STUDIES OF A MUTANT OF MuLV

USE OF PSEUDOTYPES OF VESICULAR STOMATITIS VIRUS IN STUDIES OF A TEMPERATURE SENSITIVE MUTANT OF MOLONEY MURINE LEUKEMIA VIRUS

By

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ABSTRACT

A mutant of Moloney murine leukemia virus (MoLV), designated ts_3, was recently shown to have a temperature-sensitive defect associated with the release of mature virus particles budding from the cell membrane (Wong and McCarter, 1974). In an attempt to determine whether the defective function resides in an envelope component of the virion, the formation of pseudotypes between VSV and ts_3 was studied under non-permissive and permissive conditions of ts_3 infection. Whereas similar levels of phenotypic mixing were observed between VSV and wild type MoLV at both 39°C and 34°C, the level of pseudotypes formed between VSV and ts_3 was found to be considerably lower at 39°C (non-permissive temperature) than at 34°C (permissive temperature). The results of temperature-shift experiments indicate that two separate blocks to VSV (ts_3) pseudotype production may occur depending on the length of time ts_3-infected cells are incubated at the non-permissive temperature. Pre-incubation of ts_3-infected cells for 24 hours at 39°C, prior to super-infection with VSV at 39°C, introduces an irreversible block to VSV (ts_3) pseudotype production. In contrast, brief incubation at 39°C, coincident with VSV infection,

introduces a reversible block which prevents the release of VSV (ts_3) pseudotypes from the cell membrane. Complementation of ts_3 through ts_3 (VSV) pseudotype production was not detected at the non-permissive temperature.

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INTRODUCTION

1. General Introduction

From an historical perspective, the RNA tumour viruses have been the most extensively studied of the oncogenic virus groups. Since the first demonstration of an avian tumour virus (Rous, 1911), RNA viruses possessing similar morphological, physico-chemical, and immunological characteristics have been isolated from reptile, amphibian, bovine, feline, murine and primate tumours (Temin, 1971). Whereas many of these viruses have been shown to induce tumours, some as yet, have an undetermined oncogenic potential.

Of particular concern has been the widespread origin of RNA tumour viruses. The genetic transmission of avian and murine RNA tumour viruses (Weiss, 1973; Rowe *et al.*, 1971), the spontaneous release of RNA tumour viruses from stable cell lines, and the numerous reports of chemical and/or physical induction of these viruses has provided support for the theory that all vertebrates contain genetic elements of endogenous RNA tumour viruses.

Unlike more cytopathic RNA viruses (Vesicular Stomatitis Virus and Newcastle Disease Virus), RNA

tumour viruses generally do not kill and need not replicate in the cells which they infect. With the advent of appropriate cell cultures, it has been determined that RNA tumour virus infections result in either: (1) cell transformation with concomitant virus replication; (2) cell transformation without virus replication; or (3) virus replication without cell transformation. To account for these observations, it has been proposed that the group of RNA tumour viruses contains genes for both virus replication and maintenance of cell transformation. In addition to 'complete' particles, it would, therefore, appear that there are viruses which contain or express genetic information for only one of these functions. Nevertheless, RNA tumour virus infection is always marked by the integration of virus genetic information into host cell chromosomes.

The development of inbred strains of mice with a high incidence of leukemia (MacDowell and Richter, 1935; Cole and Furth, 1941) has been largely responsible for the emergence of murine leukemia viruses as a model for studying the replicative function of mammalian RNA tumour viruses. Although much has been learned about the replication of these viruses, very little is known about the mechanism(s) controlling their release from infected cells. Recently, a temperature-sensitive

mutant of the Moloney strain (Moloney, 1960) of murine leukemia virus, designated ts_3, has been isolated and found to be defective for the release of mature virus budding at the cell membrane (Wong *et al.*, 1973; Wong and McCarter, 1974; Wong and MacLeod, 1975). As during the maturation process, budding virus particles acquire an envelope of virus-directed membrane-associated proteins, the present study attempts to correlate these proteins with the ts_3 defect.

