 1973). Kato et al.(195), however, observed that rabbit fibroma and myxoma viruses did not cause cell destruction in human amnion (FL strain) even up to 10 days p.i. Also, persistent fibroma virus infection has been seen in RK cells (Padgett and Walker, 1970) supporting the conclusion that cell death is not an inevitable consequence of poxvirus infection.

Early cell rounding (ecr) has been correlated with virus-specified protein synthesis as evident from the application of appropriate metabolic inhibitors (Bablanian, 1968; Bablanian et al., 1978; Appleyard et al., 1962; McFadden et al., 1979; Ueda et al., 1969). It was found that the ecr was inhibited by actinomycin D (5 µg/ml),an RNA synthesis inhibitor, and cycloheximide (300 µg/ml), a protein synthesis inhibitor. It was not inhibited by hydroxyurea (10^{-3}M), a DNA synthesis inhibitor. These results suggested that de novo protein synthesis is required for the appearance of ecr. An early cpe has been ascribed to a cytotoxin from input virus especially with abnormally high virus inocula (Burgoyne and Stephen, 1979). This cytotoxin, believed to be a monomer of the "surface tubule" (ST) protein, is not the mediator of ecr described by Bablanian (1968). It may, however, play an important role in the terminal events leading to cell death (Burgoyne and Stephen, 1979).

During productive infection, the ecr phenomenon leads to irreversible and profound disturbances in host cell function and structure. There is an increase in the permeabilities of the plasma and lysosomal membranes of cells infected with rabbit poxvirus (Schumperli et al., 1978) or vaccinia virus (Carrasco and Esteban, 1982). Poxvirus infection was also shown to increase cell agglutinability by concanavalin A. This is attributed to the dispersion of surface tubular elements (Mbuy and Bubel, 1978) within the plane of the cell membrane.

In contrast, the abortive infection in cells by molluscum contagiosum virus in vitro is characterised by transitory cell rounding. This cpe is reversed in 1-3 days, at the time when the early surface antigens become dissipated and the host cells resume their normal morphology (McFadden et al., 1979).

Modification of the Cell Membrane

Three types of antigen are detectable on cell membranes after poxvirus infection. The first are the virion antigens derived from the fusion of the viral envelope with the plasma membrane during adsorption and penetration into cells (Armstrong et al., 1973; Chang and Metz, 1976; Hapel et al., 1980; Oie and Ichihashi, 1981). The second consists of virus-coded antigens expressed early in infection (Ueda et al., 1976; Ada et al., 1976; Jackson et al., 1976) while the third are late viral antigens expressed during or after DNA

synthesis. An example of late viral antigen is the non-virion haemagglutinin which is expressed by orthopoxviruses (Ichihashi and Dales, 1971; Shida and Dales, 1981, 1982). The haemagglutinin has also been demonstrated in the envelope of extracellular vaccinia virus particles (Payne and Norrby, 1976). Another late antigen was seen at the cell surface in a benign tumour in rabbits inoculated with Shope fibroma virus (Tompkins et al., 1970).

Using several techniques such as irradiation, western blotting, immunoprecipitation and SDS-PAGE analysis, Mallon and Holowczak (1985) and Mallon et al. (1985) identified a number of virus polypeptides expressed in, or associated with, the plasma membranes of vaccinia virus-infected cells. In follow-up experiments (Mallon et al., 1985) it was suggested that these antigens might be involved in the formation of target-antigen-complexes (TAC) which render infected cells susceptible to lysis by VV-CTLs. This is consistent with, and expands, earlier reports by Ada et al. (1979), Jackson et al. (1976a; 1976b) and Oie and Ichihashi (1981). It was also shown by Mallon et al. (1985) that trypsin removes or modifies the polypeptides that become associated with the plasma membrane but does not affect the recognition by the VV-CTLs. It was noted by Mallon et al. (1985) that polypeptides derived from extracellular and intracellular virus and detected on the surface of infected cells were of the same molecular weight but the authors could not be certain that they were identical proteins. However, cells infected with both types were lysed with equal efficiency as measured by an in vitro ^{51}Cr-release assay,

which suggests that the polypeptides derived from them produce the same haplotopes or epitopes recognised by VV-CTLs. The molecular weights and temporal appearance of these antigens have been discussed in detail earlier.

Metabolic Changes

Cellular protein synthesis is inhibited after vaccinia virus infection (Salzman and Sebring, 1967; Esteban and Metz, 1973). Depending on the multiplicity of infection, host macromolecular synthesis is switched off within 30 min after infection with vaccinia virus (Pennington, 1974). It was shown by Bablanian *et al.* (1981b) that the shut-off of cellular protein synthesis is associated with low molecular weight viral-induced RNA (50-100 nucleotides). Rice and Roberts (1983) showed that shut-off of cell-specified protein synthesis was achieved by a mechanism by which the virus induced the rapid degeneration of cellular mRNA. It was also shown by Mbuy *et al.* (1982) that the STs purified from vaccinia virus can inhibit host protein synthesis at a concentration of 50 μg/2x10^5 HEp-2 cells suggesting that input virus may inhibit protein synthesis in the absence of transcription. However, 50 μg of STs per 2 x 10^5 cells equates to approximately 5 x 10^5 particles per cell which is vastly more virions than would normally be used in a one-step growth curve or in a natural infection. There is also a rapid inhibition of nuclear DNA replication as exemplified in several orthopoxvirus/cell systems (Kato *et al.*, 1962; Magee *et al.*, 1960; Kit and Dubbs, 1963; Pogo and Dales, 1973,

Moss, 1974) and milker's node virus (Thomas et al., 1980). In contrast, poxviruses that elicit proliferation in response to infection, as in the case of fowlpox, Shope fibroma and Yaba viruses, host nuclear DNA synthesis is stimulated prior to the onset of cytoplasmic virus-related DNA replication (Cheevers et al., 1968; Tompkins et al., 1969).

The effect of vaccinia virus growth factor (VVGF), a processed form of the polypeptide expressed early in infection (Venkatesan et al., 1982), on cells was examined by Twardzik et al. (1985) and Stroobant et al. (1985). It has been detected in infected cells and also infected cell culture medium (Twardzik et al., 1985; Stroobant et al., 1985). Since it bears homology to the EGF and TGF-1 and binds to EGF receptors and stimulate its autophosphorylation it might have a mitogenic effect (Stroobant et al., 1985). The production of EGF-like growth factors by virally-infected cells and their secretion into the extracellular fluid could account for the proliferative diseases associated with members of poxvirus family such as Shope fibroma virus, Yaba tumour virus and molluscum contagiosum virus (Brown et al., 1985).

2.7 Genetics and Gene Mapping

Studies of the genomes of orthopoxviruses and other poxviruses reveal both regions of great heterogeneity and regions of strict conservation. Restriction maps of the genomes within the orthopoxvirus genes show a highly

conserved central region and variations near or within the terminal regions. The variations within groups could be attributed to genetic mutation or recombination.

Mutations

When a single strain of vaccinia virus was serially propagated in cell culture, alterations accumulated in some progeny genomes (Panicali et al., 1981). The analysis of these naturally occurring mutants of vaccinia and other poxviruses has enabled the mapping of some viral phenotypes to specific regions of the genome.

For instance, in some poxviruses, such as rabbitpox virus (Fenner and Sambrook, 1966; Moyer and Rothe, 1980), cowpox (Archard and Mackett, 1979) and monkeypox (Dumbell and Archard, 1980) alterations in genome structure have been correlated to changed phenotypes. Most orthopoxviruses produce haemorragic or red pocks on the chorioallantoic membranes (CAM) of embroyanated eggs. However, some of these viruses produce an occasional white pock. White pock variants of cowpox (Fig. 2-18) contain a deletion of 11-12% of "red" cowpox DNA and this deletion includes a portion of the terminal repitition. Some of these variants have lost 32-38 kbp from their right-hand ends and the deleted sequences have been compensated for by the insertion of inverted copies of regions from 21-50 kbp from the left hand end of the genome. This has the effect of increasing the size of the ITR by an amount equivalent to the size of the insert (Archard and

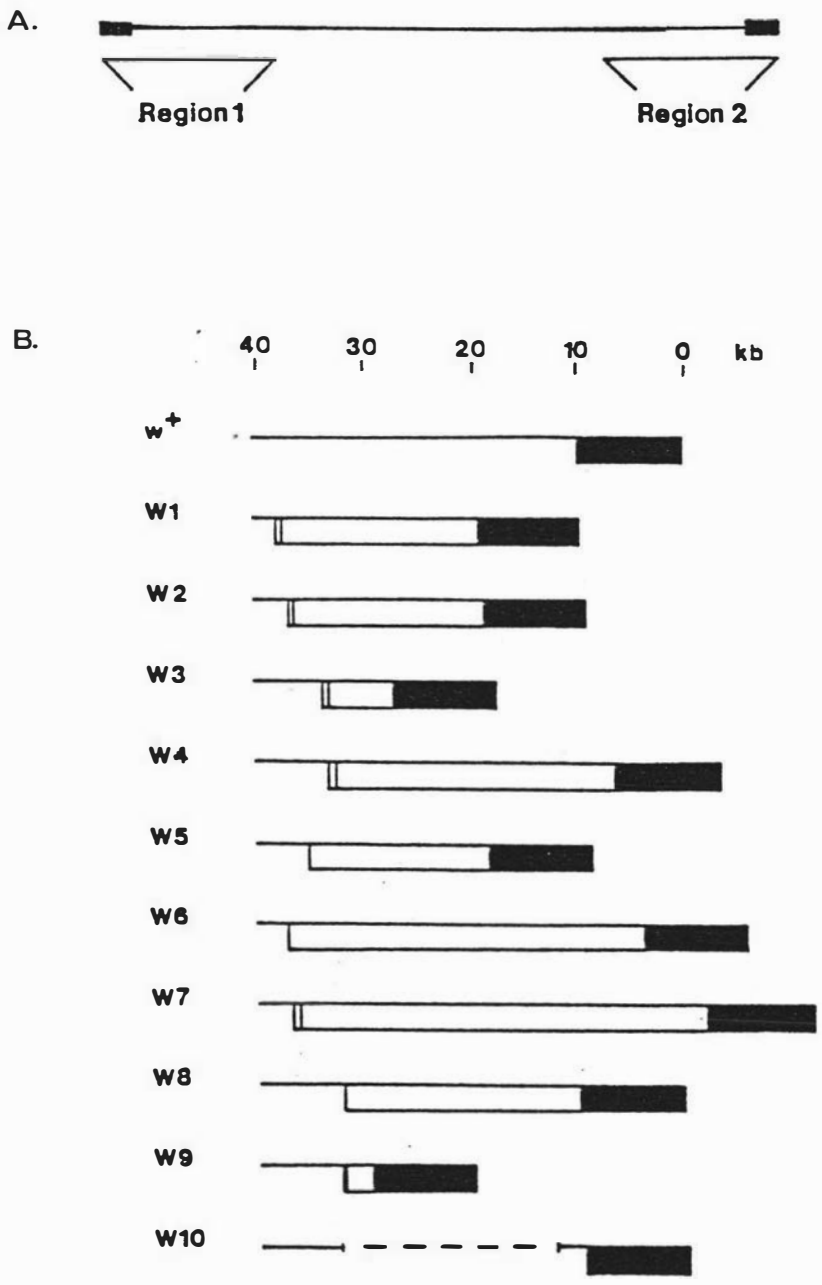


Fig. 2-18. Cowpox virus mutants. A. The genome of CPV-BR. The two flanking regions are designated as region 1 and 2. The black rectangles represent the ITRs. Size of each flanking region is about 40 kbp. B. Structures of region 2 of the white-pock variants. W^+ indicates the genome of the wild type CPV-BR. The open rectangles correspond to region 1 DNA inserted in an inverted orientation relative to the other end of the genome (From Pickup et al., 1984)

Mackett, 1979; Pickup et al., 1984). White pock mutants have been found in other orthopoxviruses. White pock (h) mutants of monkeypox show deletions as well as rearrangement of the terminal sequences. White pock mutants of rabbitpox contained deletions ranging from 5-33 kbps in the far left hand regions of the DNA near or within the ITR (Moyer and Rothe, 1980; Moyer and Graves, 1981b).

A vaccinia-host range mutant was found to contain a deletion of approximately 18 kbp pairs at the left hand end of the genome and by subcloning of the corresponding fragment of the wild type DNA and recombination experiments a 5.2 kbp EcoR1 fragment was found to be sufficient to restore the normal host range (Gillard et al., 1985). TK^- mutants (Kit et al., 1963; Dubbs and Kit, 1964; Weir and Moss, 1983) which obviously cannot express a functional TK gene have been mapped to the 5 kbp HindIII J fragment of the vaccinia genome (Weir et al., 1982; Bajszar et al., 1985). The use of this non-essential gene has played an important role in the development of vaccinia gene vectors.

An unstable variant of the WR strain of vaccinia virus has also been analysed. It contained a 9 kbp deletion of the genome which starts within or just beyond the HincII E/ EcoR1 B/ HindIII C fragment and ends within the N fragment (Moss et al., 1981). This encodes at least 8 mRNAs which apparently are not necessary for infectivity. However, the authors suggested that since the WR strain does not release significant amounts of extracellular virus it might have lost

some elements necessary for this function. Payne (1978) has detected 8 polypeptides unique to the extracellular virus.

The presence of spontaneous mutations can often be detected by the appearance or disappearance of certain restriction endonuclease cleavage sites compared with the wild type genome (Wittek et al., 1978a; McFadden and Dales, 1979; Moss et al., 1981). They may be in the form of excisions (McFadden and Dales, 1979) which involve only a few basepairs, to more extensive deletions involving several thousand basepairs (Archard and Mackett, 1979). These deletions are most commonly detected within or near the ITR sequences (Mackett and Archard 1979; Wittek et al., 1978a, Garon et al., 1978). The smaller (S variant) virus contains a 6.3 x 10^6 deletion of unique DNA sequences of the 123 x 10^6 MW genome (L variant virus). This deletion (Fig. 2-19) was mapped at 6.85 x 10^6 from the left terminus of the vaccinia virus genome just outside the ITR (Nakano et al., 1982). Some individual mutations, due to only a single base alteration, have been determined by the use of restriction endonuclease analysis (McFadden et al., 1980; Schumperli et al., 1980) , Southern transfer (Cooper and Moss, 1978) and R-loop mapping (McFadden et al., 1980; Schumperli et al.,1980). The effect of such alterations on genome expression can be determined by changes in the polypeptide patterns seen in two dimensional SDS-PAGE (Essani and Dales, 1979; McFadden et al., 1980). Mirror-image or symmetrical deletions at both inverted termini have been observed in vaccinia virus DNA. These deletions involved about 250 bp but have not been found to be correlated to any phenotypic property of the virus (McFadden and Dales, 1979).

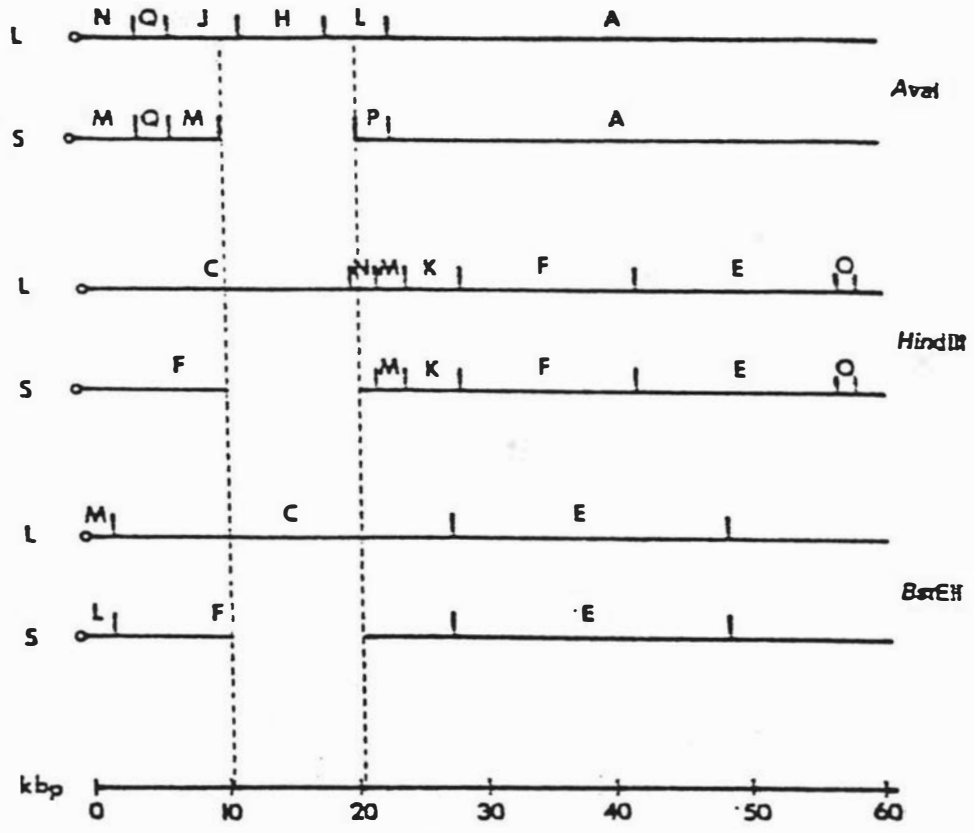


Fig. 2-19. L and S variants of vaccinia virus. Restriction endonuclease cleavage maps of the prototype L variant vaccinia genome and its S variant deletion mutant. The physical locations of the DNA fragments generated by cleavage with AvaI, HindIII, and BstEII and localized within the left terminal 39.1-Mdal SstIIB fragment of the prototype vaccinia genome are shown. (From Nakano et al., 1982).

Using Southern blot and marker rescue techniques, Schumperli et al., 1980) noted a novel EcoRI restriction site in the EcoRI D fragment of the vaccinia genome. This novel restriction site is believed to be associated with a temperature-sensitive mutation.

The spontaneous appearance of haemagglutinin-negative (HA^-) variants has been observed and correlated with the absence of both glycosylation of the HA polypeptide (Weintraub and Dales, 1974; Shida and Matsumoto, 1983) and acquisition of the capacity to induce cell-to-cell fusion (Ichihashi and Dales, 1974).

Mutations may also be induced by the use of chemical mutagens. An α-amanitine-resistant mutant (α-27)was produced by growing wild-type virus in the presence of an inhibitory level of the drug (6 ug/ml). Alpha-27 does not differ from the wild type in growth kinetics although it can grow in enucleated cells. It is also more efficient than the wild-type virus in catalysing proteolytic processing of two major polypeptides, VP62 and VP60. Restriction enzyme analysis revealed that an Xho1 cleavage site was altered in the α-27 DNA molecule (Villareal et al., 1984).

Gene Mapping

Mapping of poxvirus proteins with known or specific functions to discrete locations in the genome began only a few years ago. This was accelerated by the advent of marker rescue and cell-free transcription and translation systems. The

location of some of these genes in the vaccinia virus genome are shown in Fig. 2-12. The first gene to be mapped was the TK gene. This is located within the HindIII J fragment (5,000 bp) approximately 80 kbp from the left hand of the 18 kbp vaccinia genome (Weir et al., 1982; Hruby and Ball, 1982; Bajszar et al., 1983).

The gene coding for DNA polymerase is located within the 15 kbp HindIII E fragment. Subsequent subcloning pin-pointed the exact location to a 2.9 kbp EcoR1 site which is about 45-57 kbp from the left hand of the genome (Jones and Moss, 1984; Traktman, 1985).

The gene coding for the 11k major late structural polypeptide (a phosphopolypeptide) was also located in the HindIII E fragment (Wittek et al., 1984). Early transcripts coding polypeptides with MWs of 110K, 36K,32K, 26K, and 19K as well as some late transcript were mapped within a 7.1 kbp EcoR1 F fragment situated between the HindIII J and H fragments (Mahr and Roberts, 1984a; 1984b). Four polypeptides with MWs of 55K, 30K, 20K and 10K have been mapped to a 5.0 kbp EcoR1 fragment within HindIII MNK region of the vaccinia genome (Morgan and Roberts, 1984). Two major core polypeptides, 4a and 4b, have been mapped to the left hand end of the HindIII A fragment of 50 kb, 105 kbp from the 180 kbp vaccinia genome (Wittek et al., 1984).

The gene responsible for producing red or white pocks on the CAM of eggs infected with cowpox virus has also been mapped (Pickup et al., 1984).

2.8 RECOMBINATION AND REACTIVATION

Genetic recombination has been demonstrated in most orthopoxviruses. So far, recombination has been demonstrated only between members of the same genera (Woodroofe and Fenner, 1960). The recombination frequencies in vaccinia and rabbitpox viruses range from <1% to >75% depending on the particular mutant pair used in the cross (Padgett and Tompkins, 1968; Chernos et al., 1978).

There are two types of genetic reactivation that may occur in viruses. When the inactivated parental genomes are of the same strain it is called multiplicity reactivation. When one parent is inactivated and the other is infectious virus of a related species the process is called cross-reactivation or marker rescue. At the molecular level this latter phenomenon has been used to rescue the deleted DNA sequence by the S variant virus (Nakano et al., 1982) and in mapping genes (Jones and Moss, 1984).

Poxviruses are also capable of undergoing non-genetic reactivation known as the Berry-Dedrick phenomenon (Berry and Dedrick, 1936). This involves the reactivation of viruses whose transcriptases have been destroyed by heat or chemicals, but contain a functional genome, by a virus from a different genus whose genome and transcriptase is intact. This allows the reactiviting virus to synthesize early mRNA which uncoats the products of the undamaged genome.

2.9. POXVIRUSES AS CLONING VECTORS

This topic has been extensively dicussed by Mackett et al. (1982) and Smith and Moss (1984) and thus only an outline is given here to give an indication of the current interest in this area. The use of vaccinia as a gene vector may revolutionise vaccination against some human and animal diseases. At the very least the information gained by the use of such vectors will increase our knowledge of antigens and immune responses in general.

Adequate immunity to many viruses can be achieved only by the use of vaccines containing attenuated virus. The use of this type of vaccine poses certain hazards and problems. However, recent advances in genetic engineering now make possible the use of vaccines prepared from recombinants of an innocuous virus with a foreign gene from a pathogen. These recombinants can express the immunogenic genes of the pathogen without invoking the pathogenic effect.

The vaccinia virus genome can accommodate up to 25,000 base pairs of foreign DNA and still retain its infectivity (Mackett et al., 1982; Smith and Moss, 1983). This property makes it a good cloning and expression vector for foreign genes. Although any non-essential region of the genome can be used, segments containing the gene for the TK is the preferred site because the recombinants (TK^- phenotypes) can easily be selected by plaque formation in the presence of BUdR (Panicali et al., 1983).

Recombinant vaccinia virus containing a cloned haemagglutinin (HA) gene from influenza virus has been constructed (Panicali et al., 1983) by inserting the gene into the non-essential region (Fig. 2-19). Recombinants carrying the hepatitis B surface antigens have also been constructed by inserting the gene into the non-essential TK gene region (see Fig. 2-12) (Smith et al., 1983, Paoletti et al., 1984). Vaccinia virus recombinants expressing the herpes simplex virus glycoprotein D (Paoletti et al., 1984), rabies virus glycoprotein (Wiktor et al., 1984; Kieny et al., 1984), vesicular stomatitis virus genes (Mackett et al, 1985), HTLV-III envelope gene (Chakrabarti et al., 1986) and AIDS virus envelope gene (Hu et al., 1986) have also been constructed. Data from these experiments indicate that the genes expressed by the recombinants are immunogenic and can protect against the particular diseases in experimental animal models.

It has been the steady accumulation of knowledge of the fundamental properties of the poxviruses, and in particular vaccinia virus, which has led to these remarkable discoveries. It is hope that the information presented in this thesis will add to that knowledge and lead to further significant discoveries in this field.

CHAPTER 3

ORF VIRUS CULTIVATION AND VIRAL ASSAY

3.1 INTRODUCTION

Cultivation

Biochemical studies of a virus are facilitated when the virus can be grown to high titre and for most viruses this can be achieved either in laboratory animals or cell culture. For orf virus, most laboratory animals have proved to be refractory to infection. Mild infection in rabbits has been reported (Abdussalam,1957; Wheeler and Cawley, 1956; Darbyshire, 1961) although others have been unsuccessful in infecting this species (Glover, 1928; Howarth, 1929; Boughton and Hardy, 1935; Greig, 1956). Further, unlike the orthopoxviruses which readily grow in chick embryos, orf virus does not propagate in eggs (Newsom and Cross, 1934; Webster, 1958; Darbyshire, 1961). Sheep and goats remain the most susceptible experimental animals, the sheep still being used to grow the virus for vaccine production.

Propagation of orf virus in cell culture was first reported by Greig (1957) who grew the virus in embryonic sheep skin cells. Webster (1958), who also used embryonic sheep skin cells, did not observe a cpe but observed viral multiplication. Other cell types have been tested for their ability to support orf virus multiplication. Nagington and Whittle (1961) used primary monkey kidney cells with success

but failed to infect HeLa, Mk2 and AH9 cell lines. Orf virus has also been successfully grown in primary bovine testis (Plowright et al., 1959, Nagington and Whittle, 1961; McDonald and Bell, 1961) bovine spleen (Hessami et al., 1979; Raffi and Burger, 1985) and bovine lung (Gassmann et al., 1985) cells.

While cell culture-adapted orf virus can be propagated in a number of cell types, virus from clinical material shows a preference for primary cells of sheep or human origin (Nagington, 1968) and for fibroblastic rather than epithelioid cells (Plowright et al., 1959; Sawhney, 1966). This specificity appears to be lost after several passages when the virus becomes adapted to the cell system.

Isolation and propagation of New Zealand strains confirms many of these early observations. The New Zealand strains were able to be isolated directly from scab material, or from virus purified in sodium diatrizoate gradients, using primary lamb testis cells (Robinson et al., 1982). Some strains failed to adapt to cell culture and those that did gave low virus yields. Re-inoculation onto live sheep skin was necessary to produce yields high enough to enable sufficient quantities of virus to be recovered for biochemical studies and radioisotopes were needed to detect viral molecules of interest in cell cultures.

The use of primary cells has other limitations. Calf and lamb testes are available only at certain times of the year and cannot be maintained beyond a limited number of passages.

Viral Assay

As well as being able to cultivate a virus it is also of fundamental importance to be able to assay viral infectivity. As discussed, laboratory animals and egg embryos are refractory to,or unsuitable for, orf virus infection. Erythrocytes from several animal species failed to agglutinate in the presence of orf virus while haemagglutination and haemadsorption assays have been unsuccessful (A. J. Robinson, personal communication).

This chapter describes, firstly, the ability of various cell lines to support the growth of two orf virus isolates and, secondly, experiments done to find a suitable method of quantitating infectious orf virus particles in cell culture systems.

3.2 MATERIALS AND METHODS

Cell Culture and Media. Primary lamb (LT) and bovine testis (BT) cells, foetal bovine lung (FBL) cells, ROK (lamb kidney cells that had undergone at least 30 passages), RK13, MDBK, MDCK and Vero cells were grown in either Eagle's minimum essential medium containing non-essential amino acids (MEM, GIBCO Cat. No. 410-1500) or MEM plus 0.5% lactalbumin hydrolysate (MEM-LAH). Penicillin (100 I.U./ml), Streptomycin (50 µg/ml), Kanamycin (100 µg/ ml) and foetal calf serum (FCS) were added to the medium prior to use. Generally, 2% FCS was used for maintainance and 10% FCS for growth of cells.

Virus Strains. Two virus strains, NZ2 and NZ7, were used. NZ2, which had undergone at least 20 passages in lamb testis cells (Robinson et al., 1982), was used in the form of a crude cell lysate. NZ7 was a vaccine strain from Cooper's Animal Health Laboratory, Upper Hutt: it had been plaque-purified twice in this laboratory.

Propagation of Virus

1. Attempts to propagate NZ2 and NZ7 in different types of cells. Cells were grown to confluence in flasks with base areas of 25 cm^2 and confluent monolayers were infected with 1 ml of NZ2 virus stock. After adsorption of virus for 1 hr at 37°C, MEM containing 2% FCS was added. The cells were incubated at 37°C and were checked daily for cpe. Cells were frozen after 3-4 days at which time complete cpe was observed. The cultures were frozen and thawed 3 times and 1 ml of cell lysate was used as stock virus for subsequent passages.

In one experiment, two roller bottles, each with a surface area of 490 cm^2, one containing BT cells and the other Vero cells, were each infected with 3 ml of NZ7 virus stock. After virus adsorption, infected cultures were incubated at 37°C with MEM-LAH containing 2% FCS. After 2-3 days the cells were frozen and thawed 3 times and sonicated. Attempts to demonstrate the amount of virus in each culture were undertaken as follows. The sonicated cell lysates were centrifuged for 30 min at 3,000 x g in a Sorvall SS34 rotor. The supernatant of each sample was then layered over a 5 ml cushion of 36% sucrose and centrifuged at 65,000 x g in a

Beckman SW27 rotor for 1 hr at 5°C. The resulting pellets were each resuspended in 1 ml amounts of ET buffer, pH 8 (0.025 M Tris and 0.001 M EDTA). These suspensions were layered onto 20 ml volumes of 5%-20% sodium diatrizoate gradients and centrifuged for 16 hr at 65,000 x g at 5°C in a SW25 Beckman rotor. The gradients were then examined for the presence of bands of virus at the expected positions (Robinson et al., 1982).

2. Propagation of NZ7 in BT cells using either MEM-FCS or MEM-LAH-FCS. Using strain NZ7, cultures were infected as above. After adsorption for 1 hr infected cells were incubated at 37°C with either fresh MEM containing 2% FCS or with MEM-LAH containing 2% FCS. When complete cpe was observed the cultures were frozen. After freezing and thawing 3 times, each culture was assayed for viral infectivity as described below.

Assay of Viral Infectivity

1. Quantal Assay. Primary cells were grown in 96-well microtitre plates in MEM-LAH containing 10% FCS. When confluent, monolayers were washed twice with phosphate buffered saline (PBS), pH 7, and 8 replicates were each inoculated with 50 µl of 10-fold serial dilutions of NZ2. Dilutions of the virus were made in MEM-LAH containing 1% FCS. Control cells were mock-infected with an equal volume of the diluent medium. After 1 hr adsorption at 37°C in an atmosphere of 5% CO_2-in-air the inoculum was removed and fresh MEM-LAH containing 2% FCS added to each well. The cells were incubated for 7 days after which time each well was

checked for the presence of viral cpe. Uninfected control monolayers were still healthy after 7 days incubation although some rounded cells could be found. NZ7 was assayed in the same manner using 24-well plates and 4 replicates for each dilution were used (L.B. Fastier, personal communication). In the higher dilutions, wells showing a diffused cpe or presence of plaques were considered positive. The 50% endpoint was determined by the "moving averages" method described by Thompson (1947) and titres were expressed as tissue culture infective dose$_{50}$/ml ($TCID_{50}$/ml).

2. **Plaque Assay.** BT cells were grown in 24-well plates in MEM-LAH containing 10% FCS and antibiotics. When the cells were confluent, duplicate wells were each inoculated with 200 µl of 10-fold serial dulutions of NZ2. After 1 hr adsorption the virus inoculum was aspirated and each well was overlayed with MEM containing 1% agarose and 2% FCS. Cultures were incubated for a further 6 days. On the 6th day, an agarose overlay containing 0.01-0.02% Neutral Red was placed over the first overlay. Plaques were counted microscopically or macroscopically the following day. NZ7 was also titrated in the same way. Titres were expressed as plaque forming units/ml (pfu/ml).

3. **Fluorescent Focus Assay.** Vero cells were grown on glass cover-slips in sterile plastic vials flooded with MEM-LAH containing 10% FCS. When confluent growth was obtained, duplicates were inoculated with serial dilutions of NZ7 grown in BT cells in MEM-LAH containing 2% FCS. After 1 hr adsorption at 37^{o}C the monolayers were washed and incubated

further in MEM-LAH containing 2% FCS. At selected times samples were taken and fixed in acetone. Infected cells were stained first with orf antiserum prepared in rabbits as described below and then with fluorescein-labelled goat anti-rabbit serum (Cappel Laboratories, Malvern, PA., U.S.A.). A standard procedure for staining was followed. Cells were fixed in acetone (two changes) for 10 min and washed 3 times in phosphate buffer, pH 8, (5.5 ml of 0.1 M KH_2PO_4 and 94.5 ml of 0.1 M Na_2HPO_4). The monolayers were drained, air-dried, flooded with antiserum against orf virus for 1 hr, washed thoroughly with phosphate buffer, pH 8, and then stained with fluorescein-labelled goat anti-rabbit serum for 30 min. The cells were again washed thoroughly, mounted on glass slides and examined by U.V. microscopy.

Preparation of rabbit antiserum against orf virus. Ten mg of pooled scab material from several sheep were triturated in TE buffer, pH 8 (10 mM Tris, 1 mM EDTA). The suspension was given a low speed centrifugation to remove cell debris and the supernatant divided into 2 aliquots. One aliquot was mixed with Freund's complete adjuvant and inoculated intramuscularly into two rabbits. After 1 month the rabbits were inoculated intramuscularly with the other aliquot of virus mixed with Freund's incomplete adjuvant. The rabbits were bled after 2 months, the serum separated and stored at $-20^{o}C$.

3.3 RESULTS

Propagation of Virus

1. Propagation of NZ2 and NZ7 in different cell types. If a cpe could be elicited at each successive passage of the virus, this was taken as evidence of the ability of a cell type to support viral growth. Results are shown in Table VII. When primary cells of sheep and bovine origin were infected with strain NZ2, rapid and progressive cpe was observed on the first and subsequent passages and complete cpe was seen in 1-3 days. Rounding of cells was followed by clumping and the cells detatched from the plastic on prolonged incubation. When continuous cell lines were used, a rapid cpe was seen on first passage but on subsequent passages the effect on the cells gradually decreased until no cpe was seen after the third or fourth passage. Clumping of rounded cells was not as pronounced as that seen when primary cells were used.

Vero cells did not support virus growth to any significant level. When NZ7 grown in roller bottles containing Vero cells was partially purified on sodium diatrizoate gradients, no distinct band of virus was visible. Conversely, when the virus grown in roller bottles of BT cells and purified in diatizoate gradients a visible band of virus was seen.

2. Propagation of NZ7 in MEM-FCS and MEM-LAH-FCS. In three experiments, virus grown in MEM-LAH-FCS showed a more extensive cpe and gave higher yields than did virus grown in MEM-FCS. Virus grown in MEM-LAH-FCS gave an average yield of

2.7×10^7 pfu/ml with a range of 0.5 - 5×10^7. Virus grown in MEM-FCS alone gave an average yield of 2×10^6 pfu/ml, the range being 1 - 3×10^6 pfu/ml. The method used for plaque titration is given below.

Assay of Viral Infectivity

1. Quantal Assay. Results obtained from the quantal assay using 96-well micotitre plates were inconclusive. Even after one week of incubation complete cpe was never detected in those dilutions that were expected to contain a few particles or a single particle. On microscopic examination of monolayers, cell rounding at the expected end point dilutions could not be distinguished from rounding in control cells. The very small surface areas of the wells contributed to the difficulty in detecting a cpe. A better assessment of cpe could be made using the 24-well plates although complete cpe was never detected in those wells containing high dilutions of virus. In some cases, however, plaques could be seen at higher dilutions. Wells containing plaques or cell rounding were considered virus-positive. Results obtained by this assay are shown in Table VIII.

2. Plaque Assay. Fig. 3-1 shows orf virus plaques in BT cells after one week of incubation. Some plaques were large enough to be counted macroscopically while others required the use of a microscope and Neutral Red staining of the monolayers before they could be identified with certainty. Titres obtained by this method are shown in Table VIII.

Table VII. Propagation of Orf Virus NZ2 in Different Types of Cells.

Passage No.	Days	Presence of CPE						
		LT	BT	FBL	ROK	MDBK	MDCK	RK13
1	1	++	++	++	++	++	++	++
	2	+++	++	++	++	+++	+++	+++
	3		++	+++	++			
	4		+++					
2	1	++	++	++	++	++	++	++
	2	++	+++	++	++	++	++	++
	3	+++		+++	++	++	++	++
	4				++	++	++	++
3	1	++	++	++	++	+	+	+
	2	+++	+++	++	++	+	+	+
	3			+++	++	+	+	+
	4							
4	1	++	++	++	++	-	-	-
	2	+++	+++	+++	++	-	-	-
	3				++	-	-	-
	4				++	-	-	-

\+ less than 50%
++ more than 50%
+++ 90-100%

LT primary lamb testis cells
BT primary bovine testis cells
FBL foetal bovine lung cells

Table VIII. Comparison of Orf Virus Titres By Quantal and Plaque Assay

Virus		Quantal Assay ($TCID_{50}$/ml)	Plaque Assay (pfu/ml)
NZ2		inconclusive	2×10^5
NZ7	(a)	5×10^6	10^6
	(b)	9×10^6	2×10^6

3. Fluorescent Focus Assay. The result of an experiment using the fluorescent antibody method to detect virus-infected cells is shown in Fig. 3-2. At 24 hr and 72 hr p.i. the 10^{-1} dilution shows that almost all cells were showing fluorescence. At 72 hr p.i. monolayers infected with higher dilutions of virus showed minute foci of fluorescing cells. These were considered to be equivalent to a plaque. No attempt was made at this stage to titrate the virus by this method.

3.4. DISCUSSION

These results extend the observations of Nagington and Whittle (1961) that continuous cell lines infected with orf virus fail to support viral growth to significant levels and confirms other reports of the preference of orf virus for primary cells. It was also shown that the LAH had an enhancing effect on virus growth. This effect is probably due to a higher cell density being achieved in culture or, alternatively, to the the cells being able to support virus multiplication for longer incubation periods. This latter effect was commented on by Hessami *et al.*(1979) although these workers did not present data to support this. It was decided to use BT cells and MEM-LAH containing FCS in subsequent experiments, unless indicated otherwise.

Two strains, NZ2 and NZ7, were used in the above experiments. Extensive research had already been done on the DNA structure of NZ2 (Robinson, personal communication) and transcription

Fig. 3-1. **The titration of orf virus by plaque assay.** Bovine testis cells were infected with NZ7. Monolayers were stained with 0.01% Neutral Red 6 days after infection.

Fig. 3-2. **Fluorescent antibody staining of orf virus-infected cells.** Serial dilutions of virus were inoculated onto vero cells were fixed in acetone at different times, after infection and stained with orf antiserum and fluorescein-labelled goat anti-rabbit serum. A. 10^{-1}, 24 hr p.i.; B. 10^{-1}, 72 hr p.i.; C. 10^{-4}, 24 hr p.i.; D. 10^{-4}, 72 hr p.i.

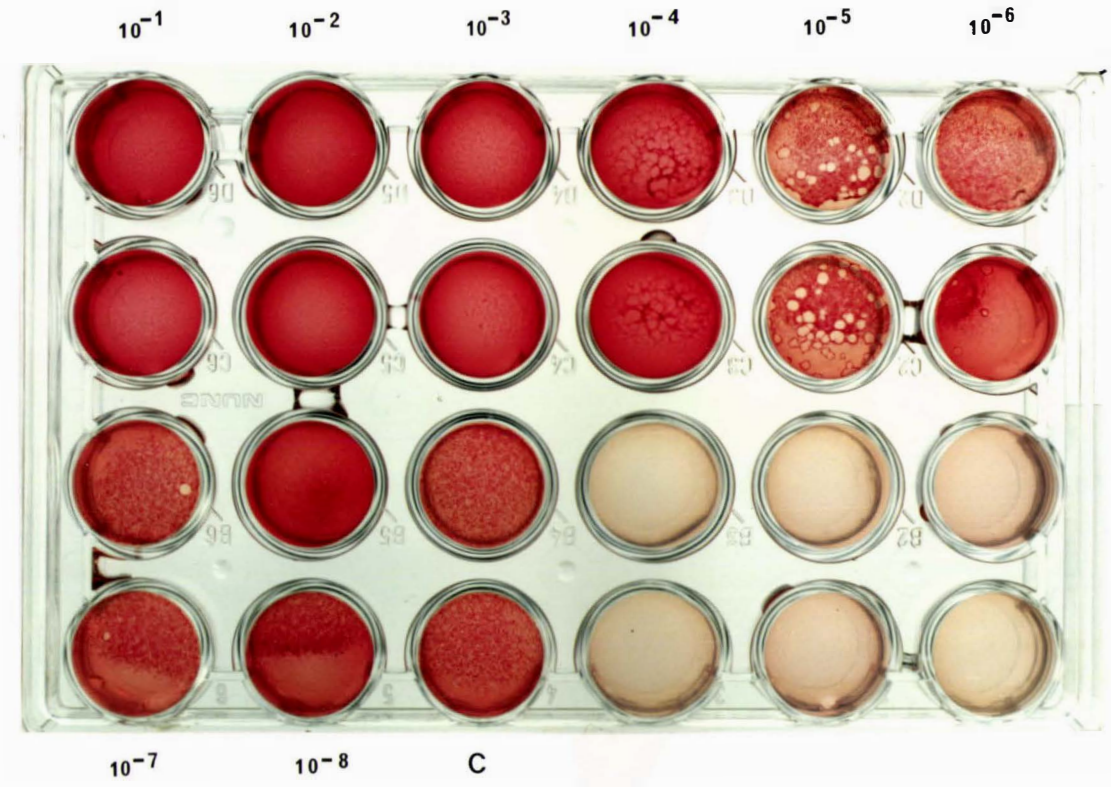


Fig. 3-1

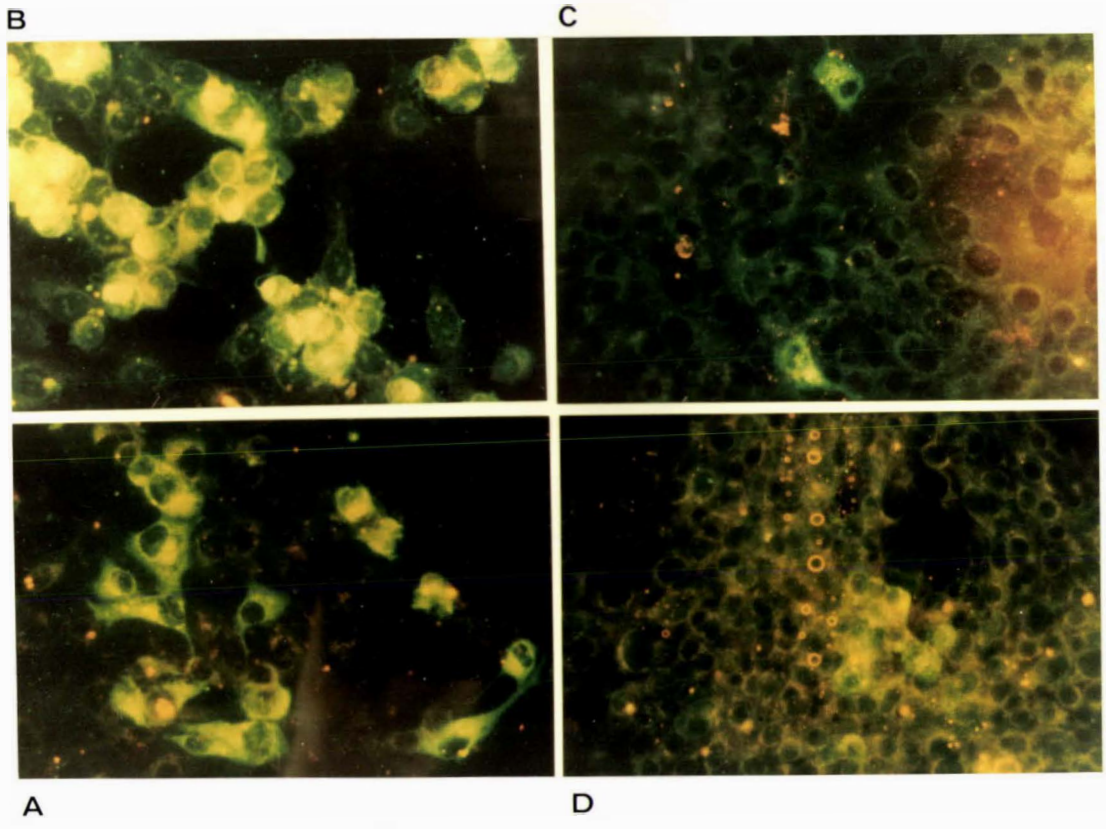


Fig. 3-2

studies are planned. It was therefore desirable that studies on the biology of orf virus be undertaken using this particular strain. It was used initially but difficulties in obtaining high titred stock virus restricted its usefulness. NZ2 gave yields of 5×10^5/ml or lower while NZ7 gave yields as high as 5×10^7/ml. It was thus decided to use NZ7 for subsequent experiments.