Over the past few years an increasing number of investigators have used Vesicular Stomatitis Virus (VSV), a non-oncogenic cytocidal RNA virus, as a probe for studying the properties of a coinfecting virus. In 1972, Zavada demonstrated that VSV participates in phenotypic mixing with murine leukemia viruses during maturation at the cell membrane. These phenotypically mixed virions of VSV or 'pseudotypes' contain the genome of VSV in envelope proteins derived from the leukemia virus. Accordingly, it was reasoned that pseudotype production would be useful in examining the defect associated with ts_3 release.

As a background to the present study, the following pages will be concerned with the general properties of murine leukemia viruses and VSV, the isolation and characterization of ts_3, and the formation and properties of VSV pseudotypes.

2. Murine Leukemia Viruses

The murine leukemia viruses have been classified as C-type particles on the basis of their morphology in thin section electron microscopy (Bernhard, 1958;1960). These early observations revealed roughly spherical structures, approximately 100 nm in diameter, containing a central nucleoid core (Sharp *et al.*, 1952; Bernhard *et al.*, 1958). Although the morphology remains unclear, some structure has been resolved using the techniques of freeze-etching, negative staining and freeze drying (Nermut *et al.*, 1972). In summary, murine leukemia viruses are enclosed in a unit membrane from which projects the knob-like structures of glycoprotein. Inside the unit membrane lies a core shell consisting of a protective capsid with icosohedral symmetry and a core envelope. This core envelope in turn encloses what appears to be a filamentous ribonucleoprotein of helical symmetry.

Although C-type particles appear to contain several species of cellular RNA (Bauer, 1966; Bonar *et al.*, 1967; Imai *et al.*, 1966; Gay *et al.*, 1970; Erikson *et al.*, 1973), the bulk of evidence suggests that the viral genome is a 60-70S single-stranded RNA with a molecular weight of 10-12 x 10^6 (Robinson and Baluda, 1965; Duesberg and Robinson, 1966). Upon denaturation, this molecule

dissociates into 35S and 4S subunits (Duesberg, 1968; Bader and Steck, 1969; Erikson, 1969; Montagnier *et al.*, 1969; Manning *et al.*, 1972; Erikson and Erikson, 1971) and it has since been proposed that the viral genome consists of 35S subunits joined together by hydrogen bonding with associated 4S 'linker' molecules. Recently, Riggin *et al.* (1975) have shown that the 60-70S RNA of Moloney leukemia virus contains two 35S subunits.

At present, seven structural proteins are recognized within the murine leukemia virion (see review by Bolognesi *et al.*, 1974 and also Ikeda *et al.*, 1975; Ihle *et al.*, 1975). These have been classified according to molecular weight as internal proteins p10, p15, and p30 and surface proteins or glycoproteins p12, p15E, gp45 and gp69/71. Glycoprotein 69/71 comprises two antigenically related molecules of molecular weights 69,000 and 71,000 (Strand and August, 1973) and interferes with murine leukemia virus infection (Hunsmann *et al.*, 1974).

In accordance with Temin's provirus hypothesis (1964) that the viral genome replicates through a DNA intermediate, an RNA-dependent DNA polymerase (Reverse Transcriptase) activity was demonstrated for the virions of RNA tumour viruses (Baltimore, 1970; Temin and Mitzutani, 1970). The first characterization of virion-associated reverse transcriptase demonstrated its

capacity to use endogenous 60-70S viral RNA as template for the synthesis of complementary DNA (Garapin *et al.*, 1970; Rokutanda *et al.*, 1970; Spiegelman *et al.*, 1970; Manly *et al.*, 1971). However, since then, the enzyme has been found capable of synthesizing DNA using a variety of exogenous template-primers (see review by Temin and Baltimore, 1972). Moreover, because the enzyme is primarily associated with the group of RNA tumour viruses, reverse transcriptase activity has been widely used in their detection.

The present understanding of RNA tumour virus replication comes largely from studies carried out with avian tumour viruses. A brief summary of these investigations and the proposed model of replication are presented below.