In the quantal assay, infected monolayers were incubated for one week before reading the results and end points were assessed by the presence of cpe and/or plaques. Since a single infectious particle did not produce complete destruction of the monolayers in the quantal assay it was assumed that neither NZ2 nor NZ7 spread rapidly between cells.

1t also took one week to produce recognizable plaques using the 2 strains of virus in the plaque assay. By comparison, vaccinia virus produces visible plaques in 48 hr in BT cells (personal observation) or in CV-1 cells (Panicali and Paoletti, 1982). It was observed that orf virus could occassionally produce plaques in the absence of a solid overlay, as seen in the quantal assay, but plaques could only be produced consistently under solid media.

Titres obtained in the quantal assay were slightly higher than those obtained in the plaque system. Higher estimates of titres are often obtained when quantal assays are used (Goodheart, 1969).

The fluorescent focus assay (FFA) gave encouraging results and may prove to be a more sensitive assay than either the quantal or plaque assay for detecting infectious foci early post-infections. However, some problems were encountered with the FFA. One was that primary cells did not grow consistently well on glass surfaces. The use of Vero cells which, surprisingly, seemed to give positive fluorescing cells in the virus-infected monolayers might be worth exploring in more detail even though they could not be used for propagating virus. Whether the fluorescing cells were undergoing a fully infectious cycle or only an abortive infection was not ascertained. This is a potential problem when using the fluorescent antibody test as an assay for viral infectivity. Unlike the plaque assay which will detect only the production of virus, The FFA will detect both antigen and infectious virus in an infected cell. A careful comparison of the plaque assay and FFA would need to be done to see if pfus correlated with fluorescent foci. Another problem was that staining of individual coverslips was a tedious procedure and consumed expensive reagents. The use of compartmentalised plastic microscope slides suitable for microassay might improve the technique.

The plaque assay gave results as consistent as those determined with the quantal assay, and was more reproducible. This led to its being used to determine virus titres in all subsequent experiments.

CHAPTER 4

THE EFFECTS OF PHYSICAL TREATMENTS ON VIRUS TITRES

4.1 INTRODUCTION

Effects of Physical Treatments on Virus Titres

With many poxviruses, the majority of the virus particles remain cell-associated at the end of the growth cycle and can be released by subjecting the cells to multiple cycles of freezing and thawing (Green et al., 1942; Joklik, 1962b; Moss and Rosenblum, 1973) and sonication (Stern and Dales, 1974). It has been shown that these physical methods not only release the virus from the cells but also disrupt aggregates of virus thereby effectively increasing the titre of the virus stock.

Trypsin treatment can also increase the titres of vaccinia (Planterose et al., 1962; Joklik, 1962b; Zwartouw et al., 1968; Gifford and Klapper, 1967) and influenza viruses (Tobita et al., 1975; Lazaro and Chopin, 1975; Klenk et al., 1975).

In our experience, New Zealand field isolates of orf virus do not give high yields of virus when grown in BT cell culture. The titres of field isolates in early passages are about 10^5/ml. The vaccine strain, NZ7, grows relatively better than do the field isolates and gives titres similar to that reported for other strains of orf virus isolated in

other countries. For instance, NZ7 can grow to about 10^8 $TCID_{50}$/ml (L.B. Fastier, personal communication), whereas a human orf virus (Shoe) isolated in North America gave yields as high as 1.2 x 10^8 $TCID_{50}$/ml (Hessami et al., 1979). The reasons why New Zealand field isolates of orf virus appear to grow so poorly have not been established but if the virus remains strongly cell-associated in culture it may be possible to maximize virus titres by vigorous disruptions of the cells.

Extracellular and Intracellular Virus

It is therefore important to know to what extent the virus remains cell-associated. In vaccinia-infected cells, the proportion of extracellular and intracellular virus depends on the strain used. With the WR strain a large proportion of progeny virus remains cell-associated and only 10% or less is released into the medium in most cell culture systems (Furness and Younger, 1959; Smith and Sharp, 1960). When the IHD-J strain is grown in RK13, 60% is released as extracellular virus and when grown in L cells only about 30% is released into the medium (Mallon and Holowczak, 1985).

This chapter describes, firstly, the effect of various physical treatments on the titre of orf virus grown in BT cells and, secondly, the amounts of virus seen extracellularly and intracellularly.

4.2 MATERIALS AND METHODS

Cells and Virus. BT cells were grown in 10 x 35 mm^2 dishes with surface areas of 96 cm^2. For plaque titration, primary BT cells were grown in 24-well plates in MEM-LAH containing 10% FCS. Virus stock (NZ7) was grown in BT cells in 175 cm^2 flasks in MEM-LAH containing 2% FCS.

Effect of Physical Treatments on Virus Titres:

(a) Freezing and Thawing with or without Sonication. NZ7 was grown in BT cells for 48 hr. The infected cells were scraped into 30 ml of MEM-LAH containing 2% FCS. Five ml of the virus suspension were frozen and thawed 3 times. The remaining cell suspension (25 ml) was centrifuged at 11,950 x g in a Sorvall SS34 rotor for 15 min. The pellet was resuspended in 2.5 ml of MEM-LAH containing 2% FCS. The cell pellet was frozen and thawed 3 times and sonicated 6 times, each for 10 sec, on ice. Sonication was done with a soniprobe (Dawe Instrument Ltd., London, England) at maximum setting. A 10-fold dilution was made for each sample and titrated using the plaque assay.

(b) Sonication and centrifugation. Virus stock grown in BT cells was given 3 cycles of freezing and thawing and divided into 4 aliquots of 5 ml each. One aliquot was sonicated and then centrifuged at 470 x g for 2 min, another was sonicated but not centrifuged, and a third not sonicated but centrifuged. The fourth aliquot was neither sonicated nor centrifuged. Serial dilutions were made of each aliquot and their virus titres determined by plaque assay. The low-speed

centrifugation was included in this experiment to determine whether or not a significant proportion of the virus was lost during centrifugation of the virus suspension.

Determination of the Proportion of Extracellular and Intracellar Virus

The proportions of extracellular and intracellular virus were determined in 2 ways.

(1) NZ7 was propagated in BT cells grown in 10 x 35 mm dishes for 48 hr. Cell-associated virus and virus that had been released into the medium were separated by centrifugation of the infected cells at 500 x g. The pelleted cells were then disrupted by 3 cycles of freezing and thawing before being sonicated for 3 min. The virus titres of both the supernatant and of the disrupted cells were determined by plaque assay.

(2) NZ7 was propagated in BT cells grown in 150 cm^2 flask for 48 hr. The infected cells were centrifuged at 500 x g. The pelleted cells were processed as described above. The supernatant was centrifuged at 45,000 x g for 30 min. The virus titres of both the supernatant and the disrupted cells were determined by plaque assay.

4.3 RESULTS

The Effect of Physical Treatments on Virus Titres

(a) Freezing and thawing with or without sonication. The effects on virus titres of freezing and thawing alone or with

subsequent sonication are shown in Table IX. Freezing and thawing of virus-infected cells coupled with sonication gave a higher virus titre than did freezing and thawing alone. There was about a 2-fold increase in titre when sonication was included. (It should be noted that the results, expressed in pfu/ml, take into account the different volumes of cell suspension used in the two experiments).

Table IX. Effect of Freezing/Thawing With or Without Sonication On Virus Titres

Treatment	Titre (pfu/ml)
Freeze/Thaw	5×10^6
Freeze/Thaw plus sonication	1.2×10^7

(b) Sonication and centrifugation. Sonication of freeze/thawed infected BT cells increased the virus titre 10-fold irrespective of whether or not the sonication was followed by centrifugation (Table X). There was a slight decrease in titre when centrifugation was included in the method.

Determination of the Proportions of Extracellular and Intracellular Virus

The proportions of extracellular and intracellular virus were determined at the end of a 48 hr incubation. The results are

determined at the end of a 48 hr incubation. The results are shown in Table XI. In the two experiments 6% and 0.5% of the virus yield was released from infected cells (extracellular) while 94% and 99% respectively remained cell-associated (intracellular).

Table X. Effect of Sonication and Centrifugation on Viral Infectivity

Treatment	Titre (pfu/ml)
Sonication / centrifugation	2.5×10^7
Sonication / no centrifugation	3×10^7
No sonication / centrifugation	3.5×10^6
No sonication / no centrifugation	4.5×10^6

Table XI. Extracellular and Intracellular Virus After One-Step Growth Cycle of Orf Virus in BT Cells.

Expt.No.	Extracellular (% total)	Intracellular(% total)
1.	3×10^5 (0.5%)	8×10^7 (99.5%)
2.	4×10^6 (6.5%)a	6.1×10^7 (93.5%)

(%) percentage of total (a) concentrated virus

4.4 DISCUSSION

A significant increase in virus titre was noted when frozen/thawed lysates of virus-infected cells were subsequently sonicated and this is in agreement with results obtained with other poxviruses (see Introduction). The slight decrease in virus titre associated with low-speed centrifugation might be due to the continued cell-association of a number of virus particles.

Over 90% of newly synthesised orf virus remains cell-associated during the viral growth cycle. This level of cell-associated virus is similar to that observed with the WR strain of vaccinia virus (Easterbrook, 1962) and the Shoe isolate of orf virus grown in bovine spleen cells (Hessami et al., 1979). It would be interesting to see whether or not the extracellular and intracellular forms of orf virus differ in such features as infectivity or polypeptide structure, such differences having been noted for vaccinia (Boulter and Appleyard, 1973; Payne and Norrby, 1978; Payne, 1978).

In summary, the above experiments showed that the titre of orf virus can be enhanced by freezing and thawing and subsequent sonication of the infected cells. The results also provided evidence that most of the virus produced remains cell-associated which is consistent with the need for the above treatments to artificially release the virus from host cells.

CHAPTER 5

ANALYSIS OF VIRION POLYPEPTIDES

5.1 INTRODUCTION

Comparison of New Zealand orf isolates by DNA restriction endonuclease analysis provided evidence of strain variation (Robinson et al., 1982). Heterogeneity in DNA structure has also been detected among European and North American strains (Sawhney, 1966; Precausta and Stellman, 1973; Wittek et al., 1980; Gassman.,et al., 1985; Raffi and Burger, 1985). Using SDS-PAGE, Buddle et al. (1984) examined the structural polypeptides of North American isolates and found differences mainly in those polypeptides with MWs of 37K-44K.

The aims of the work reported in this chapter were, firstly, to compare by SDS-PAGE analysis the number and MWs of polypeptides obtained from 6 New Zealand orf isolates, secondly, to identify virion surface polypeptides separated from core components by treatment of virions with Nonidet P40 (NP40) and 2-mercaptoethanol (2-ME) and, thirdly, to separate and identify the surface tubule (ST) polypeptide(s) from other virion components.

5.2 MATERIALS AND METHODS

Cells and Media. BT cells up to the tenth passage were grown in medium 199 (GIBCO) buffered with Tricine (T199) or in

methionine-free MEM (based on GIBCO Eagle's MEM formulation). The media contained either 2% or 10% FCS according to need.

Viruses. Six field isolates, identified as NZ2, NZ7, NZ9, NZ10, NZ12 and NZ19 were used and all had been adapted and plaque-purified twice in primary LT cells. Virus stocks, which had undergone at least 20 passages, were grown in BT cells.

Labelling of Virion Polypeptides with ^{35}S-Methionine. Primary BT cells were grown in 75 cm^2 flasks. When confluent growth was achieved, the cells were washed with methionine-free MEM warmed to 37°C. Cells were infected with untitrated, concentrated virus stocks of NZ2, NZ7, NZ9, NZ10, NZ12 and NZ19 suspended in methionine-free MEM. As a control, a flask of uninfected BT cells was mock-infected with 1 ml of methionine-free MEM. After 1 hr adsorption at 37°C the inocula were removed and the cultures were incubated with MEM containing one-third of the recommended quantity of unlabelled methionine (in the GIBCO Eagle's MEM formulation) plus 100 µCi/flask of ^{35}S-methionine (Amersham International, Bucks, England., specific activity >12,000 Ci/mmol) and 2% FCS. Infected cultures were further incubated at 37°C until 90% - 100% cpe was detected after which time they were stored frozen at -70°C.

Purification of Labelled Virus. ^{35}S-methionine-labelled orf virus was purified by a combination of the methods of Joklik (1962b) and of Esposito *et al.* (1978), with some modifications. Isotope-labelled cultures were given 3 cycles

of freezing and thawing followed by sonication for 4 min using a Dawes soniprobe at maximum setting. Cell debris was removed by centrifugation in a Sorvall SS34 rotor at 3,000 x g for 30 min. The virus was pelleted from the resulting supernatant by centrifugation through a cushion of 36% sucrose in a Beckman SW27 rotor at 65,000 x g. The pellet was resuspended in 1 ml of ET buffer, pH 8 (0.025M Tris, 0.001M EDTA). The virus suspension was then layered onto a 25% - 50% sodium diatrozoate gradient (Robinson et al., 1982). After overnight centrifugation at 65,000 x g at 5°C, fractions were collected from the gradient and samples were assayed for radioactivity. Fractions containing peak radioactivity were pooled and diluted to a final volume of 4 ml with ET to reduce the density of the gradient. This was centrifuged through a cushion of 36% sucrose at 45,000 x g in a SW 50.1 rotor. The pellet was resuspended in 1 ml of ET buffer and dialysed against 3 changes of the same buffer. Samples from each virus preparation were counted in a Beckman liquid scintillation counter to determine the total amount of ^{35}S-methionine incorporated. Control uninfected cells were processed in a similar manner.

SDS-PAGE of Virion Polypeptides. The discontinuous buffer system of Laemmli (1970) was used to analyse virus polypeptides by SDS-PAGE. Aliquots containing 100,000 or 300,000 cpm were taken from the purified virus preparations, lyophilised and resuspended in 50 µl of SDS-PAGE buffer (see Appendix). The samples were then immersed in boiling water for 2 min. Proteins were resolved in either 10% gels or 5% - 20% gradient slab gels, each with a 3% stacking gel.

Electrophoresis was performed at 150V for stacking and then at 200 - 250V when the tracking dye had reached the resolving gel. Electrophoresis was continued until the dye reached about 1 cm from the bottom of the gel. Gels were fixed and stained for 2 hr with 0.25 Coomassie Brilliant Blue R (see Appendix) and destained for 16 - 20 hr by diffusion in destaining solution (see Appendix). Molecular weight markers (SDS 70 and SDS 200, Sigma Chemical Co., St. Louis, MO., U.S.A.) were included in every gel. Staining was done for three reasons. One, to give a quick assessment of the quality of the gel, two, to correlate the positions and number of stained polypeptides and, three, to locate the MW markers. The gels were dried in an LKB gel dryer for 1 hr and then autoradiographed using Kodak X-OMAT X-ray film. Autoradioraphy was done at -70^{o}C for up to 2 months.

Effect of Protease Inhibitors on Virion Polypeptides. In order to test whether or not proteases present in host cells cause some degradation of virion polypeptides during virus purification, an experiment was done which included the presence of the protease inhibitors phenylmethylsulphonyl-fluoride (PMSF) or soybean trypsin inhibitor throughout the purification process of ^{35}S-methionine-labelled virus. PMSF (0.1 mM) and soybean trypsin inhibitor (100 µg/ml) were present in the virus suspension throughout the purification process. Processing of virus-infected and uninfected cells followed the procedure described above.

Identification of Surface Polypeptides by Controlled Degradation of Orf Virus. Virus cores can be released from viral lipid envelopes by non-ionic detergents. The method described by Easterbrook (1966) was used with some modification (Thomas et al., 1980; Buddle et al., 1984). Purified NZ7 (150,000 cpm) in 1 ml ET buffer was treated with 1% NP40 and 0.1% 2-ME for 60 min at 37°C with constant agitation. The detergent-treated virus suspension was sonicated using a Dawes soniprobe at maximum setting for 10 sec. The suspension was then centrifuged at 30,000 x g for 30 min at 4°C and the resulting pellet was resuspended in 0.001 M Tris, pH 8. The resuspended pellet and the supernatant were dialysed against distilled water and lyophilised.

Identification of the Surface Tubule Polypeptide(s). The method of Stern and Dales (1976) with some modification was used to purify surface tubules (ST). After treatment of 1 ml of NZ7 virus suspension with NP40 and 2-ME and sonication, the suspension was layered over 800 µl of 1.17 M sucrose in 0.001 M Tris, pH 8, and centrifuged at 100,000 x g for 20 min. The supernatant and interface fractions were pooled, dialysed against water and lyophilised. The freeze-dried fractions were resuspended in SDS-PAGE buffer and boiled for 2 min. The samples, whole virus preparations and SDS markers were loaded into separate wells in a 10% gel and electrophoresed as described above. After staining and destaining, the gel was subjected to fluorography using the enhancer Amplify (Amersham), according to the manufacturer's recommendations. The gel was soaked in Amplify for 20 min

with continuous agitation by rocking. The gel was dried and then exposed to sensitised Dupont Cronex X-ray film (Cat no.568-361-21A). The film was sensitised by pre-exposure to a brief flash of light according to Laskey and Mills (1975). For NZ2, NZ9, and NZ12, STs were partially purified by detergent treatment as above but without sonication. After treatment with NP40 and 2-ME, the treated virus suspensions were each layered onto 800 ul of 1.17 M sucrose cushions in ET buffer. These step-gradients were centrifuged at 100,000 x g for 20 min in a Sorvall HB4 rotor. Each pellet was resuspended in 50 ul SDS-PAGE buffer while the supernatant and interface were pooled, diluted with 4 ml of ET buffer to reduce density and centrifuged at 100,000 x g in a Beckman SW 50 rotor for 16 hr. The pellet was resuspended in SDS-PAGE buffer and, in parrallel with the pellet from the first centrifugation and a whole virus preparation of each strain, was boiled for 2 min. The samples were loaded into separate wells of a 5% - 20% gradient gel with a 3% stacking gel. After electrophoresis the gels were stained in 0.25% Coomassie Brilliant Blue R, destained and dried. The dried gels were autoradiographed using Kodak X-Omat x-ray film. Exposure time was 2 weeks to 2 months at -70^{o}C.

Electron microscopy. To monitor the controlled degradation of orf virions, samples were taken from the detergent-treated NZ7 virus before sonication and after fractionation and lyophilisation, The samples were applied directly to Formvar-coated copper grids. Samples were stained with 0.5% uranyl acetate, blotted, washed with water, blotted, air dried and examined in a Hitachi HUII electron microscope.

5.3 RESULTS

SDS-PAGE of ^{35}S-Methionine-Labelled Virion Polypeptides. Six New Zealand strains of orf virus were propagated in the presence of ^{35}S-methionine. SDS-PAGE electrophoresis of purified virus showed 25-33 well-resolved ^{35}S-methoinine-labelled polypeptides with MWs ranging from 130K - 10K. Autoradiograms of the different strains on linear 10% and 5 - 20% gradient gels are shown in Figs.5-1 and 5-2, respectively. The polypeptide profiles of strains NZ2, NZ7, NZ9, NZ10 and NZ12 were similar except for differences in some minor bands. Strain NZ19 showed more marked difference from the other 5 strains especially in the region of MW about 38.5K. Table XII shows the MWs of the polypeptides and differences between the isolates. Each isolate gave a different pattern due to the presence or absence of certain bands. Uninfected cells used as a control did not yield any significant peak of ^{35}S-methionine-labelled material in the diatrizoate gradients. The total counts recorded from uninfected cells and finally available for gel analysis was less than 200 cpm. As shown in Fig. 5-3 no bands were detected in uninfected BT cells incubated in the presence of ^{35}S-methionine and processed in the same manner as the virus-infected cells. This indicates that the polypeptide bands detected were of virus origin. Repeated gel preparations of the same samples gave the same results although time did not permit the labelling experiment to be repeated.

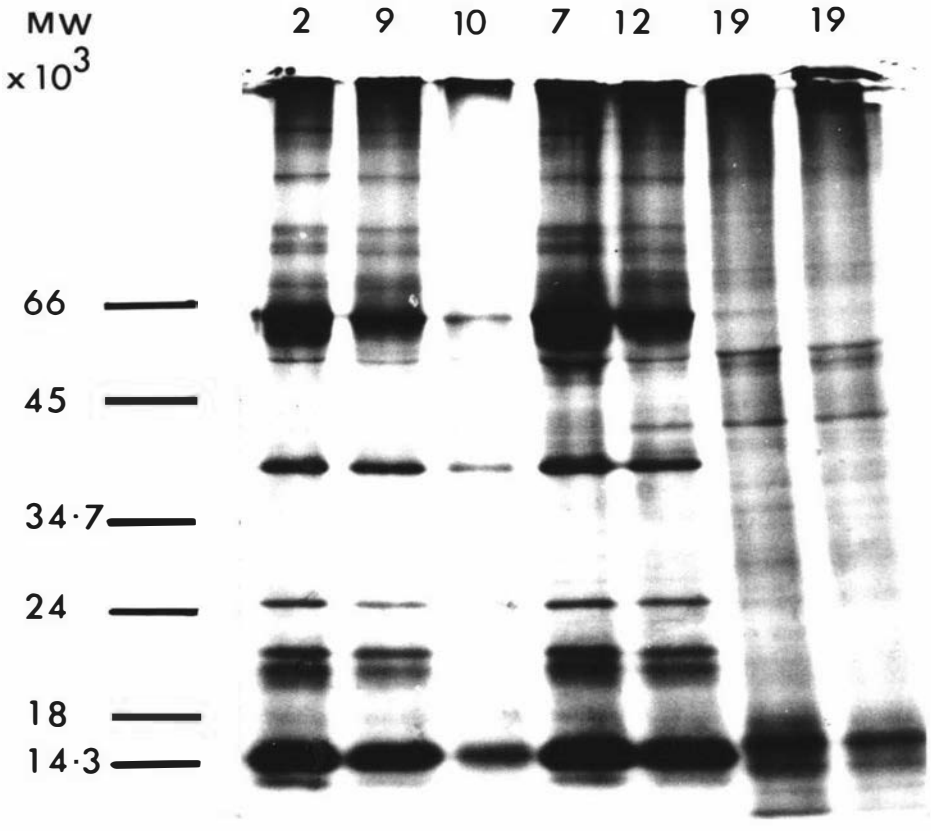


Fig. 5-1. **Autoradiogram of the polypeptides of six NZ orf virus strains labelled with ^{35}S-methionine.** Samples each containing 100,000 cpm were analysed in a 10% polyacrylamide gel.

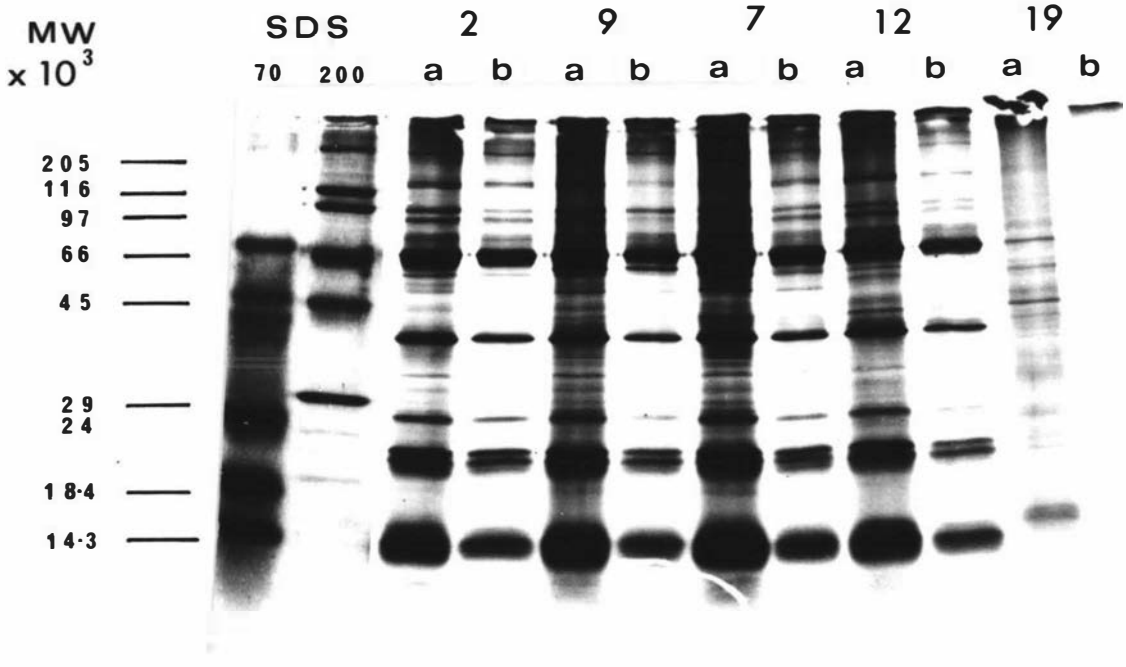


Fig. 5-2. **Autoradiogram of the polypeptides of five NZ orf virus strains labelled with ^{35}S-methionine.** Samples were analysed in a 5-20% polyacrylamide gradient gel. (a) 300,000 cpm per well (b) 100,000 cpm per well. (SDS) molecular weight markers.

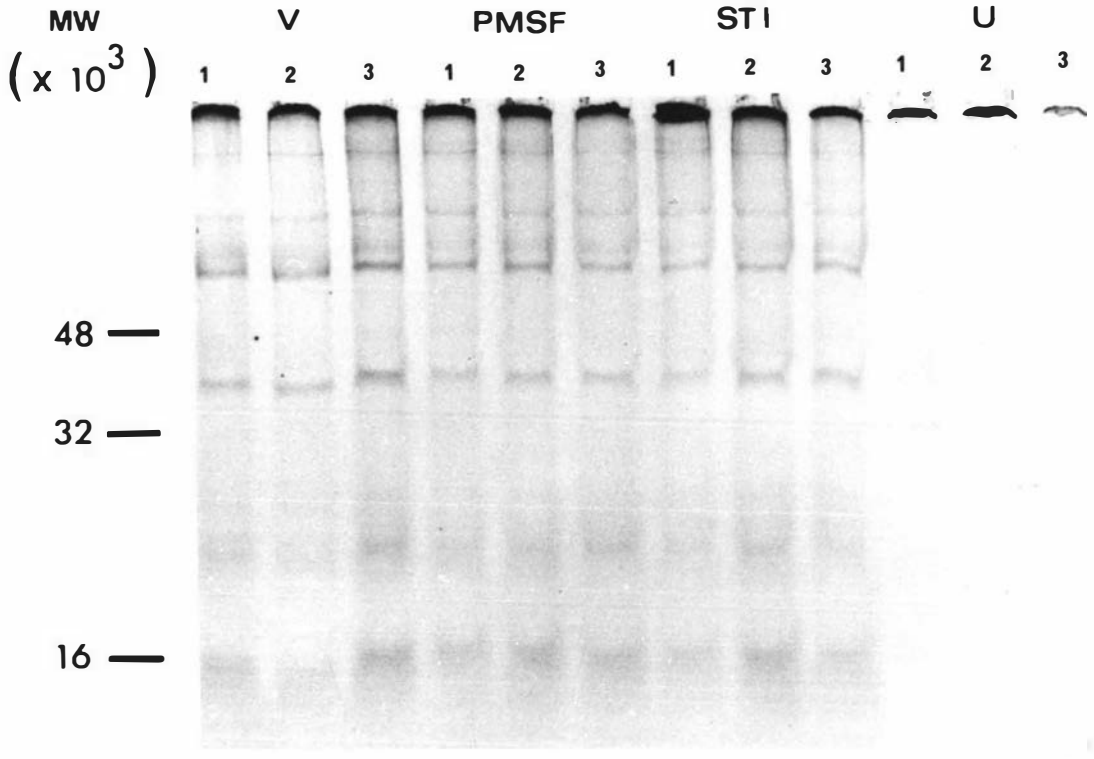


Fig. 5-3. **Autoradiogram of ^{35}S-methionine-labelled polypeptides of orf virus purified in the presence of protease inhibitors.** The SDS-PAGE analysis was done in a 10% gel. (V) untreated virus; (PMSF) phenylmethylsufonylfluoride; (STI) soybean trypsin inhibitor; (U) uninfected cells.

Effect of Protease Inhibitors on Virion Polypeptides. No differences were detected between the polypeptides from PMSF- and soybean trypsin inhibitor-treated virus and those from untreated virus (Fig. 5-3).

Identification of Polypeptides Solubilised in NP40 and 2-ME. When ^{35}S-metionine-labelled NZ7 was treated with NP40 and 2-ME and sonicated for 10 sec, 12 isotope-labelled polypeptides were released from the core as shown by SDS-PAGE analysis of the different fractions (Fig. 5-4). Some of these polypeptides were either not detectable in the pellet fraction or their concentrations (relative to those of whole orf virus) were reduced as indicated by the intensity of the bands. The estimated MWs of the core (pellet) and surface (supernatant) polypeptides are shown in Table XIII.

Attempts to Visualise the Surface Tubule Polypeptide(s). Further purification of the detergent-solublized proteins produced enhancement of a polypeptide with a MW of approximately 38.5K (Fig. 5-4). Controlled degradation and purification of the STs of NZ2, NZ9 and NZ12 also showed enhancement of a polypeptide with a MW of about 38.5K after SDS-PAGE analysis (Fig. 5-5). The 38.5K dalton polypeptide is believed to be the component of the ST. However, EM examination of fractions after treatment with NP40 and 2-ME failed to reveal morphological evidence of surface tubules in the sample preparations.

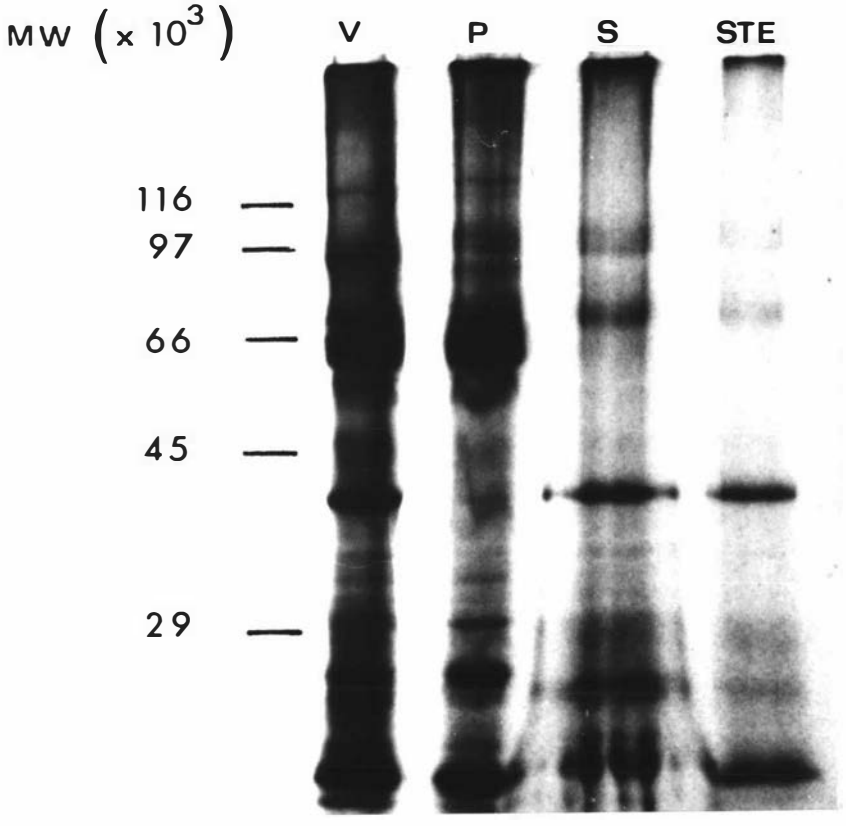


Fig. 5-4. **Autoradiogram of orf virus NZ7 polypeptides released after treatment with NP40 and 2-ME and sonication.** The 10% polyacylamide gel was treated with Amplify and exposed to Cronex X-ray film. (V) whole virion; (P) pellet; (S) supernatant; (STE) surface tubular element after partial purification.

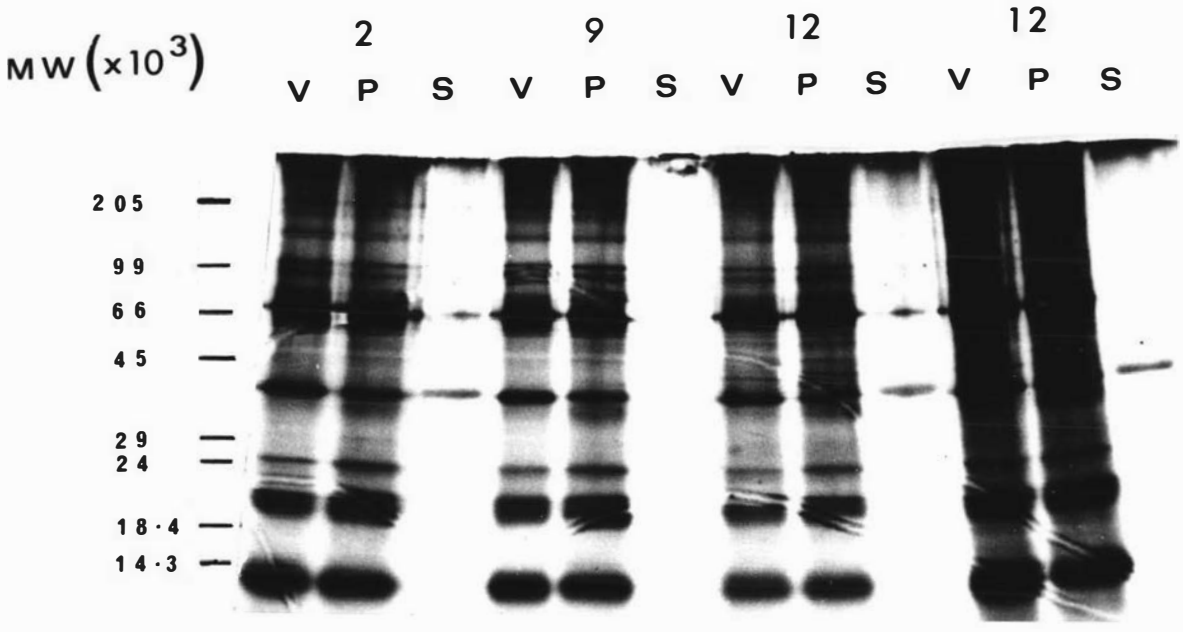


Fig. 5-5. **Autoradiogram of ^{35}S-methionine-labelled polypeptides of orf virus strains after treatment with NP40 and 2-ME, samples were fractionated according to Stern and Dales (1976) and analysed by SDS-PAGE in a 5-20% gradient gel.** (V) whole virion; (P) pellet fraction; (S) supernatant fraction.

Table XII. Comparison of the Structural Polypeptides of NZ orf virus isolates

Band No	Approx MW($x10^3$)	ISOLATE NZ2	NZ7	NZ9	NZ10	NZ12	NZ19
1	130	+	+	+	+	+	-
2	125	+	+	+	+	+	-
3	118	+	+	+	+	+	-
4	105	+	+	+	+	+	+
5	104	+	+	+	+	+	+
6	93	-	-	-	-	+	+
7	90	+	+	+	+	+	+
8	86	+	+	-	+	-	-
9	83	+	+	-	-	-	-
10	82	+	+	+	+	+	-
11	78	-	-	-	-	+	-
12	75	+	+	+	+	+	+
13	74	+	+	+	-	+	+
14	66.5	-	-	+	-	-	-
15	64.5	+	+	+	+	+	+
16	58	+	+	-	-	+	-
177	57	-	-	+	-	-	-
18	55	+	+	+	+	+	+
19	52	+	+	+	+	+	+
20	50.5	+	+	+	-	+	+
21	47	-	+	-	-	+	+
22	44	+	+	-	-	-	-
23	41.5	+	+	+	+	+	+
24	41	+	+	-	-	+	-
25	40.5	+	-	-	-	-	+
26	38.5	+	+	+	+	+	-
27	36.5	-	-	-	-	-	+
28	33.25	+	+	+	+	-	+
29	31	+	+	-	+	-	+
30	28.5	+	+	+	+	-	+
31	25.75	+	+	+	-	+	-
32	24	+	+	+	+	+	+
33	23.5	+	+	+	-	+	+
34	21.5	+	+	+	+	+	+
35	20.5	+	+	+	+	+	+
36	19.5	+	+	+	+	+	+
37	18.25	+	+	+	-	-	-
38	17	-	-	-	+	+	+
39	16.25	+	+	+	+	+	-
40	16	+	+	+	+	+	-
41	15.5	+	+	+	+	+	-
42	14	+	+	+	-	+	-
43	13	-	-	-	-	-	+
44	12.5	-	-	-	-	-	-
45	12	-	-	-	-	-	-
46	10	-	-	-	+	-	+
	TOTAL	33	33	28	25	28	26

Table XIII. Location of Structural Polypeptides of orf Virus No 7

Approximate Molecular weight ($x10^3$)	Core (Pellet fraction)	Surface (Supernatant fr.)	Surface Tubule
130	+		
125	+		
118	+		
105	+	+	
104	+	+	
90	+		
86	+		
83	+		
82	+		
75	+	+	
74	+	+	
64.5	+		
58	+		
55	+		
52	+		
50.5	+		
47	+		
44	+		
41.5	+		
38.5		+	+
33.25	+		
31	+	+	
28.5	+		
25.75	+	+	
24	+	+	
23.5	+		
21.5	+		
20.5	+	+	
19.5		+	
18.5	+		
16.25	+	+	
15	+	+	
14	+	+	

5.4 DISCUSSION

Two patterns of polypeptide bands were identified from 6 New Zealand strains of orf virus. One pattern was common to NZ2, NZ7, NZ9, NZ10, and NZ12. NZ19 differed from this pattern, the main difference being in the 38.5 Kd region. Differences have also been observed in the polypeptides of North American isolates, especially in those with MWs of 37K-44K (Buddle et al., 1984). Comparison of the structural polypeptide profiles of the New Zealand strains and American isolates was not possible because of the different analytical techniques used. Buddle et al. (1984) described polypeptides with MWs of 37K, 39K and 44K as the polypeptide components of the STs of the North American strains. Whether the 38.5K dalton polypeptide detected in the present study is one of these polypeptides is not known. Classification of isolates by the ST polypeptides, as suggested by Buddle et al. (1984), would place the New Zealand isolates into two groups. Thus strains NZ2, NZ7, NZ9, NZ10 and NZ12, which showed the same polypeptide associated with the putative ST could be classified in one group while strain NZ19, which lacked a polypeptide with MW of 38.5K, in another. Studies on more New Zealand isolates may reveal other differences in the ST polypeptide(s). Buddle et al. (1984) suggested that the ST polypeptide can be correlated with the antigenicity of the virus. This would suggest that strains NZ2, NZ7, NZ9, NZ10 and NZ12 are antigenically related while strain NZ19 is in a different antigenic group. Although DNA restriction endonuclease analysis revealed differences between the New Zealand strains (Robinson et al., 1982) there was no obvious correlation between

restriction endonuclease fragment patterns and the SDS-PAGE profiles of the two groups.

All the polypeptides detected by staining with Coomassie Blue were also detected by autoradiography. However, some polypeptides were detected by autoradiography which were not detected by staining. This justifies the use of the radioactive label.

Subpassage did not alter the DNA profile of NZ2 passaged about 20 times in ovine and bovine cells and later repassaged back in sheep when compared with that of DNA isolated from original scab materials (Robinson et al., 1982). There was also no difference observed between the passage 8 and passage 32 orf virus in the polypeptide studies of Buddle et al. (1984). This indicates that the differences detected among the isolates in this study are unlikely to be due to differing cell culture passages. A slight difference, however, between the DNA fragment patterns of low passage (P8) and high passage (P108) orf virus isolates has been detected (Wittek et al., 1980) suggesting that changes may eventually occur.

In one experiment, protease inhibitors did not appear to affect the MWs of polypeptides during the extraction procedure although the intensity of the bands was low. The bands that were visible did not appear to be significantly altered in size in the treated or untreated lanes. This, coupled with the observation that bands in subsequent gels

were consistently sharp, gave us confidence that proteases were not substantially altering the apparent size of virion bands.

CHAPTER 6

ONE-STEP GROWTH CYCLE: APPEARANCE OF INFECTIOUS VIRUS PARTICLES AND KINETICS OF DNA SYNTHESIS

6.1 INTRODUCTION

The virus growth cycle in vaccinia-infected cells is a rapid event (Salzman, 1960; Joklik and Becker, 1964; Esteban and Holowczak, 1977). Using a very high multiplicity of infection DNA synthesis starts 1.5 - 2 hr p.i. and is virtually complete by 6 hr p.i. at which time new infectious particles appear. Virus replication is almost complete by 12 hr (see Fig. 2-9). Other poxviruses show slower growth cycles (Ewton and Hodes, 1967; Yohn et al., 1970; Gafford et al., 1972). With milker's node virus, DNA synthesis starts 4-8 hr p.i. and infectious particles are first detected at 20 hr p.i. (Thomas et al., 1980). In their studies of human orf isolates in bovine spleen cells, Hessami et al. (1979) have detected infectious particles at 8 hr p.i. with the maximum infectivity titre being seen 36 hr p.i. It was important to therefore determine the rate of orf virus replication in BT cells in preparation for biochemical studies.

In most viral cell cycles the onset of viral nucleic acid replication heralds the onset of structural polypeptide synthesis or so-called "late" events. The period before the onset of nucleic acid replication has been termed "early". Early events are characterised by the production of enzymes

and other non-structural polypeptides and late events by the production of virion structural polypeptides. Early non-structural polypeptides have been implicated as being targets for the immune response against poxvirus infection (Mallon et al., 1985) and thus detection of the onset of DNA replication is important in the study of molecular events in orf virus-infected cells.

In this chapter, two methods were employed in the study of orf virus DNA synthesis. The first method was based on the measurement of total incorporation of ^{3}H-thymidine in infected cells. The second method involved a quantitative analysis of orf-specific DNA appearing at various times after infection. In this latter method total cellular DNA was adsorbed to a nitrocellulose filter using a "dot-blot" apparatus and the relative amount of viral DNA present at different times was assessed by hybridization with radiolabelled orf DNA.

Another set of experiments described in this chapter determined the levels of DNA synthesis inhibitors required to inhibit orf DNA synthesis in BT cells. This knowledge is important for studying early events in orf virus-infected cells. The arabinosyl nucleosides, adenine arabinofuranoside (ara-A) and cytosine arabinofuranoside (ara-C), hydroxyurea (HU) and bromouracil deoxyribose (BUdR) are known to inhibit cellular and viral DNA synthesis (Fenner et al., 1974). By inhibiting DNA synthesis in poxvirus-infected cells early events are prolonged (Kates and McAuslan, 1967c).

In summary, this chapter describes experiments designed, firstly, to define the kinetics of infectious particle production and DNA synthesis during one-step growth cycles of orf virus in BT cells at 4°C and 37°C and, secondly, to determine the levels of ara-A, ara-C, HU and BUdR that are required to inhibit DNA synthesis.

6.2 MATERIALS AND METHODS

Cells and Media. BT cells which had undergone 18 passages were grown in MEM-LAH containing either 10% or 2% FCS as required.

Virus Stock. NZ7 grown in BT cells and vaccinia virus (Elstree strain) which had been passaged in BT cells once were used as cell lysates. The titre of NZ7 was 6.5 x 10^7 pfu/ml and that of the vaccinia virus was 1.25 x 10^7 pfu/ml.