Following adsorption to specific cell receptors, infecting virions penetrate the cell membrane and are transported to the cell nucleus (Dales and Hanafusa, 1972). At some stage during or after viral transport (Hatanaka *et al.*, 1971; Dales and Hanafusa, 1972), reverse transcriptase copies 60-70S viral RNA into a double-stranded DNA 'provirus' which, by an unknown mechanism, becomes covalently integrated into host chromosomes (Hill and Hillova, 1972; Varmus *et al.*, 1972, 1973a and b). After cell division, the provirus is transcribed into viral RNA (Humphries and Temin, 1972) possessing the

same base sequences as 60-70S virion RNA (Parsons *et al.*, 1973; Cannani *et al.*, 1973). It has been suggested that these RNA transcripts serve as a source of both messenger for the synthesis of virus-specific proteins and new viral genomes for maturing virus particles. During the final stages of replication, maturing particles migrate to a position just beneath the cell surface and upon budding through the cell membrane acquire a unit membrane envelope containing virus-specific glycoproteins.

3. Spontaneous Temperature Sensitive Mutant of Moloney Leukemia Virus - ts_3

In 1970, Bassin *et al.* reported that $S^+ L^-$ cells (cells transformed by a murine sarcoma virus which replicates non-infectious particles [Bassin *et al.*, 1971]) superinfected with murine leukemia virus become rounded up and readily detach from monolayer culture. Based on this observation, Wong *et al.* (1973) devised a procedure for the selection of spontaneous temperature-sensitive mutants of Moloney murine leukemia virus. Briefly, selection involved discarding those $S^+ L^-$ cells which round up and detach after infection with wild type virus at the non-permissive temperature (39°C). Cells remaining firmly attached could then be induced to round up and release temperature-sensitive mutants by incubation at the permissive temperature (32°C). One

such mutant, designated ts_3, was then harvested and subsequently enriched and purified by repeated selection and cloning. The final isolate showed a differential titre of approximately 500-fold following growth in TB cells at the permissive and the non-permissive temperature (Wong and McCarter, 1974).

Recent studies using the scanning electron microscope (Wong and MacLeod, 1975) have clearly revealed that the temperature-sensitive defect of ts_3 prevents the release of mature virus budding at the cell membrane (Wong and McCarter, 1974). A significantly greater number of budding particles was observed on the surface of TB cells infected with ts_3 at the non-permissive temperature (39°C) than at the permissive temperature (34°C). Furthermore, following infection at 34°C, the number of budding particles observed at the cell membrane remained more or less constant, whereas at 39°C, the number of budding particles observed at the cell membrane progressively increased from 8 to 48 hours after infection. After this time, no further increase was detected. That these observations reflect a temperature sensitive phenomenon is indicated by the fact that approximately 90% of these particles dissociate from the cell membrane within 1 hour of a shift from 39°C to 34°C. These observations agree with earlier temperature-shift studies by Wong and McMarter (1974) who reported that a temperature shift from 32°C to 39°C immediately

blocks the release of infectious virus from ts_3-infected TB cells. Furthermore, ts_3-infected cells pre-incubated for 42 hours at 39°C were found to release large quantities of virus immediately following shift to the permissive temperature. Actinomycin D and cycloheximide, inhibitors of RNA and protein synthesis respectively, had very little effect on the quantity of virus released during the first hour after temperature shift. In addition, the temperature-sensitive defect of ts_3 has been shown not to affect the somatic properties of the virus because the inactivation kinetics of ts_3 are indistinguishable from those of wild type virus at 40°C.

Additional studies by Wong and McCarter (1974) have examined the ability of ts_3 to rescue murine sarcoma virus (MuSV) from a MuSV-infected non-producer cell line. Whereas ts_3 was found to rescue MuSV at the permissive temperature, a shift to the non-permissive temperature immediately blocked the release of both viruses. Moreover, a shift from the non-permissive to the permissive temperature resulted in a simultaneous release of both ts_3 and MuSV. As for ts_3, actinomycin D and cycloheximide were found to have very little effect on the quantity of MuSV rescued during the first hour after temperature shift.

4. Vesicular Stomatitis Virus

Vesicular Stomatitis Virus (VSV) is a cytolytic, bullet-shpaed rhabdovirus, 175 nm in length and 65 nm in diameter, consisting of a helically wound ribonucleoprotein core contained within a unit membrane envelope (see review by Howatson, 1970). The infectious component or 'B' particle contains a single-stranded RNA genome (Prevec and Whitmore, 1963) of molecular weight 3-4 x 10^6 (Huang and Wagner, 1966) and a virion-associated transcriptase (Baltimore *et al.*, 1970).