One-step Growth Cycles. One-step growth cycles were established using adsorption temperatures of 4°C and 37°C.
(a) Adsorption at 4°C. BT cells were grown in 25 cm^2 flasks and when confluent were infected with at least 5 pfu/cell of NZ7. Virus was allowed to adsorb at 4°C for 1 hr with continuous rocking. After adsorption the infected monolayers were brought to 37°C and 15 min later the medium was replaced with fresh MEM-LAH containing 2% FCS. Infected monolayers were taken every 2 hr for the first 24 hr and then at 36 hr and 48 hr p.i. and frozen at -70°C. The infected cells were

frozen and thawed 3 times, sonicated and 10-fold serial dilutions made. Plaque titrations were done on BT cells grown in MEM-LAH plus 10% FCS in 24-well plates.

(b) Adsorption at 37°C. BT cells were grown in 10 x 35 mm dishes. When confluent, each monolayer was infected with 15 pfu/cell of NZ7 virus stock. Virus was adsorbed at 37°C in a 5% CO_2-in-air atmosphere for 1 hr. After adsorption of virus the inoculum was washed off and the cells were incubated with fresh MEM-LAH plus 2% FCS. Infected cells were incubated further at 37°C in a 5% CO_2-in-air atmosphere and 2 dishes were frozen every 4 hr. Samples were processed as above for plaque titration.

^{3}H-Thymidine Labelling of DNA. BT cells were grown in 35 x 10 mm plastic dishes. Monolayers were infected with either 15 pfu/cell of NZ7 virus, 10 pfu/cell of vaccinia virus or were mock-infected with culture medium. After adsorption for 1 hr at 37°C the cultures were washed with PBS, pH 7, and the medium replaced with MEM-LAH containing 5 µCi/ml of ^{3}H-thymidine (Amersham, specific activity 5 Ci/mmol) and 2% FCS. At various times after infection, infected and mock-infected cells were processed as follows. The medium was aspirated and the cells were scraped into 1 ml of 0.002 M Tris, pH 9. About 100 µl of each cell lysate were precipitated with 900 µl of 10% trichloracetic acid (TCA) and filtered through GF/C filter papers (Whatman Ltd). The filters were dried and their radioactivity was measured in a Philips liquid scintillation counter.

"Dot-Blot" Analysis. BT cells were grown in 24-well plates. When confluent, the monolayers were infected with at least 15 pfu/cell of either NZ7 stock or 5 pfu/cell of vaccinia. The viruses were adsorbed at 37°C for 1 hr in a 5% CO_2-in-air atmosphere. After adsorption the monolayers were washed twice with MEM-LAH warmed to 37°C. Maintenance medium (MEM-LAH containing 2% FCS) was added to each well and incubated further at 37°C. At various times after infection the monolayers were washed twice with ice-cold PBS, pH 7. The cells from each well were scraped into 800 ul amounts of TE buffer, pH 8,(0.01 M Tris, 0.001 M EDTA) and frozen and thawed once. Each sample was treated with 2.5 ul of 50 µg/ml trypsin (Sigma, 3x crystallised) and incubated at 37°C for 30 min to 1 hr. Doubling dilutions with final volumes of 200 µl were made of each sample and the DNA immobilised on nitrocellulose membrane as described below.

DNA Immobilization. DNA immobilization was done by a modification of the method of Kafatos et al. (1979). To each 200 ul of the above dilutions of infected cells, 80 µl of 1.0 M NaOH were added, mixed, and then 28 µl of 8.0 M sodium acetate was added and mixed. Each mixture was then transfered onto a nitrocellulose membrane (BA85, Schleicher and Schull, Dassel, West Germany) which had been pre-soaked in 1.0 M sodium acetate. Transfers were done using a Bio-Rad "bio-dot" microfiltration apparatus. After transfer each well was washed thoroughly with 1.0 M sodium acetate. The nitrocellulose membranes containing the DNAs were then dried at 37°C and baked at 80°C in vacuo for 2 hr. After baking, the membranes were ready for DNA hybridization.

Preparation of NZ2 DNA Probe. DNA probes were produced using the nick translatation technique (Rigby et al., 1977; Technical Bulletin TB.80/3, Amersham International). Except for the DNA substrate, the components of the system were supplied in an Amersham Nick Translation Kit PB.5100 (Amersham International). The reaction was done on ice in a total volume of 50 µl. Six µl of TE buffer, pH 8.0, containing 0.5 µg of DNA were added to 10 µl of the nucleotide buffer containing 100 µM each of the unlabelled deoxytri-phosphates of adenosine, guanosine and thymidine. To this mixture was added 5.0 µl of a solution containing 25 µM deoxycytidine 5'-[$-^{32}P$] triphosphate (Amersham, specific activity 410 Ci/mMol). Enough distilled water was added to obtain the desired final volume of 50 µl. Finally , 5.0 µl of the enzyme solution containing 500 units of DNA polymerase/ml and 10^{-2} µg/ml of DNAse I were added and the reaction mixture incubated at 14°C for 80 min. The mixture was then passed through a 1.0 ml column of G50 Sephadex (Pharmacia, Uppsala, Sweden). The first radioactive peak was collected and the labelled DNA concentrated by ethanol precipitation. This was done by adding 5.0 µl of 5.0 M NaCl and 10 volumes of 95% ethanol to the collected fraction and incubating at -20°C for 30 min. The mixture was then centrifuged in a Beckman microfuge at full setting (approximately 13,000 x g) for 5 min. The precipitate was harvested and resuspended in 50 µl of TE buffer, pH 8. The specific radioactivity was estimated by measuring Cerenkov radiation in a Philips liquid scintillation counter.

DNA Hybridization. DNA hybridization was done by the method of Denhardt (1966). The dried nitrocellulose membrane containing the immobilised DNA was prehybridized for 2 hr at 45°C in a shaking water bath. Prehybridization was carried out in a sealed plastic bag containing 50% prehybridization mix (see Appendix) and 50% formamide (BDH, recrystalised at 0°C). After 2 hr the prehybridization mix was discarded and replaced with hybridization mix (see Appendix) containing 2×10^6 cpm of *in vitro*-labelled NZ2 DNA. Hybridization was done for 16-20 hr at 45°C with continuous shaking. The membrane was washed 3 times at room temperature with a solution containing 3.0 M NaCl, 0.3 M sodium citrate (SSC) and 0.1% sodium dodecyl sulphate (SDS) and then washed twice at 45°C in a solution containing 0.3 M NaCl, 0.03 M sodium citrate and 0.1% SDS for about 15 min each wash. The membrane was dried and autoradiographed at -70°C for up to 16 hr using Dupont Cronex X-ray film.

^{3}H-thymidine Incorporation into DNA in the Presence of DNA Synthesis Inhibitors. BT cells were grown in 24-well plates in MEM-LAH containing 10% FCS. When confluent, the cells were infected with 15 pfu NZ7/cell. After 1 hr adsorption at 37°C in a 5% CO_2-in-air atmosphere the monolayers were fed with fresh medium containing 2% FCS , 5.0 µCi/ml of ^{3}H-thymidine (Amersham, specific activity 5 mCi/mMol) and varying levels of either ara-A, ara-C, HU or BUdR (Sigma Chemical Co.) The cells were incubated for 48 hrs. The cells were then harvested and filtered on GF/C filters (Whatman Ltd) using a vacuum apparatus. The filters were washed thoroughly with distilled water, dried at 37°C, placed in scintillation vials

with 5 ml amounts of scintillant (see Appendix) and the amount of radioactivity measured in a Philips liquid scintillation counter.

6.3 RESULTS

One-step Growth Cycle

a. Adsorption at 4°C. The result of the one-step growth cycle experiments when adsorption was done at 4°C are shown in Fig. 6-1. There was a decreasing titre from 4 hr to 12 hr p.i. after which time the titre started to increase. Fifty per cent of the final virus yield was reached 36 hr p.i.

b. Adsorption at 37°C. The results shown in Fig. 6-2 are the averages of 2 samples for each time point when adsorption was done at 37°C. New infectious particles were detected between 16 and 20 hr p.i., the time when a rapid increase of DNA synthesis was also observed. About 50% of the total virus yield was reached at 34 hr p.i. corresponding with the time of peak DNA synthesis. The growth cycle was probably complete by 48 hr.

^{3}H-Thymidine Incorporation. For the measurement of ^{3}H-thymidine incorporation into viral DNA in NZ7-infected cells, samples were taken at 5 hourly intervals from 1 hr p.i. to 40 hr. For vaccinia-infected cells, samples were examined at 0.5, 1, 2, 3, 4, 6, 8, 10, 12 and 26 hr p.i. Results are shown in Fig. 6-3.

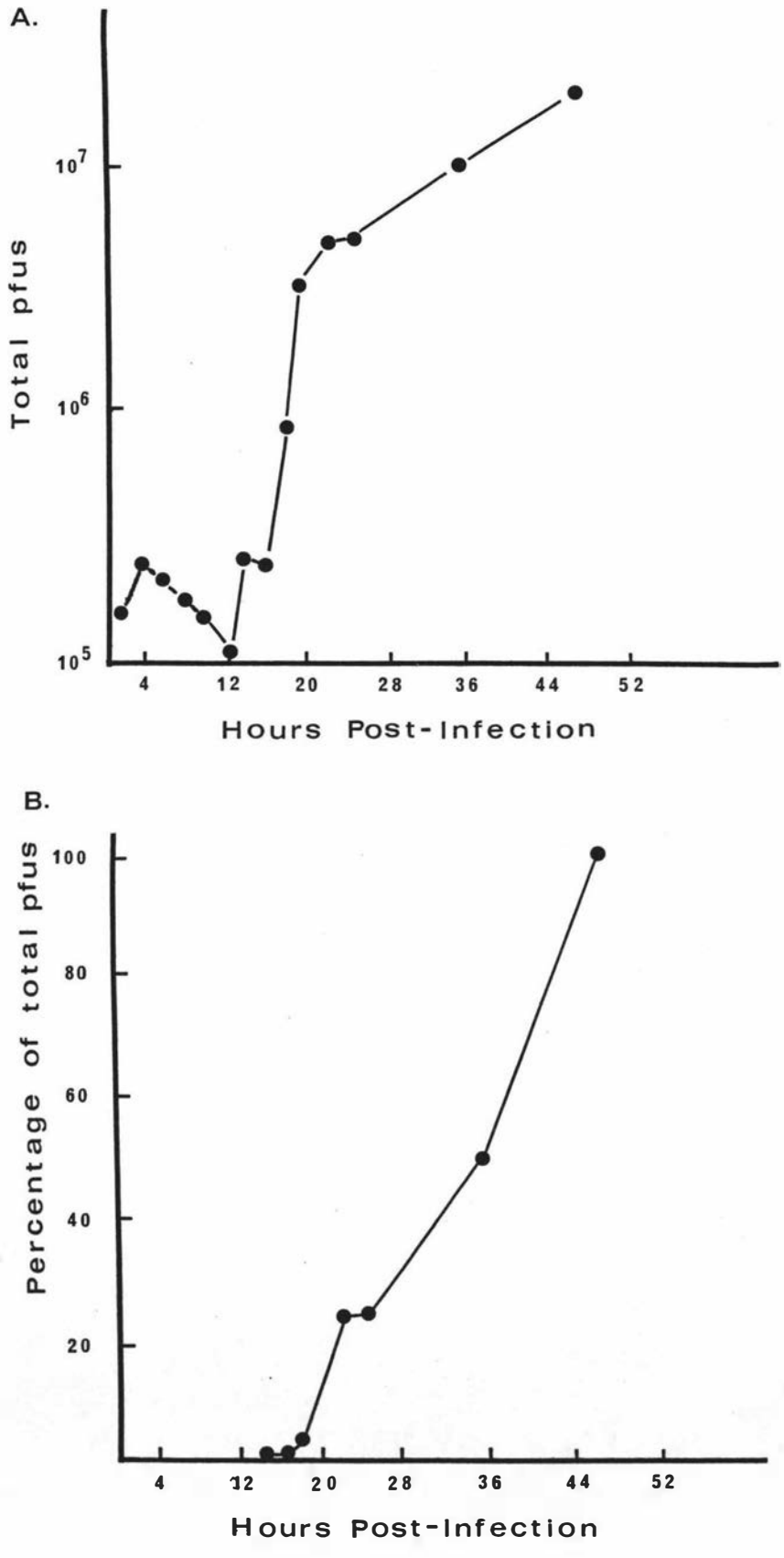


Fig. 6-1. **One-step growth cycle- appearance of new infectious particles.** NZ7 was adsorbed to bovine testis cells at 4°C. A. Results represent total plaque-forming units (pfu). B. Results represent percentage of total pfus.

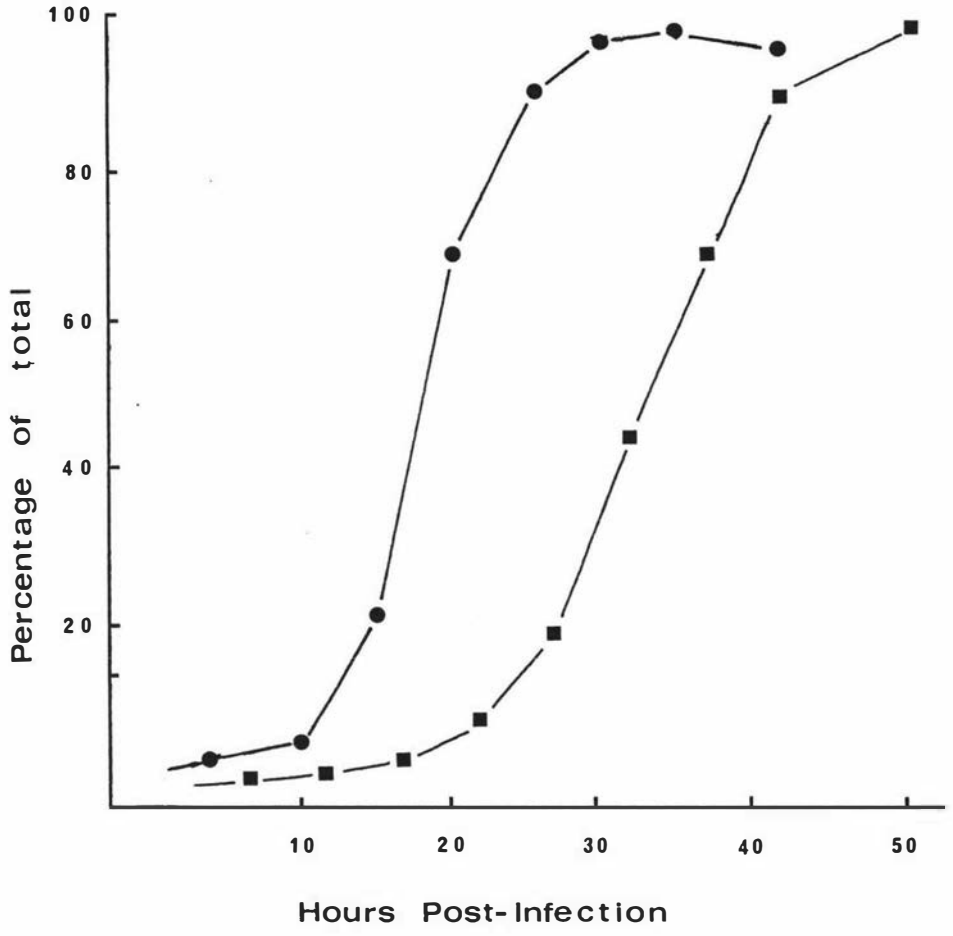


Fig. 6-2. **One-step growth cycle.** NZ7 was adsorbed to bovine testis cells at 37 ^{o}C DNA synthesis ●-●; appearance of new infectious particles ■-■. Total ^{3}H-thymidine incorporation into DNA at 35 hr p.i. was 2.5 x 10^{6} counts per minute (cpm). Total infectious particles at 48 hr was 5 x 10^{7} plaque-forming units (pfu).

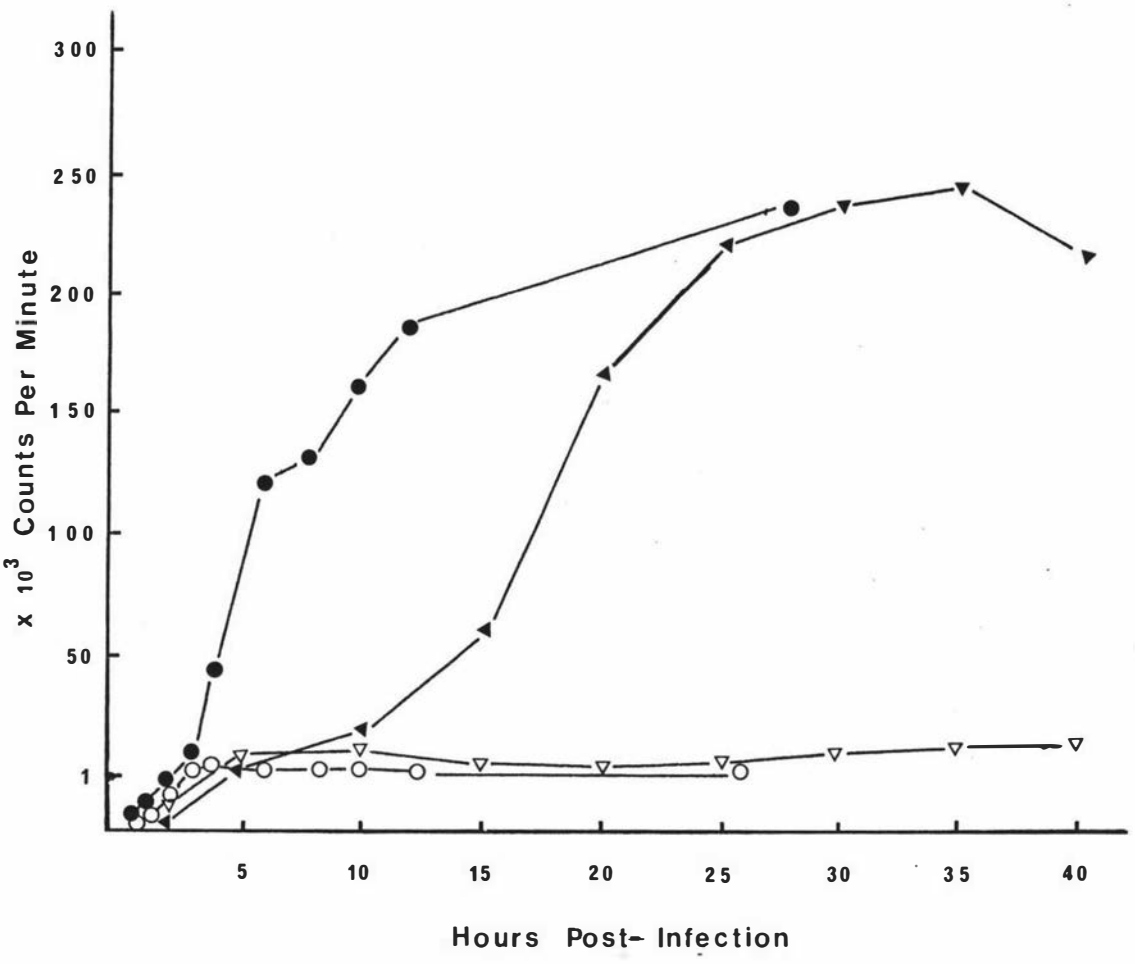


Fig. 6-3. **^{3}H-thymidine incorporation orf virus- and vaccinia virus-infected bovine testis cells.** NZ7-infected cells ▼-▼; vaccinia virus-infected cells ●-●; NZ7 control cells ▽-▽; vaccinia control cells o-o.

The incorporation of ^{3}H-thymidine by cells infected with NZ7 increased by 3-fold at 10 hr p.i. compared with the amount incorporated by uninfected cells. At 15 hr there were 40 times more counts in the infected cells than in the uninfected cells. Fifty percent incorporation was reached at about 18 hr p.i. ^{3}H-thymidine incorporation continued for 30 -35 hr. Incorporation of ^{3}H-thymidine into cells infected with vaccinia virus could be first detected between 3-4 hr p.i. There was a 2-fold increase in incorporation at 2 and 3 hr p.i. when compared with the amounts incoporated by uninfected cell. Fifty percent incorporation of ^{3}H-thymidine was reached after about 6 hr and incorporation had almost ceased by 12 hr p.i.

"Dot-blot" Analysis. Fig. 6-4 shows a time course of DNA synthesis as determined by hybridizing ^{32}P-labelled orf DNA to a filter containing blots of orf virus-infected cells. There was a slight increase in intensity of some dots at 4-6 hr but the most rapid increase in intensity occured from 12-16 hr p.i.

Incorporation of ^{3}H-Thymidine into DNA in the Presence of Inhibitors of DNA Synthesis. ^{3}H-thymidine incorporation into NZ7-infected BT cells was inhibited by ara-C, BUDR and HU (Fig. 6-5). Ara-C at a level of 5-10 µg/ml was sufficient to block ^{3}H-thymidine incorporation by about 92%, BUdR at 25 µg/ml resulted in 90% inhibition, and HU at 10 mM caused 87% inhibition. Ara-A caused 50% inhibition at a level of 10 µM.

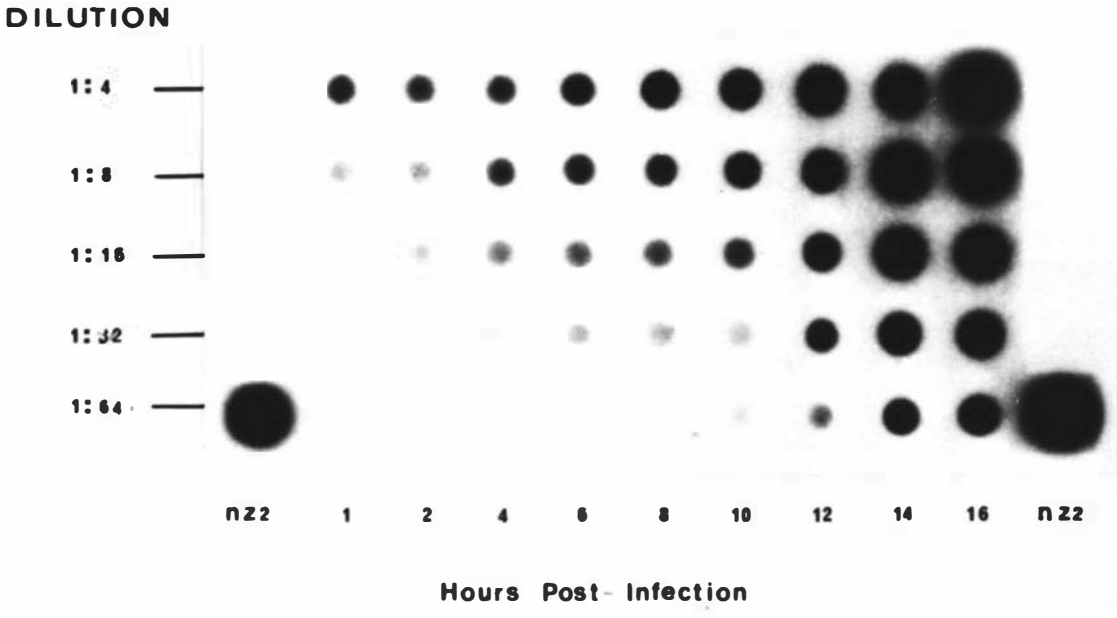


Fig. 6-4. **"Dot-blot" analysis of orf virus-infected bovine testis cells.** Cells infected with NZ7 were processed as described in Materials and Methods. Dilutions of cell lysates obtained at various times after infection were immobilised onto nitrocellulose paper and hybridised with nick-translated NZ2 DNA.