The five polypeptides which comprise VS virion are designated L,G,N,Ns and M (Wagner *et al.*, 1972) and have molecular weights of 190,000, 69,000, 50,000, 45,000 and 29,000 respectively. Proteins L,N and Ns in conjunction with virion RNA constitute the ribonucleoprotein core (Bishop and Roy, 1972; Emerson and Wagner, 1972; Szilagyi and Uryvayev, 1973) and are believed necessary for viral transcriptase activity (Emerson and Wagner, 1972, 1973). Protein G, a glycoprotein (Burge and Huang, 1970) comprises the envelope spikes (Cartwright *et al.*, 1970a and b; McSharry *et al.*, 1971) and contains the antigenic determinant recognized by neutralizing antibody (Kang and Prevec, 1970). Protein M forms the structural matrix underlying the viral envelope (Cartwright *et al.*, 1970b).

5. VSV Pseudotypes of Murine Leukemia Viruses

In 1972, Zavada reported that VSV grown in cells pre-infected with murine leukemia virus contains a proportion of virus resistant to anti-VSV serum. These 'pseudotypes' contain the genome of VSV but have acquired the envelope components of the coinfecting murine leukemia virus. As a consequence of their envelope properties, VSV pseudotypes are susceptible to neutralization by murine leukemia virus-specific antiserum and generally exhibit a low infectivity for cells known to contain contaminating RNA tumour-like virus particles.

Analysis of the virus produced upon infection with VSV pseudotypes has determined that progeny virus express the neutralization and host range specificities characteristic of normal wild type VSV. Hence, pseudotype formation results from a phenotypic mixing between viruses as VSV pseudotypes replicate the wild type genome.

By virtue of their foreign envelope, VSV pseudotypes provide a method of separating functions determined by viral envelope components from functions determined by other viral elements. Accordingly, the following study has used VSV pseudotype production in order to determine whether the viral envelope components of ts_3 express the temperature-sensitive function which prevents the release of budding virus particles.

MATERIALS AND METHODS

MATERIALS

1. Cell Lines

TB cells (a continuous line established from the bone marrow and thymus of CFW/D mice [Ball *et al.*, 1964]) were kindly provided by Dr. J.A. McCarter, Cancer Research Laboratory, University of Western Ontario.

The established line of Swiss·NIH/3T3 mouse embryo cells (Todaro and Green, 1963) was obtained from Dr. C. Pringle, Institute of Virology, Glasgow, Scotland. NIH·3T3 cells chronically infected with N-tropic strain of Friend leukemia virus (FLV) were provided by Dr. Sutapa Sengupta, Department of Pathology, McMaster University.

XC cells (a transformed, non-producing cell line derived from a rat tumour induced by Rous Sarcoma Virus [Svoboda *et al.*, 1963]) were provided by Dr. A. Axelrad, University of Toronto.

2. Viruses

HR-LT strain of the Indiana serotype of VSV was obtained from Dr. L. Prevec, Department of Biology, McMaster University. This strain was originally selected from IND-ST stock by its resistance to high temperature (45°C, 3 hr) (Nakai and Howatson, 1968). Highly infectious virus used in this study was prepared by plaque picking twice on L-cell monolayers.

Moloney murine leukemia virus (MoLV) and a spontaneous temperature-sensitive mutant, designated ts_3 (Wong *et al.*, 1973), were obtained from Dr. J.A. McCarter as cultures of chronically infected TB cells.

3. Chemicals and Solutions

(a) Phosphate Buffer Saline (PBS) (Dulbecco and Vogt, 1954)

NaCl	8	gm
KCl	2	gm
$NaHPO_4$	1.15	gm
KH_2PO_4	2	gm

formulated to 1 liter in glass distilled water pH 7.5.

(b) Toluene-Based Scintillation Fluid

PPO	4	gm
POPOP	0.3	gm
Toluene	1	liter

4. Biological Compounds and Radiochemicals

Polybrene was purchased for the Aldrich Chemical Co., Milwaukee, Wisconsin and Acti-dione (cycloheximide) was purchased from the Upjohn Company, Kalamazoo, Michigan.

Complete Freund's adjuvant was obtained from Difco.

Yeast RNA was purchased from Mann Research Laboratories, New York, N.Y.

Polyriboadenylic acid: oligothymidylic acid [Poly (rA): oligo $(dT)_{(12-18)}$], for use as template-primer in reverse transcriptase assays, was purchased from Collaborative Research Inc., Waltham, Mass. The RNA-DNA hybrids were contained in a buffer concentation of 0.1 M Tris HCl pH 7.4, 0.15 M NaCl.

Thymidine 5'-triphosphate [^{3}H-methyl], of specific activity 15 Ci/mmole, was obtained from Schwarz/Mann, Orangeburg, N.Y.

METHODS

5. Cell Culturing

TB and XC cells were grown in monolayer culture in Eagle's Minimal Essential medium (MEM) containing 10% New Born Calf Serum (NBCS), penicillin 100 units/ml, and streptomycin 100 μg/ml. NBCS was heat-inactivated at 56°C for 1 hour prior to use. Monolayer cultures of 3T3 cells were maintained in McCoy's 5a medium supplemented with NBCS and antibiotics as indicated above.

All cell cultures were grown in 16 oz Brockway bottles at an initial density of 2-5 x 10^5 cells in 30 ml complete medium. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. All cultures were maintained by routine trypsinization and subculturing every 3-4 days once full cell monolayers had formed. Trypsin-EDTA stock solution containing 0.5% (w/v) trypsin and 0.2% (w/v) EDTA in normal saline was diluted ten-fold prior to use. Cultures were generated from frozen stock as necessary and generally never carried more than 30 consecutive passages.

All growth media and supplements were purchased from Grand Island Biological Company (GIBCO), Grand Island, New York.

6. Viral Assays

(a) VSV plaque assay

The number of infectious particles or plaque forming units (PFU) present in a VSV stock was determined by plaque assay. Briefly, 0.1 ml of appropriately diluted virus was added directly to susceptible cell monolayers grown overnight in 60 mm Falcon petri dishes (Falcon Plastics). After 30 minutes virus adsorption at 37°C, infected cell monolayers were overlaid with 4 ml of a solution containing MEM, 5% NBCS, and 0.9% Noble agar (DIFCO). Plaques could be observed following 20-24 hours incubation at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cell sheets were then treated with Carnoy's fixative, plaques counted and titre of initial virus stock expressed as PFU per ml.

(b) Murine leukemia virus-XC assay

The XC assay is based on the observation of Klement *et al.* (1969) who noticed that when mouse embryo cells releasing murine leukemia virus (MuLV) were mixed with XC cells, the XC cells fuse to form plaques of syncytia. Rowe *et al.*, (1970) developed this into a sensitive plaque assay in which individual cells

supporting the replication of MuLV are detected by the addition of XC cells.

The procedure used was briefly as follows: Cultures of NIH/3T3 cells were seeded at 2×10^5 cells in 60 mm Falcon petri dishes. The following day, cells were washed with PBS and incubated for 1 hour at 37°C in the presence of 2 ml of polybrene (Aldrich Chemical Co.) at 10 μg/ml. Polybrene was then removed and cells exposed to 0.1 ml of appropriately diluted virus for 45-60 minutes at 37°C. To ensure a uniform distribution of infected cells, the virus inoculum was rotated over the cell sheet at regular intervals. After adsorption of virus, cells were grown at 37°C in 4 ml of McCoy's 5a medium containing 10% NBCS. The medium was changed after 3 days and on the fifth day post-infection, medium was removed and cells UV-irradiated at 67 ergs/mm^2/sec. Cultures were then overlaid with 10^6 XC cells in 4 ml of MEM containing 10% NBCS. The medium was changed after 2 days and cells were fixed with 70% methanol after 3 days additional growth. Staining with Giemsa revealed syncytial plaques and titre of initial virus was expressed as PFU per ml.

7. Production of Virus Stocks

Concentrated stocks of MoLV and ts_3 were prepared from persistently infected TB cells designated

Mol-TB and TB-ts_3, respectively. Mol-TB cells were grown at 37°C whereas TB-ts_3 cells were grown at 34°C, the temperature permissive for virus release (Wong *et al.*, 1973). Virus containing supernatants were harvested every 24 hours from confluent monolayer cultures prepared in 16 oz Brockway bottles. Harvests were cleared of cellular material by centrifugation at 400 x g for 20 minutes. The supernatant was recollected and virus pelleted by centrifugation in a Type 19 rotor for 165 minutes at 18,000 rpm at 5°C. Virus was resuspended in PBS to 1/100 the original volume and stored at -70°C until use.