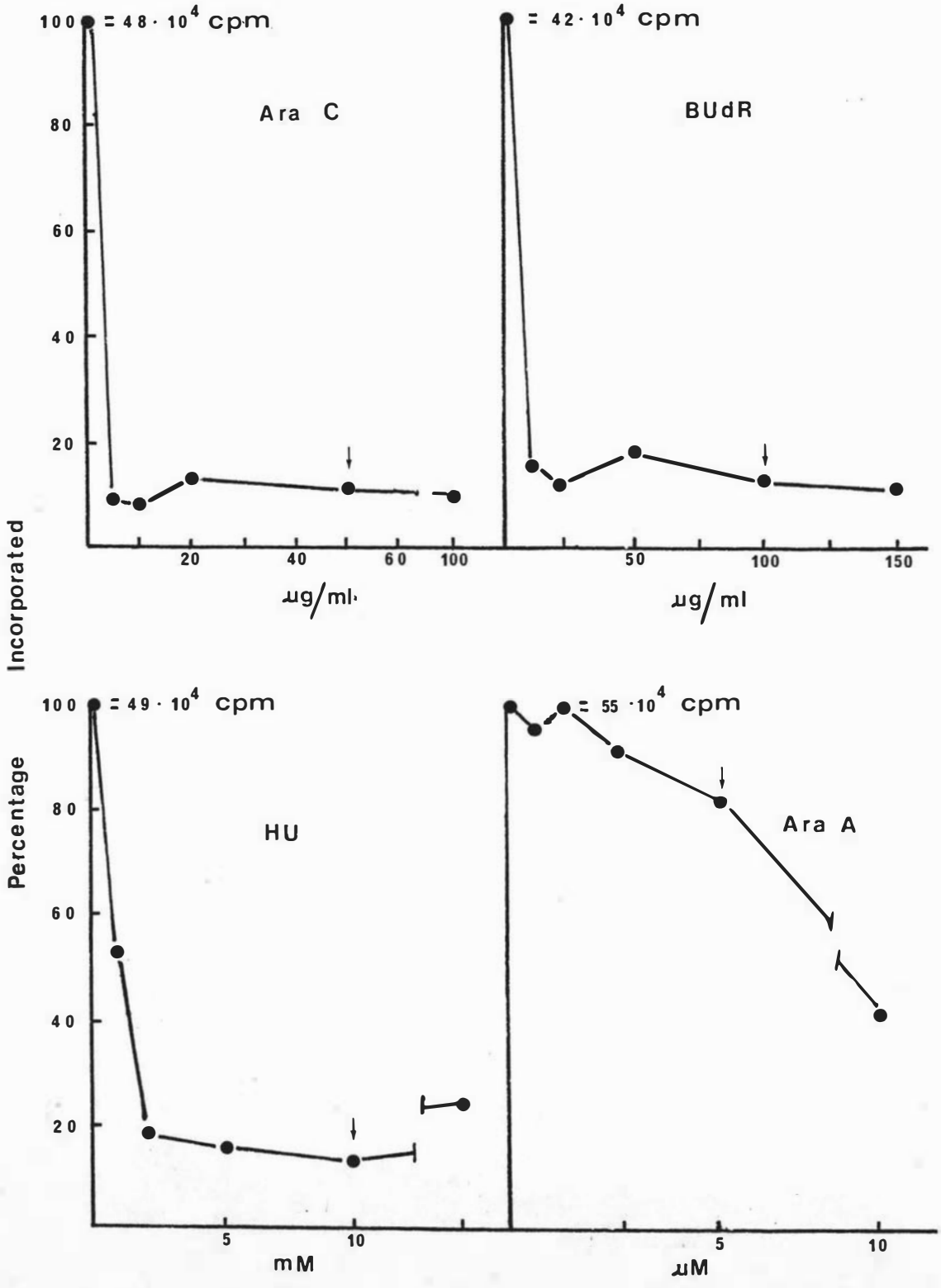


Fig. 6-5. **^{3}H-thymidine incorporation into orf virus-infected bovine testis cells in the presence of inhibitors of DNA synthesis.** The arrows represent the concentrations commonly used for the inhibition of DNA replication in other viral systems.

6.4 DISCUSSION

Adsorption and penetration of virus to cells are temperature-dependent events. While viruses are able to adsorb to cells at 4°C, they are unable to penetrate at this temperature; subsequent elevation to 37°C allows penetration to proceed. Thus adsorption at 4°C ensures a synchronous infection of cells (Dales, 1973). However, a near synchronous infection can be achieved at 37°C by increasing the multiplicity of infection. This might explain the very slight difference in growth seen between that of virus adsorbed at 4°C and 37°C since 3 times the number of pfus/ml were used at the higher temperature. This apparent slight difference in growth rate could also be due to sampling error. Only one experiment was done at 4°C while the figures shown for the 37°C were averages of 2 samples. Whether adsorption was done at 4°C or 37°C the time of appearance of new infectious particles did not differ significantly.

The one-step growth experiments showed that new orf virus particles appeared at 14-16 hr p.i. and continued to increase in number for up to 48 hr. Hessami et al. (1979) reported that production of orf virus in bovine spleen cells starts at 8 hr and finishes at 48 hr p.i. In these respects the growth curve of orf virus is similar to that of milker's node virus (Thomas et al., 1980).

Data from the one-step growth cycles provided evidence that the orf virus growth cycle is slower than that reported for vaccinia virus and give a basis for comparing the temporal

appearance of newly synthesised orf viral DNA and proteins with those of other viruses.

From the above experiments DNA synthesis in orf virus-infected BT cells started between 4-6 hr p.i. and continued to 35-40 hr p.i. whereas vaccinia virus DNA replication in BT cells was first detected between 3-4 hr p.i. and was almost complete by 24 hr. The rate of development of vaccinia noted in these experiments was slower than that which has been reported by other investigators (Esteban and Holowczak, 1980). This might have been due to the different type of cell used or to the lower multiplicity of infection employed. The slow and prolonged orf virus DNA replication is more closely related to that seen with milker's node virus (Thomas et al., 1980), fowlpox virus (Randall et al., 1972), Yaba virus (Yohn, et al., 1970) and rabbit fibroma virus (Ewton and Hodes, 1967) than to vaccinia virus.

Stimulation of host DNA synthesis by virus infection has been noted in some virus systems. Polyoma virus or SV40 markedly stimulate the rate of cellular DNA synthesis (Dulbeco et al., 1965), the increase being dependent upon several factors such as the initial state of the cells and the multiplicity of infection (Vogt et al., 1965). In the poxviruses, inhibition as well as stimulation of DNA synthesis has been observed. In the HeLa cell-vaccinia system, at high input multiplicity, cellular DNA synthesis is rapidly inhibited by either active or U.V.-irradiated virus (Joklik and Becker, 1964). On the other hand, using rabbit fibroma virus, which produces localised tumour in rabbits, host DNA synthesis in infected

cell cultures may become arrested for long periods and then begin again after the peak of viral multiplication (Tompkins et al., 1969). No attempt was made to separate cytoplasmic and nuclear fractions as has been done with vaccinia-infected and other virus-infected cells (Esteban and Holowczak, 1977; Thomas et al., 1980). This approach was justified as there was good correlation between the "dot-blot" hybridization and the ^{3}H-thymidine incorporation. Also, the lack of ^{3}-thymidine incorporation in uninfected cells makes it unlikely that host DNA was significantly affecting the result. It appears that orf virus DNA replication in BT cells begins at 4 - 6 hr p.i. and that this time is the likely transition period between early and late events of orf virus multipication.

Although ara-C, BUdR, and HU are equally effective as inhibitors of orf virus DNA synthesis it appears that ara-C is the most effective choice of inhibitor because it is effective at low concentrations. However, the selection of the appropriate inhibitor in the biochemical studies will depend on the cell system under study and other experimental requirements (Furth and Cohen, 1968; Furlong and Graham, 1971; Fenner et al., 1974; Sussenbach and van der Vliet, 1973). As effective inhibition was obtained with ara-C, BUdR or HU, the failure of ara-A to inhibit DNA synthesis was not investigated further.

CHAPTER 7

ANALYSIS OF VIRUS-INDUCED PROTEIN SYNTHESIS

7.1 INTRODUCTION

There are no reports in the literature describing the temporal synthesis of orf virus proteins in infected cells, most studies having concentrated on the molecular biology of vaccinia virus as a representative of the Poxviridae. A one-step growth cycle for vaccinia virus is a very rapid event, but as shown in earlier experiments (Chapter 6) DNA synthesis and the appearance of infectious progeny virus after orf virus infection is a much slower process. It can be assumed that protein synthesis will also be relatively delayed. To confirm this view, and to gain basic information on the orf virus cell cycle, an analysis of the sequential appearance of polypeptides in orf virus-infected cells was undertaken by pulse-labelling virus-induced polypeptides with ^{35}S-methionine.

Pulse-labelling for 15-30 min with either ^{35}S-methionine or with ^{14}C-labelled amino acids is sufficient to label proteins in vaccinia virus-infected cells (Pennington, 1974). Preliminary experiments, however, showed that when orf virus-infected cells were pulse-labelled for 15 min with ^{35}S-methionine, label could not be detected in the cell lysates when they were analysed by SDS-PAGE and exposed to auto-radiography for one week. Only 2 or 3 polypeptide

bands were detected even after autoradiography for 2 months. Therefore, before a study of protein synthesis in orf virus-infected cells could be made it was necessary to determine whether or not increasing the ^{35}S-methionine pulse-labelling period enabled the induced polypeptides to be detected by SDS-PAGE and fluorography.

7.2 MATERIALS AND METHODS

Determination of Optimum Pulse-Labelling Time

Cells and Media. Secondary BT cells were grown in MEM-LAH containing 10% FCS. Infected cells were maintained in MEM-LAH containing 2% FCS. Pulse-labelling was done in methionine-free MEM (see below).

Virus Stock. Strain NZ7 was grown in BT cells and used as a cell lysate, the titre of which was approximately 1.5 x 10^7 pfu/ml.

Infection of Cells with Virus. Confluent monolayers of BT cells grown in 10 x 35 mm plates were washed twice with PBS, pH 7, and infected with 25 pfu/cell. The virus was adsorbed for 1 hr at 37^{o}C in an atmosphere of 5% CO_2-in-air. After adsorption the inoculum was washed off and the medium was replaced with MEM-LAH containing 2% FCS.

Determination of Optimum Pulse-Labelling Time. Infected cells and uninfected control cells were pulse-labelled for 30 min, 1 hr and 2 hr at 12 hr, 24 hr and 36 hr p.i. Cultures

were washed twice with warm (37^{o}C) methionine-free MEM and then replaced with methionine-free MEM containing 35 μCi of ^{35}S-methionine (Amersham, specific activity 885 Ci/mmol). After 2 hr incubation the medium from each dish was aspirated and the cells were scraped into 400 μl of 0.001 M Tris, pH 9, and frozen at -70^{o}C until analysed.

Each cell lysate was thawed and boiled for 2 min in 10% SDS containing 1% 2-ME. About 10 ul from each lysate was precipitated with 90 μl of 10% TCA, blotted onto GF/C paper (Whatman) and assayed for radioactivity.

Gel Electrophoresis. SDS-PAGE analysis was done in a 10% resolving gel. About 300 μl of each sample were loaded onto the gel and electrophoresed using a discontinuous buffer system (see Appendix) at 150 - 200V constant current until the tracking dye reached about 1 cm from the bottom of the gel. The gel was stained with 0.25% Coomassie Brilliant Blue R (see Appendix), destained, and the protein bands detected by fluorography as described below.

Fluorography. After staining and de-staining the gel was soaked in Amplify (Amersham) for 15 - 30 min. The gel was then dried using filter paper (No. 3469, Schleicher and Schuell, Dassel, West Germany) in an LKB gel drier and autoradiographed with a pre-exposed (Laskey and Mills, 1975) Dupont Cronex x-ray film at -70^{o}C. Several exposures at 16 hr, 24hr and up to about one week were done on the gel.

Temporal Appearance of Polypetides in Orf Virus-Infected Cells. Two sets of experiments were done. In the first set of experiments, BT cells that had undergone 2 subculture passages (P2) were grown in 10 x 35 mm dishes in MEM-LAH containing 10% FCS. When confluent growth was achieved the monolayers were infected as above. At various times after infection the infected cells were pulse-labelled for 2 hr every 2 hr starting at 10 hr and finishing at 28 hr p.i. In the second set of experiments, BT cells that had been passaged 18 times (P18) were grown in 10 x 35 mm dishes. Three monolayers were infected with the virus and pulse-labelled, one dish each at 4, 6, 8 hr p.i. The remaining monolayers were infected as above and pulse-labelled every 2 hr at 10-30 hr p.i. In both instances a standard procedure was performed as follows. The cells were washed with warm (37^{o}C), methionine-free MEM and then incubated in MEM containing 75 μCi/dish of ^{35}S-methionine (Amersham, specific activity >1000 Ci/mmol). After the labelling period the medium was aspirated and the cells from each culture were scraped into 400 μl amounts of 0.001 M TE buffer, pH 9, and frozen at -70^{o}C until analysed by SDS-PAGE.

^{35}S-Methionine-Labelling of Virion Polypeptides. The procedure described in Chapter 5.2 was followed.

SDS-PAGE. A standard procedure as described earlier was followed with some modifications. A 3% stacking gel was layered over a 10% resolving gel. Aliquots containing 50,000 cpm from each sample were solubilised by boiling in sample buffer (see Appendix) for 100 sec. Samples were centrifuged

at 10,000 x g for 5 min to remove insoluble particles before being loaded onto the gel. Electrophoresis was done at 150 V until the dye had reached the resolving gel and then at 200 - 250 V. Staining, destaining and fluorography were as described above.

7.3 RESULTS

Determination of Optimum Pulse-Labelling Time. When infected and uninfected cells were pulse-labelled for 30 min, only a few ^{35}S-methionine-labelled polypeptides were detected. Cells labelled for 1 hr showed an increased number of bands. The bands were more distinct when the labelling time was extended to 2 hr (Fig. 7-1).

Sequential Appearance of Orf Virus-Induced Polypeptides. The results of the first set of experiment are shown in Fig. 7-2. All polypeptide bands seen in the control can be seen at the other time points, except at 28 hr p.i. where some bands have disappeared. At least 15 bands appeared to be virus-specific. Five bands appeared at 10 hr p.i. 3 of which were continuously synthesised at an increasing rate, one disappeared at 20 hr p.i. and another at 26 hr. Three bands appeared at 12 hr p.i. and continued to be labelled up to 28 hr p.i. Four were first detected at 14 hr and were synthesised throughout the labelling period while 2 others were first detected as late as 20 hr. A schematic diagram of the appearance and persistence of these virus-induced

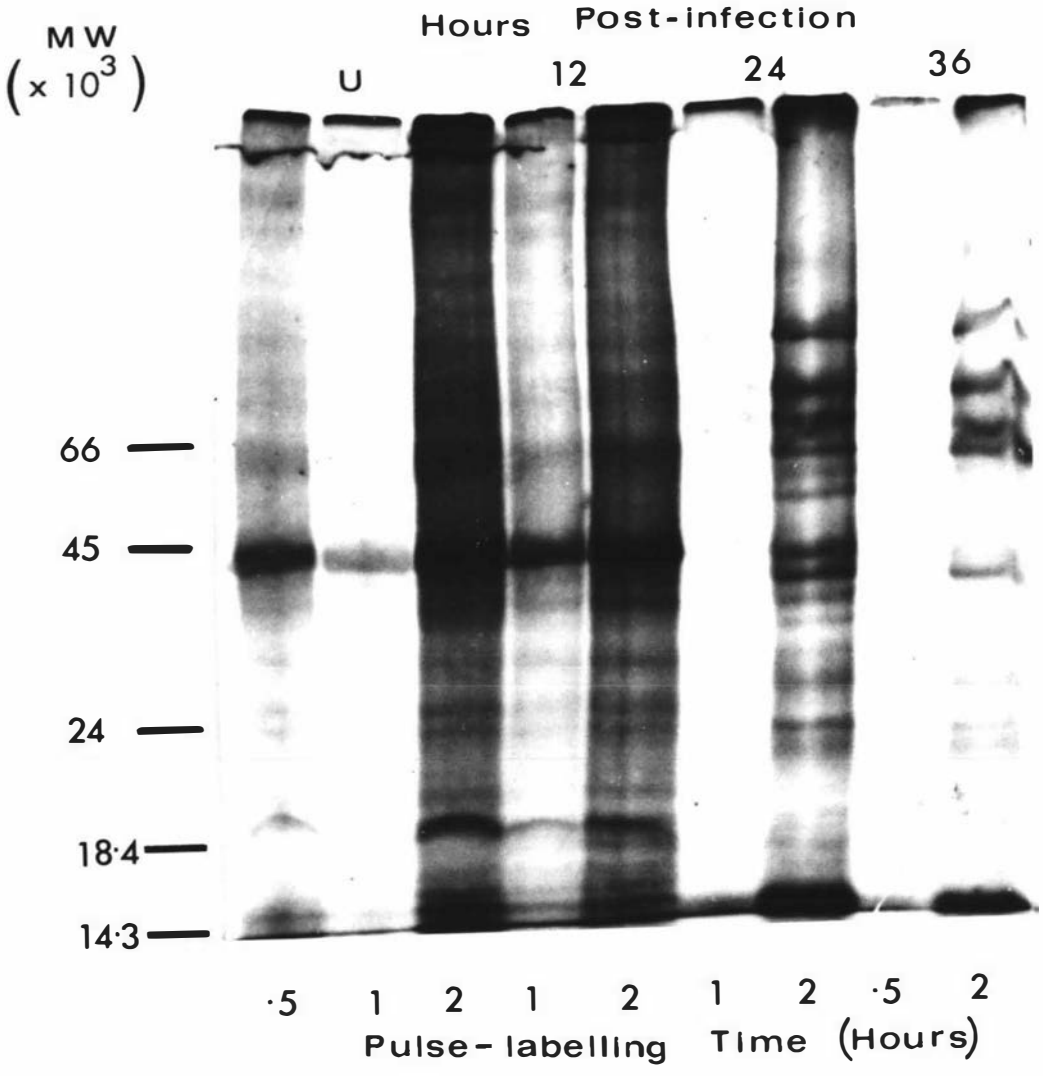


Fig. 7-1. **Determination of optimal ^{35}S-methionine pulse-labelling time in orf virus NZ7-infected BT cells.** Cells infected with orf virus NZ7 were pulse-labelled with ^{35}S-methionine for the times indicated. Cell extracts were analysed by SDS-PAGE in a 10% gel. The gel was treated with Amplify and exposed to Cronex X-ray film. (U) uninfected cells.

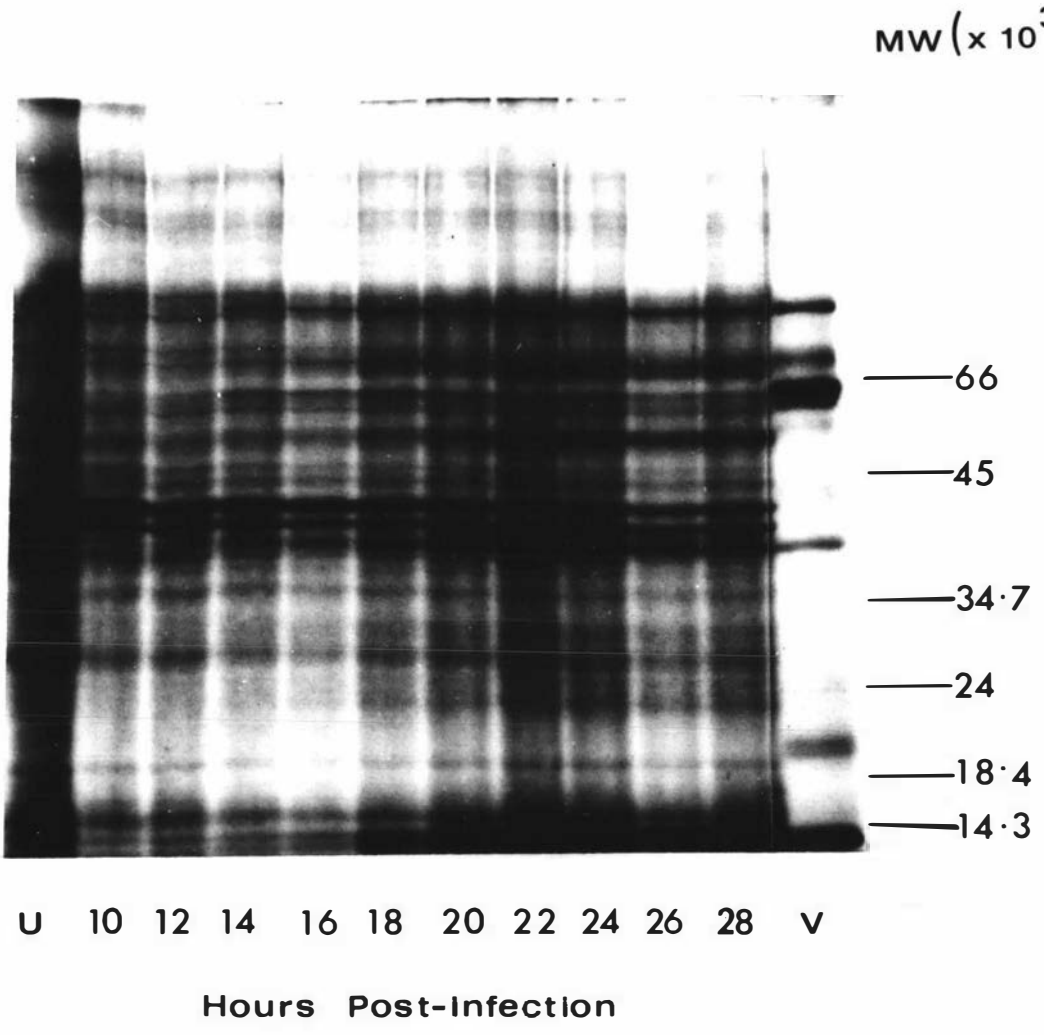


Fig. 7-2. **Autoradiogram showing the time course of polypeptide synthesis in orf NZ7-infected cells.** Polypeptides were pulse-labelled for 2 hr at the times indicated. (U) uninfected cells; (V) Virus.

polypeptides and their apparent molecular weights is shown in Fig. 7-3.

Results of the second set of experiments are shown in Fig. 7-4. There were 1 or 2 polypeptides appearing at 4 hr p.i. Most bands appeared late, some appearing as late as 14-16 hr p.i. A schematic diagram of the appearance of these polypeptides is shown in Fig. 7-5. About 30 polypeptides were detected at 20 - 30 hr p.i. Most of these were shown to be structural polypeptides. Three virion polypeptides with MWs of 93K, 20.5K, and 19.5K were not detectable in the cell extract preparations in the first experiment.

To show that the electrophoretic mobilities of these virion polypeptides were not substantially different in a cell lysate a mixture of whole virus and cell extract obtained at 36 hr p.i. was boiled in SDS/2-ME, loaded in the same well and compared with polypeptides from each sample. The result is shown in Fig. 7-6.

7.4 DISCUSSION

Clearly, a 2 hr pulse-labelling time was needed to efficiently label polypeptides in orf virus-infected cells. The need for this relatively long pulse-labelling time is likely to be due to a slower and less efficient rate of polypeptide synthesis in orf virus-infected cells compared with the rate of polypeptide synthesis in cells infected with vaccinia virus. Increasing the multiplicity of infection

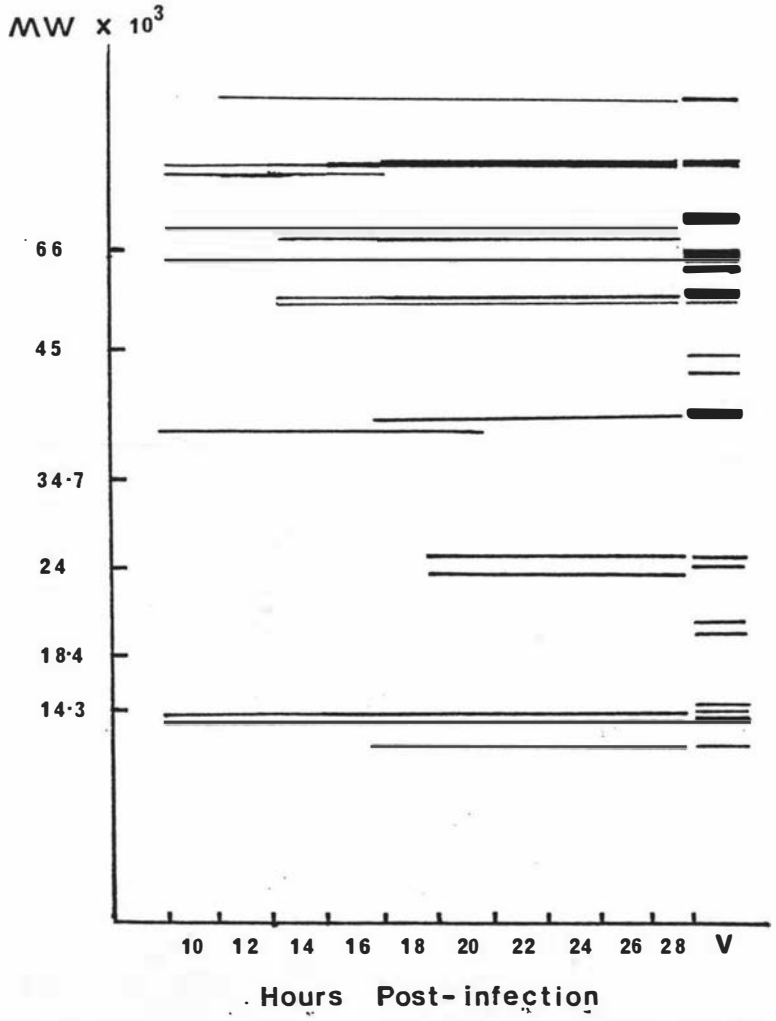


Fig. 7-3. Schematic diagram of gel depicted in Fig. 7-2.

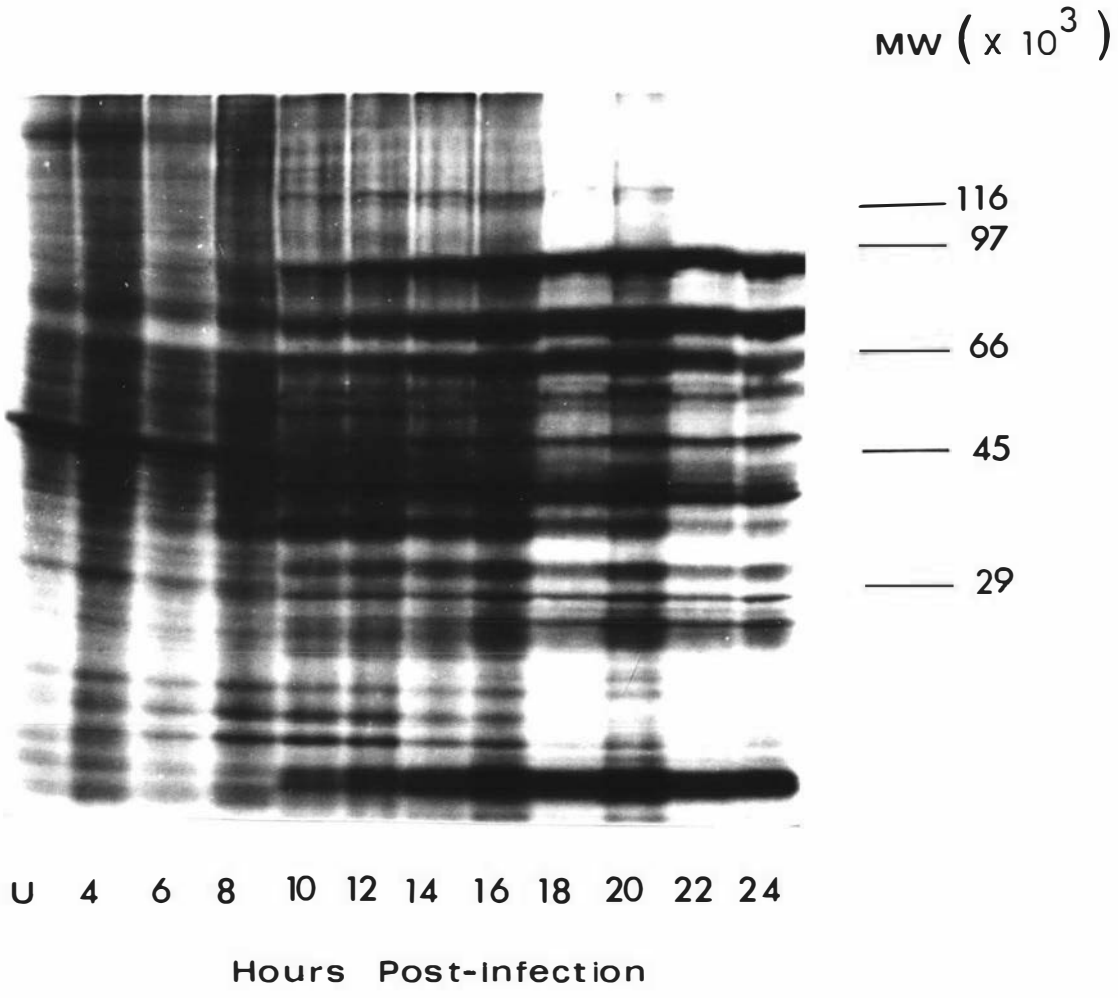


Fig. 7-4. **Autoradiogram showing the time course of polypeptide synthesis in orf-infected BT cells.** Pulse-labelling for 2 hr at times indicated. (U) uninfected cells.

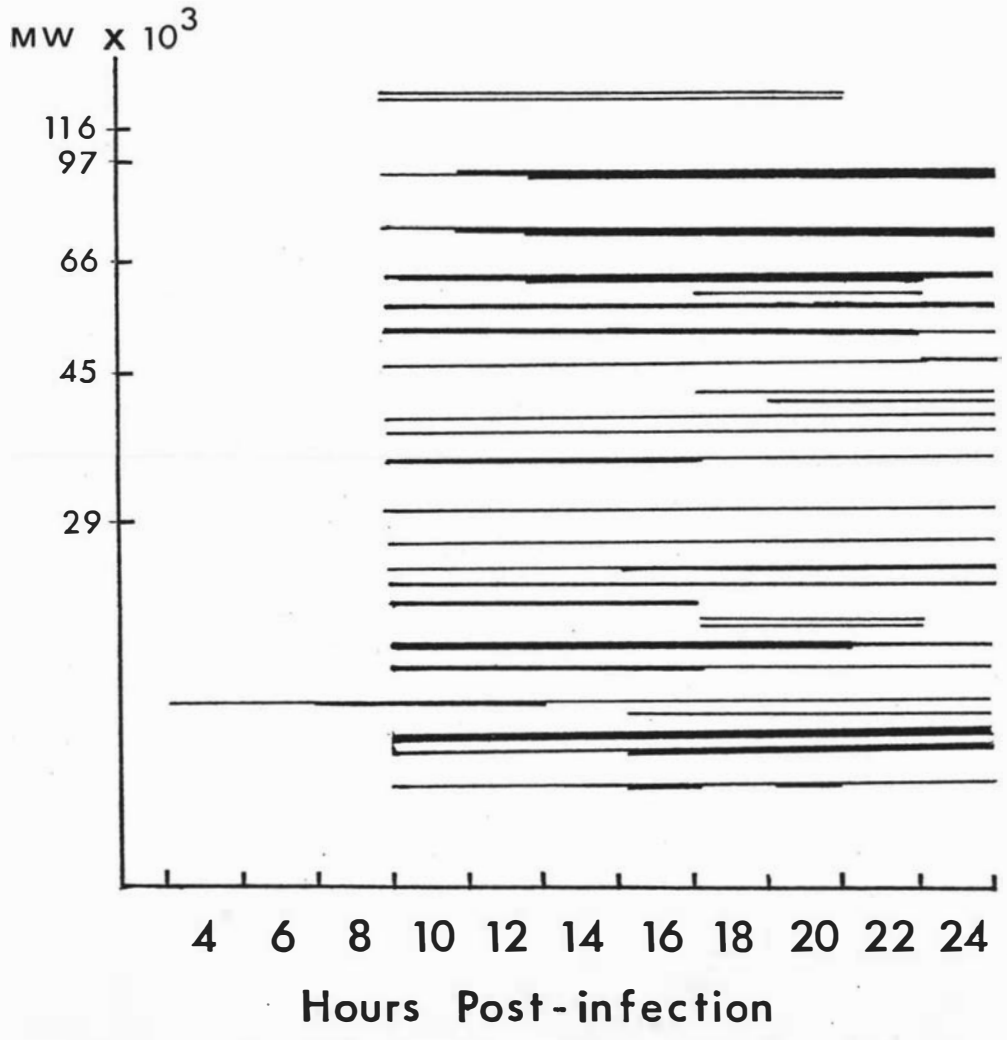


Fig. 7-5. Schematic diagram of the gel depicted in Fig. 7-4.

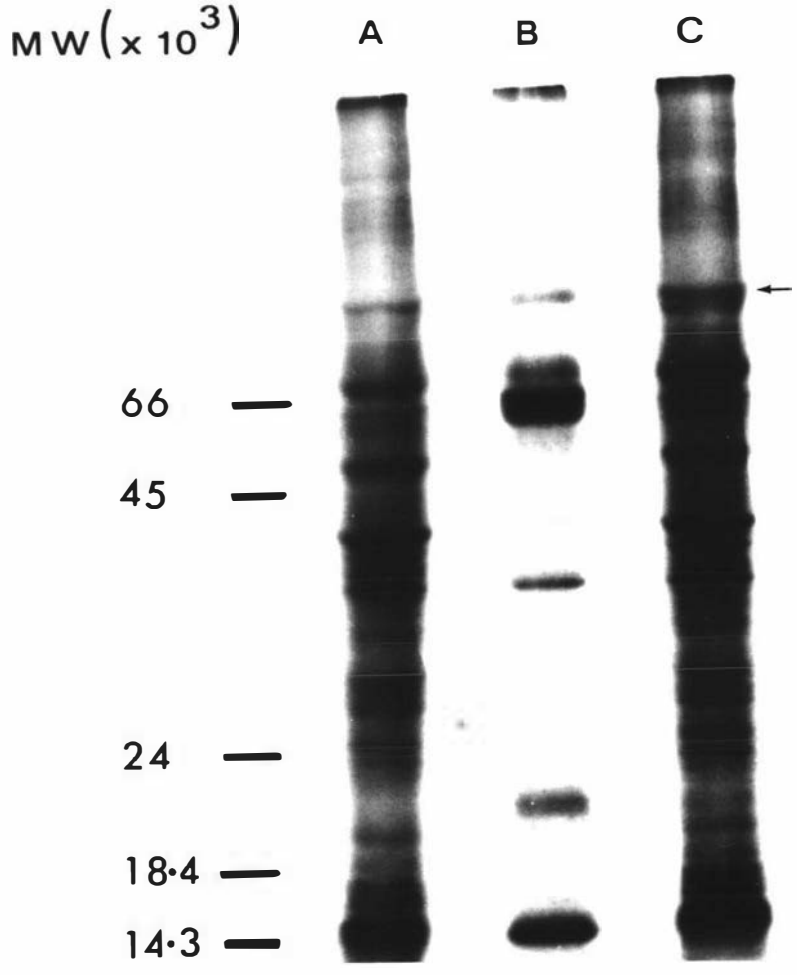


Fig. 7-6. **Autoradiogram showing the structural polypeptide that is a probable product of post-translational modification.** (A) cells pulse-labelled at 36 hr p.i.; (B) whole virion polypeptides; (C) mixture of cell extracts and whole virus. Arrow indicates the position of the presumed post-translationally modified polypeptide.

might have increased the rate of protein synthesis. Pennington (1974) used vaccinia virus at 50 pfu/cell which was twice the dose used in this experiment. However, he did not compare the rate of polypeptide synthesis at different multiplicities of infection. Difficulties encountered in getting a high-titred orf virus stock limited the use of high multiplicity of infection in these studies. A 2 hr pulse-labelling period was sufficient to label the intracellular polypeptides and thus was used in subsequent experiments.

In the first set of experiments, using P2 cells, it appeared that after pulse-labelling of orf-infected BT cells, there was little inhibition of cellular macromolecular synthesis from 10 hr up to 24 hr p.i. (Fig. 7-2). A slow or incomplete "switch off" of host cellular metabolism was also seen in the second experiment. Low passage (P2) cells, which were still in the rapidly dividing state, in the first experiment were used and although at the time of infection they were loosely confluent, the change into fresh medium after virus adsorption may have stimulated cellular metabolism causing an increase in cellular protein synthesis. Alternatively, there is a possibility that not all of the cells were infected during virus adsorption.

Higher passage cells (P18) were used in the second experiment. In this case there was an abrupt "switch off" of cellular protein synthesis at 8 hr p.i. and the appearance of new (virus-coded) polypeptides at 10 hr. Because the infection was staggered and the abrupt "switch off" coincided with the second set of infections, it is difficult to

conclude that this abrupt switch is real and the experiment needs to be repeated. However, a similar abrupt 'switch off' was observed by Pennington (1974) in vaccinia virus-infected cells when cell monolayers were infected simultaneously. His results suggest that the abrupt switch-off in orf virus-infected cells may be real.

Orf virus-infected BT cells generated a large number of polypeptides such that any early virus-coded or virally-induced protein may have been obscured by co-migrating host proteins. In order to identify "early" viral proteins an experiment blocking DNA synthesis could be performed using the optimal concentration of inhibitor for blocking DNA synthesis in orf virus-infected cells as determined above. (Chapter 6.3) this would be a relatively simple experiment to perform.

There are virus coded polypeptides that have similiar molecular weights to those of host cell polypeptides and this tended to obscure the exact time of appearance of some of the viral structural polypeptides. While the temporal appearance of some could be determined by the increase in band intensity, the time of appearance of others was difficult to determine.

There were at least three major polypeptides, with apparent MWs of 93K, 20.5K and 19.5K, that were present in virion preparations but not in the cell lysates in the first experiment. They are probably products of protein cleavage or amino acid modification. It has been demonstrated in vaccinia

virus-infected cells that at least two major structural polypeptides are products of higher molecular weight precursors (Moss and Rosenblum, 1973; Pennington, 1973). However, 20.5K and 19.5K orf virus polypeptides appeared to have been detected in the cell lysates in the second experiment. Further studies will be needed to show what post-translational changes are occuring in the virus cell cycles. This may be possible using the inhibitor rifampicin, which inhibits virus assembly and maturation, in a pulse-chase experiment.

From the gel illustrated in Fig. 7-3 , many of the virally-induced polypeptides apearing late in infection did not correspond to any of the polypeptides in the virion preparations. In this experiment only 50,000 cpm of virions were used and some bands that had been detected in previous experiments (see Fig. 5-4) were not detected on fluorography. By increasing the amount of radioactivity per well by 3-fold more polypeptides were resolved. In fact, a mixture of 50,000 cpm each of virions and cell lysates pulse-labelled at 36 hr p.i. showed a pattern similar to the whole virion polypeptide profile found in the experiments reported in Chapter 5.

Although it is clear that more work needs to be done to analyse in detail the polypeptides produced in orf virus-infected cells, the system devised has provided a groundwork for future studies.

CHAPTER 8

THE EFFECT OF ULTRAVIOLET IRRADIATION AND METABOLIC INHIBITORS ON EARLY CELL ROUNDING

8.1 INTRODUCTION

It was noted that when orf virus, isolated either directly from scab material or purified from sodium diatrizoate gradients, was grown in cell culture, a rapid cpe occurred in the first 2 or 3 passages (unpublished results). After these initial passages the virus would either disappear completely or become adapted to cell culture in subsequent passages. If the virus became adapted to cell culture a typical cpe was produced. This usually began as a rounding of cells as early as 2 hr p.i., depending on the multiplicity of infection, and was called early cell rounding (ecr) because of its similarity to a phenomenon seen in vaccinia virus-infected cells (Appleyard et al., 1962; Bablanian et al., 1978b). Cell rounding became generalised by 5 hr p.i. and at 24 hr p.i. rounded cells were clumped together. If left longer than 24 hr, cells began to detach from the surface of the culture dish. Experiments were undertaken to determine whether the ecr was likely to be due to a toxic component such as a virion polypeptide present in the virus inoculum or to a gene product expressed early during infection.

Two approaches were used to investigate these possibilities. Firstly, the effect of ultraviolet (U.V.) irradiation of virus on cell rounding and virus yield was examined. An inoculum of virus was irradiated with U.V. light and then the dose required to reduce ecr by 50% was calculated. The dose required would give an indication of whether or not the U.V. was affecting a gene. The effect of U.V. on viral infectivity was also investigated.

Secondly, the effect of metabolic inhibitors on cell rounding was investigated. These experiments were designed to determine whether or not blocking stages in the virus development would inhibit cell rounding. If they did, then it would be likely that ecr was due, either directly or indirectly, to the expression of a viral gene.

8.1 MATERIALS AND METHODS

Virus and Cells. Primary BT cells were grown in MEM-LAH containing 10% FCS. The virus inoculum, NZ2, was grown in BT cells and used as a concentrated crude cell lysate to produce generalised ecr within 4-5 hr p.i.

Metabolic Inhibitors. The metabolic inhibitors used were Actinomycin D, cycloheximide, tunicamycin, α-amanitine, Adenine arabinofuranoside (ara-A), Cytosine arabinofuranoside (ara-C), bromouracyldeoxyribose (BUdR), and hydroxyurea (HU) (Sigma Chemical Company).

U.V. Irradiation of Virus Inoculum. Two ml aliquots of virus suspension were dispersed into 60 x 15 mm petri dishes and exposed to a 15-Watt 254 nm U.V. lamp at a distance of 23cm. At various times after the commencement of irradiation petri dishes of irradiated virus were taken and stored on ice for use in subsequent experiments. Two ml of unexposed virus suspension were set aside for use as controls.

Actinometry. The energy of the U.V. light used to inactivate virus was measured by the method of Hatchard and Parker (1953) using potassium ferrioxalate as the photolyte. Fifteen ml of 0.006 M potassium ferrioxalate (see Appendix) were irradiated at the same time as the virus inocula. To minimise the effect of extraneous light the actinometric solution was placed in a plastic dish painted black and covered with a black lid immediately after exposure to the U.V. light. After irradiation the dishes were transferred to a dark-room illuminated with a Kodak OB safelight. The contents of each dish were mixed and 5 ml of the irradiated solution were added to 2 ml of a 0.1% solution of 1,10 phenanthroline hydrate and 2.5 ml of buffer solution (600 ml of N-sodium acetate and 360 ml of N-sulphuric acid diluted to 1 L). Each mixture was made up to 20 ml with distilled water and allowed to stand for 30 min. The solutions were then removed from the dark-room and the optical density of each was measured at 510 nm in a Unicam P500 spectrophotometer. The optical density of a similar volume of unexposed potassium ferrioxalate was also measured. The difference between the two optical densities was converted to the quantity of ferrous ions formed during the reaction using a

standard curve (see Appendix) determined with known quantities of ferrous ions using the same spectrophotometer. The quantity of ferrous ions formed in the total volume of irradiated solution was converted to the radiation dose using the quantum efficiency of 1.28 as recommended for the 254 nm wavelength light. The energy was then calculated using the formula $E = hc/\lambda$ erg where **h** is Planck's constant, **c** is the velocity of light and λ is wavelength in nm (Parker, 1968). From this the total energy delivered to each dish of virus could be calculated.

Effect of U.V. Irradiation on Cell Rounding and Viral Infectivity. The effect of U.V. irradiation on viral infectivity was assessed using the plaque assay described in Chapter 3. Both U.V.-irradiated and non-irradiated inocula were used to infect BT cells grown in 24-well plates.

The effect of U.V. irradiation on cell rounding was also analysed in BT cells grown to confluence in 24-well plates. Duplicate wells were infected with 10-fold serial dilutions of the irradiated and non-irradiated virus. Duplicate wells were mock-infected with culture medium alone and used as controls. After 1 hr of adsorption, infected and control monolayers were fed with fresh maintenance medium. Rounded cells were counted at 5 hr p.i. using an inverted microscope at x100 magnification. At this time p.i., the monolayers infected with non-irradiated virus were showing generalised cell rounding. Ten microscopic fields were counted from each dish. Virus-induced cell rounding was calculated as the the

difference between the average counts in the control monolayers and those in the infected cells.

Effect of Metabolic Inhibitors on Cell Rounding. BT cells were grown to confluence in MEM-LAH containing 10% FCS in 24-well plates and the monolayers infected with 200 µl of stock virus. After 1 hr the medium was removed and was replaced with MEM-LAH containing 2% FCS. Duplicate wells were treated with either cycloheximide (300 µg/ml), Actinomycin D (5 µg/ml), ara-A (5µM), ara-C (50 µg/ml), BUdR (100 µg/ml) tunicamycin (2 µg/ml) or hydroxyurea (0.01M). When ∠amanitine was used, uninfected cells were treated overnight with the drug at 10 µg/ml before infection as it was found that 18 - 24 hr of pre-treatment of the cells was required to equilibrate the effective drug dosage. The same level of drug was added during and after adsorption of virus to prevent changes in internal drug concentration (Hruby et al., 1979). Actinomycin D (5 µg/ml) was added either 30 min before or 60 min and 120 min after infection. Uninfected cells treated with each drug and some infected but untreated cells were also used as controls. Cell rounding was assessed at 5 hr p.i. at which time infected controls were showing generalised cell rounding.

8.3. RESULTS

The Effect of U.V. Irradiation on Cell Rounding and Viral Infectivity. As shown in Fig. 8-1 more than 90% of the infectivity was lost when the virus was irradiated at a dose

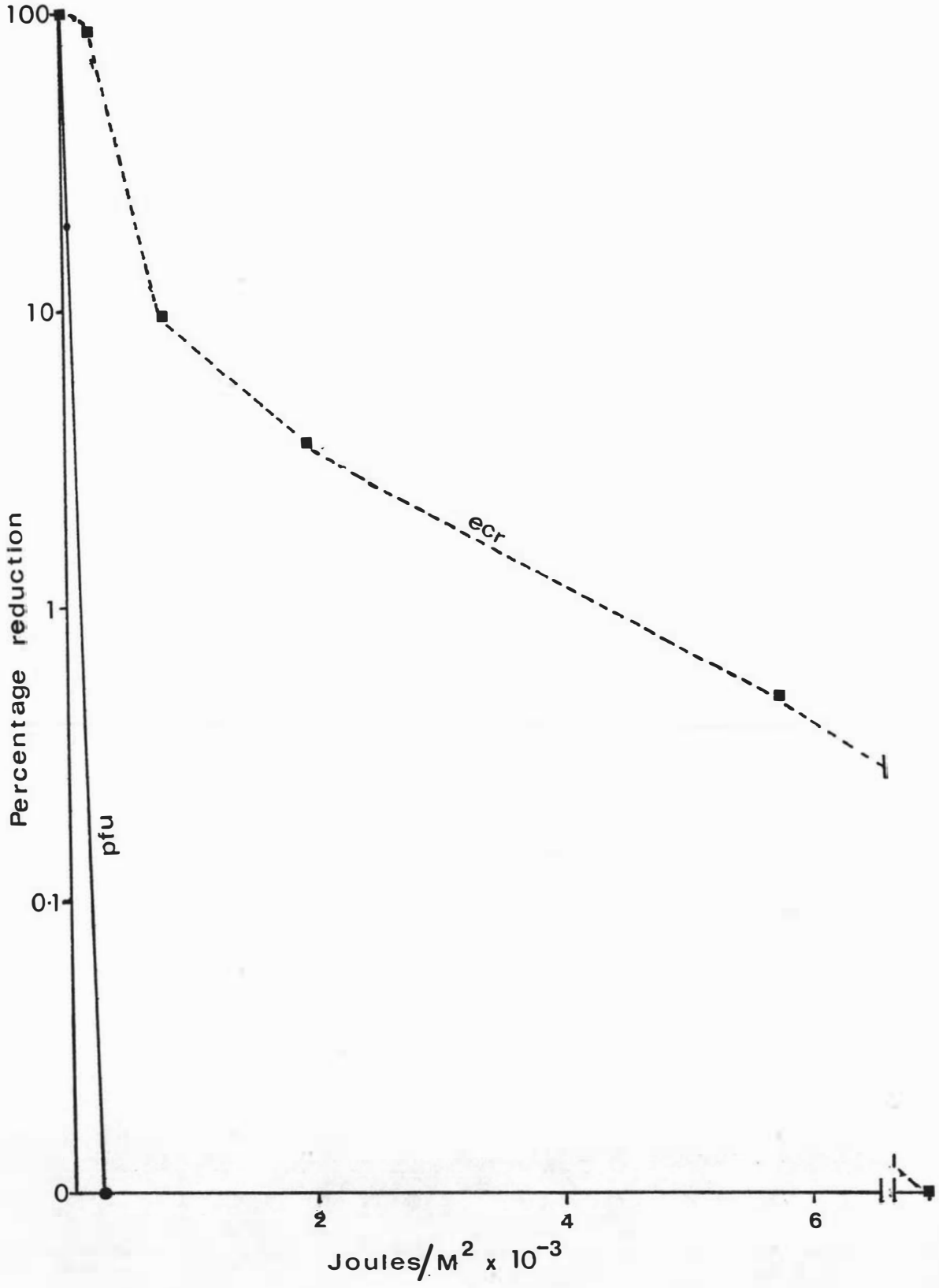


Fig. 8-1. The effect of U.V. irradiation on viral infectivity and cell rounding.

of 0.5 x 10^2 J/m^2 before being used to infect BT cells. About 50% inactivation was noted when virus was irradiated at 0.39 x 10^2 J/m^2. Ecr was reduced by 50% when virus was exposed to 0.38 x 10^3 J/m^2 of U.V. light. The results show that 10 times more U.V. was required to cause 50% reduction in ecr than to cause 50% inactivation of viral infectivity.

The Effect of Metabolic Inhibitors on Cell Rounding. The effect of the metabolic inhibitors on ecr is shown in Table XIV. BT cells infected with NZ2 in the absence of metabolic inhibitors showed cell rounding at 5 hr p.i. Cell rounding was inhibited by Actinomycin D when the drug was added either 30 min before or 60 min after infection but not when it was added 120 min after infection. Cycloheximide also inhibited ecr, cell rounding was not inhibited by either α-amanitine, tunicamycin, ara-A, ara-C, BUdR or hydroxyurea.

8.4. DISCUSSION

Sharma and Ali (1976) have shown that 0.7 x 10^6 J/m^2 is required to inactivate the activity of the enzyme tyrosinase by 50%. Also Bablanian *et al*. (1981a) did not detect any effect on vaccinia virus enzymatic activity when the virus was irradiated at a dose of 2.8 x 10^4 J/m^2. In contrast, viral infectivity and synthesis of vaccinia virus-specified polypeptides can be reduced by 50% at a dose of 0.1 x 10^2 J/m^2 and 0.26 x 10^2 J/m^2, respectively (Bossart *et al*., 1978b). It was also shown that 50% inactivation of ØX174 was

Table XIV. Effect of Metabolic Inhibitors on Orf Virus Early Cell Rounding

Inhibitors	Early Cell Rounding	
	Presence	Absence
I. PROTEIN SYNTHESIS:		
CYCLOHEXIMIDE (300 µg/ml)		+
II. RNA SYNTHESIS		
ACTINOMYCIN D (5 µg/ml)		
30 MIN. BEFORE INFECT.		+
1 HOUR AFTER INFECT.		+
2 HOURS AFTER INFECT.	+	
α-AMANITINE (10 µg/ml)	+	
III. DNA SYNTHESIS		
ARA-A (5 µM)	+	
ARA-C (50 µg/ml)	+	
BuDR (100 µg/ml)	+	
HYDROXYUREA (.01M)	+	
IV. PROTEIN GLYCOSYLATION		
TUNICAMYCIN (2 µg/ml)	+	

achieved at a U.V. dose of 0.15-0.4 x 10^2 J/m^2 (Yarus and Sinsheimer, 1964). By comparing the dose-effect of U. V. irradiation on these systems with that reported here it can be seen, for instance, that at least 2,000 more J/m^2 are required to inactivate tyrosinase activity by 50% than to cause a similar level of reduction in ecr induced by orf virus infection.

The relatively low dose of U.V. irradiation required to inactivate rounding in orf virus-infected BT cells suggests that this event is not caused by either a protein component of the virus or a factor in the inoculum. It is therefore more likely that the U.V. light dose of 0.38 x 10^3 J/m^2 required to significantly reduce ecr is due to an action on nucleic acid and inactivition of a gene. The energy required to produce 50% reduction in ecr is about the same magnitude as that required to produce 50% inhibition of vaccinia virus-induced polypeptide synthesis and is about 10 times more than that required to reduce orf virus and ØX174 infectivity and about 40 times more than is required to reduce vaccinia virus infectivity to 50%.

The slightly higher (four-fold) U.V. dose reported to be required to inactivate vaccinia virus infectivity by 50% when compared to orf virus infectivity might be explained by the different experimental technique used in each experiment and possibly to a different method of calculating U.V. dose. Differences in multiplicity reactivation and repair of U.V.-damaged virus by the host cells used (Abel, 1962; Zavadova, 1971) may also explain the discrepancy. In future experiments

it would be useful to test the effect of U.V. on vaccinia virus under the conditions described here.

Actinomycin D, an RNA synthesis inhibitor and cycloheximide, a protein synthesis inhibitor, prevented ecr while inhibitors of DNA synthesis did not. This provides evidence that an early protein induced as a result of virus infection is the mediator of ecr. This is in agreement with the findings of Bablanian (1968, 1970) and Burgoyne and Stephen (1979). The latter investigators also provided evidence that a cytotoxin, a monomer of the ST and a late gene product, is not the mediator of ecr. The cytotoxin may be responsible for cell degeneration in the latter stages of infection. However, Burgoyne and Stephen (1979) did show that a rapid cpe, which is a cytotoxic effect, in early infection could be produced when an abnormally high multiplicity of infection (1200 pfu/cell) was used and that it may be due to a high concentration of the cytotoxin from the virus inoculum. Mbuy et al. (1982) showed that purified ST (50 µg) inhibited protein synthesis in 2 x 10^5 HEp-2 cells. This may be an explanation for the rapid cpe seen in the initial passages of orf virus in cell culture. However, it is also possible that scab material contains a high proportion of inactivated virus that is able to express some early gene effect, such as ecr, but is unable to complete a virus/cell cycle. In the latter case a cpe would be seen but little virus would be produced. Tunicamycin is an inhibitor of N-glycosylation and α-amanitine is a specific inhibitor of RNA polymerase II which is necessary for nuclear synthesis. The negative results obtained with these drugs means that N-

glycosylation and nuclear RNA synthesis were not involved in ecr. Ara-A, Ara-C and BUdR are inhibitors of DNA synthesis. The absence of cell rounding in the presence of these drugs suggests that polypeptides responsible for cell rounding are synthesised early e.g. before DNA synthesis.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

In these studies orf virus isolates were adapted and plaque-purified in cell culture. The virus showed a preference for primary cells of ovine and bovine origin rather than for established cell lines from other animal species. It was shown that supplementation of the MEM with 0.5% lactalbumin hydrolysate increased virus yields. The virus titre could also be increased by several cycles of freezing and thawing of infected cells coupled with sonication. This information might allow cultivation of other New Zealand field isolates to higher yields of virus. About 96%-99% of the progeny virus remained intracellular. This information is important in future attempts to concentrate virus from large volumes of infected cell cultures.

Another important achievement of this work was the establishment of a reproducible assay for viral infectivity. The quantal and plaque assays proved to be effective ways to assay viral infectivity but preliminary experiments showed that fluorescent-antibody staining of plaques has the potential to be a more rapid alternative. However, the plaque assay was found to be a relatively simple and reproducible method of quantitating viral infectivity for further analysis of the biology of orf virus.

Using the plaque assay to establish a reliable viral titre, a one-step growth experiment showed that infectious virus particles were detectable at 16-18 hr p.i. and continued to be detectable for up to 45 hr. In another one-step growth experiment it was shown that orf virus DNA synthesis began at 4-8hr p.i. and that a rapid increase in DNA synthesis was seen at 12-14 hr and continued for up to 30-35 hr. Vaccinia virus DNA synthesis in the same cell system began at 3 hr and was almost complete by 12 hr. Orf virus DNA synthesis was inhibited by Ara-C, BUdR and hydroxyurea within the dose range required for the DNAs of other virus types.

The relatively slow growth rate of orf virus and the intracellular nature of progeny virus results in slow spread from cell-to-cell which may thus explain why plaques were sometimes difficult to obtain in cell culture. The slow growth rate and cell-associated nature of viral progeny also becomes important when attempting to grow and adapt orf virus in cell cultures. In initial isolation attempts from clinical material which might contain a high proportion of non-infectious virus there would appear to be no advantage in incubating cultures for periods longer than one virus cycle, which for orf virus would be 48 hr. Passage of virus at 2-day intervals with a freeze/thaw cycle and sonication between passages, rather than increasing the time between passages, would seem to provide the most rational way of adapting virus in cell culture.

The labelling of orf virion polypeptides with ^{35}S-methionine was another achievement of the work presented here. This

enabled a detailed comparison of the structural polypeptides of 6 New Zealand isolates to be made. Five isolates, NZ2, NZ7, NZ9, NZ10, and NZ12, showed similar profiles while strain NZ19 showed a different pattern on SDS-PAGE.

Following on from the analysis of virion polypeptides an analysis of polypeptides appearing in infected cells at various times after infection was attempted and some important parameters defined. For instance, it was found necessary to pulse-label cells for 2 hr to ensure detection of polypeptides. Also pulse-labelling of orf virus-infected cells showed that most of the virus-induced polypeptides were detected from 10 hr p.i. There was a slow or incomplete "switch-off" of cellular macromolecular synthesis which may have prevented the detection of early induced polypeptides. Most of the late polypeptides were structural entities as they were also detected in whole virion preparations. A polypeptide with a molecular weight of 93K, and which was seen also in whole virus, was probably a product of post-translational changes.

The six isolates could be grouped tentatively into two strains. This may be important in the light of future vaccine preparations. The two vaccines commercially available in New Zealand are of similar strains of virus as determined by restriction endonuclease analysis (Robinson, personal communication). Although they seem to represent the more common antigenic type e.g. in the presence of the 38.5K polypeptide, the presence of other antigenic types could pose problems in vaccination programmes. It might be reasonable

to recommend incorporation of these two strains of virus in vaccine preparations. This was tried in the United States (Buddle et al.,1984) although there has been no evidence established as to whether or not there was any improvement in vaccine efficacy as a result.

Evidence has been presented which indicates that the ecr produced in orf virus infection is produced by a gene product expressed early in infection. The results from the U.V. irradiation experiments suggested that reduction in ecr is due to U.V. action against nucleic acid rather than protein. An experiment to confirm this would be to determine the action spectrum needed to inactivate the virus and to reduce the cell rounding effect. The activity of U.V. light at 260 nm on nucleic acid is about twice that at 280 nm. Conversely, the action on protein is higher at 280 nm than 260 nm. Measuring the ratio of activity at 260 nm and 280 nm might indicate whether it is protein or nucleic acid that is the target. Another experiment that should be done is to determine whether or not ecr is related to the multiplicity of infection.

(A.J. Robinson, per com.)

Now that the orf virion genome has been cloned, it will be possible to begin mapping viral genes. The availability of the cell-free transcription/translation system might facilitate the identification of virus-coded polypeptides, in particular the immunogenic polypeptides, and perhaps the establishment of a translation map of the orf virus genome. The basic information gained on the number and size of orf

virion polypeptides will be useful in the gene mapping studies.

Immunity to poxviruses involves cell-mediated immunity. There is evidence that, for vaccinia, antigens derived from the virus and some early and late antigens expressed at the surface of infected cells are recognised by cytotoxic T-cells (Mallon and Holowczak, 1985; Mallon et al., 1985). Thus an alternative orf vaccine might consist of infected cells blocked at the early stage of infection. Initially, it was the aim of the present study to investigate those antigens occurring in the cell membrane of orf virus-infected cells as well as the virion antigens. However, early difficulties in growing enough virus to do the experiments put constraints on realising this objective. In retrospect this may have been fortuitous as the exeriments reported by Mallon and Holowczak (1985) and Mallon et al. (1985) showed that a variety of viral and cell polypeptides could be found at the cell surface. All, some, or none of these might be targets for cytotoxic T cells and thus they could not come to any clear conclusions as to which ones were important. It now seems likely that most are involved in the stimulation of a T cell response (Holowczak, personal communication).

Another approach might be to clone specific viral genes and express these in vitro or in vivo to see what effect they have on the stimulation of an immune response.

A recombinant DNA approach may be applied to develop a new vaccine. In this case, a cloned DNA containing the relevant

immunogenic genes may be used to produce sufficient immunogens *in vitro* for use in a vaccine. Alternatively, the use of a recombinant virus expressing orf genes might be possible.

The information gained from the experiments in this thesis has given a broader knowledge of the biology and behaviour of orf virus in cell culture, which in the past had been assumed to be similar or closely related to vaccinia virus. The basic parameters of orf virus infection reported here will be invaluable for further genetic studies.

APPENDIX

PREPARATIONS OF BUFFERS, SOLUTIONS, POTASSIUM FERRIOXALATE AND $FeSO_4$ CALIBRATION GRAPH

Buffers and Solutions

1. 0.25% Coomassie Blue-R (staining solution)

2.5	g	Coomassie Blue R
230	ml	40% acetic acid
454	ml	methanol
316	ml	distilled water

Filter through Whatman no. 1

2. Destaining Solution

400	ml	methanol
175	ml	40% acetic acid
425	ml	distilled water

3. SDS-PAGE Running Buffer

14.4	g	Glycine
2.5	ml	1 M Tris buffer, pH 8.8
5.0	ml	20% SDS
1000.0	ml	distilled water

Adjust to pH 8.3

4. Scintillation fluid

1000.0 ml Toluene

5.0 g PPO

0.3 g POPOP

4. Prehybridization Mix

(a) Stock PO_4 buffer (0.2 M, pH 6.5)

3 g NaH_2PO_4

7.16 g Na_2HPO_4

Dissolve to make 100 ml

(b) Prepare

50.0 ml stock PO_4 Buffer (0.2 M, pH 6.5)

4.35 g NaCl

4.41 g Na Citrate

25.0 ml water

(c) Dissolve 5 mg salmon sperm DNA in 10 ml of solution (a). Sonicate mixture 3X (1 min). Boil for 5 min. Plunge into ice.

(d) 50X Denhardt's solution

200 mg BSA

200 mg PVP

200 mg Ficol 400

2 g glycine

Dissolve with remaining solution (a)

(d) Mix solution (b) and (c). Add 5 ml of this solution to 5ml Formamide for working prehybridization solution.

5. Hybridization Mix

(a) 20 ml phosphate buffer stock

30 ml distilled water

4.35 g NaCl

4.41 g Na Citrate

(b) Dissolve 2 mg salmon sperm DNA in 10 ml of (a)

(c) Prepare 50X Denhardt's solution as above

(d) Mix (a), (b) and (c)

(e) Make up a solution of 50% Dextran sulphate (w/v). Heat and stir to dissolve.

(f) Add 40 ml of (e) to (d) and make it up to 100 ml with water.

(g) 5 ml of (f) mixed with 5 ml formamide is used for hybridization

Preparation of Pure Potassium Ferrioxalate

Potassium ferrioxalate was prepared by mixing 3 volumes of 1.5 M potassium oxalate with 1 volume of 1.5 M ferric chloride with vigorous stirring. The precipitated potassium ferrioxalate was recrystallised from warm water and dried using a warm-air hair-dryer. A 0.006 M solution was prepared by dissolving 2.947 g of the crystals in 800 ml of water. One hundred ml of 1.0 N sulphuric acid was then added and the solution was made up to 1 L with distilled water. The above procedures were done in a dark-room lit by a yellow safelight (Kodak OB).

Preparation of $FeSO_4$ Calibration Graph

Solutions required:

a. 0.4 x 10- mole/ml Fe+ in 0.1 N H_2SO_4 (prepared by dilution of standarised 0.1 M $FeSO_4$ in 0.1 N H_2SO_4

b. 0.1% 1:10 phenanthroline monohydrate

c. buffer solution (600 ml of N-sodium acetate and 360 ml of N-sulfuric acid diluted to 1 L.

Into a series of 20 ml flasks add the following volumes of solution (a): 0, 0.5, 1, ...5.0 ml. Add 0.1 N-sulfuric acid so as to make the total acidity equivalent to 10 ml of 0.1 N-sulfuric acid. Add 2 ml of solution (b), 5.0 ml of solution (c) (mixing after each addition), make up to 20 ml, mix and allow to stand for at least 30 min. Measure the optical densities at 510 nm in a 1 cm cell with a spectrophotometer. Correct each optical density for the value obtained with control. Plot the results against the amount of ferrous ions added.

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