Stocks of VSV were prepared by growth of plaque-purified virus in TB cells. The following procedure was used: Confluent monolayers of approximately 1.5 x 10^7 TB cells were prepared in 16 oz Brockway bottles. After removing culture medium, cells were exposed to 1 ml of virus inoculum at a multiplicity of infection (MOI) of 0.1 PFU/cell for 30 minutes at 37°C. After adsorption of virus, cells were maintained at 37°C in 30 ml. of MEM containing 2% NBCS. Virus-containing supernatants were collected 16-20 hours later and the harvests cleared of cellular material by centrifugation at 400 x g for 20 minutes. The cell-free virus fluid was then stored in 1.0 ml aliquots at -70°C until use. Infectious titre of stock preparations ranged from 0.5 to 5 x 10^8 PFU per ml.

8. Chronically Infected Cell Lines

Chronically infected TB cell lines were established by high multiplicity infection with concentrated MoLV stocks. Cultures of TB cells were seeded at 2×10^5 cells in 30 ml of complete medium and grown overnight in 16 oz Brockway bottles. The following day, cells were washed with PBS and incubated for 1 hour at 37°C in the presence of 2 ml of polybrene at 10 μg/ml. Cells were then exposed to 1 ml of virus inoculum at an MOI of 20 PFU per cell for 45 minutes at 37°C. After adsorption of virus, cultures were grown at 37°C in MEM containing 10% NBCS until confluent cell monolayers had formed. Cells were then passaged at high density for 5 days to avoid selecting populations of non-infected cells.

9. Preparation of Antisera

Rabbit anti-VSV serum was prepared using whole virus grown in human KB cells. Before use as antigen, the VSV inoculum was purified by sucrose gradient centrifugation and suspended to an infectious titre of 10^9 PFU/ml. The rabbit was then given intravenous and intramuscular injections of 10^8 PFU of virus, followed by an intraperitoneal injection of virus suspended in 0.4 ml of complete Freund's adjuvant. Fourteen days

after primary injections, the rabbit was started on a schedule of 3 weekly booster injections administered intramuscularly and intraperitoneally as before. One week after the last injection, the rabbit was bled and the serum collected after clotting overnight at 4°C. Serum was then complement-inactivated (56°C, 1 hour) and stored in 1.0 ml aliquots at -70°C until further use.

10. Reverse Transcriptase Assay

Baltimore *et al.* (1970) reported that after exposure to non-ionic detergents, the virions of murine RNA tumour viruses incorporate deoxyribonucleotide triphosphates into DNA. This RNA-dependent DNA polymerase (Reverse Transcriptase) activity was assayed by the incorporation of [^{3}H-methyl]-thymidine 5'-triphosphate (^{3}H-TTP), using an exogenous template-primer of poly-ribonucleic acid: oligodeoxythymidylic acid [poly (rA): oligo $(dT)_{12-18}$].

Reaction mixtures contained 76 mM Tris-HCl pH 8.0, 45 mM NaCl, 3 mM dithiothreitol, 0.2 A_{260} poly (rA): oligo $(dT)_{[12-18]}$, .045% Triton X-100, 9.1 mM magnesium acetate, 12 mM ATP, and 2.0 x 10^{-5} M ^{3}H-TTP (15 C/mmole) in a final volume of 33 μl. A 10 μl sample of virus was added and the mixture incubated at 37°C for 1 hour. The reaction was terminated by chilling in an ice bath,

adding 0.5 ml of 0.1 M Napyrophosphate, mixing, adding 0.05 ml of 5 mg/ml yeast RNA and finally precipitating with 0.5 ml of cold 10% trichloroacetic acid (TCA). After at least 10 minutes at 4°C, the samples were filtered through a Millipore filter (.45 μm) and washed 6 or 7 times with cold 5% TCA. Filters were then dried, 5 ml of toluene-based scintillation fluid added, and radioactivity measured on a Beckman LS-233 liquid scintillation counter. An unincubated reaction (0°C) containing less than 3% of the radioactivity was subtracted from all experimental values.

RESULTS

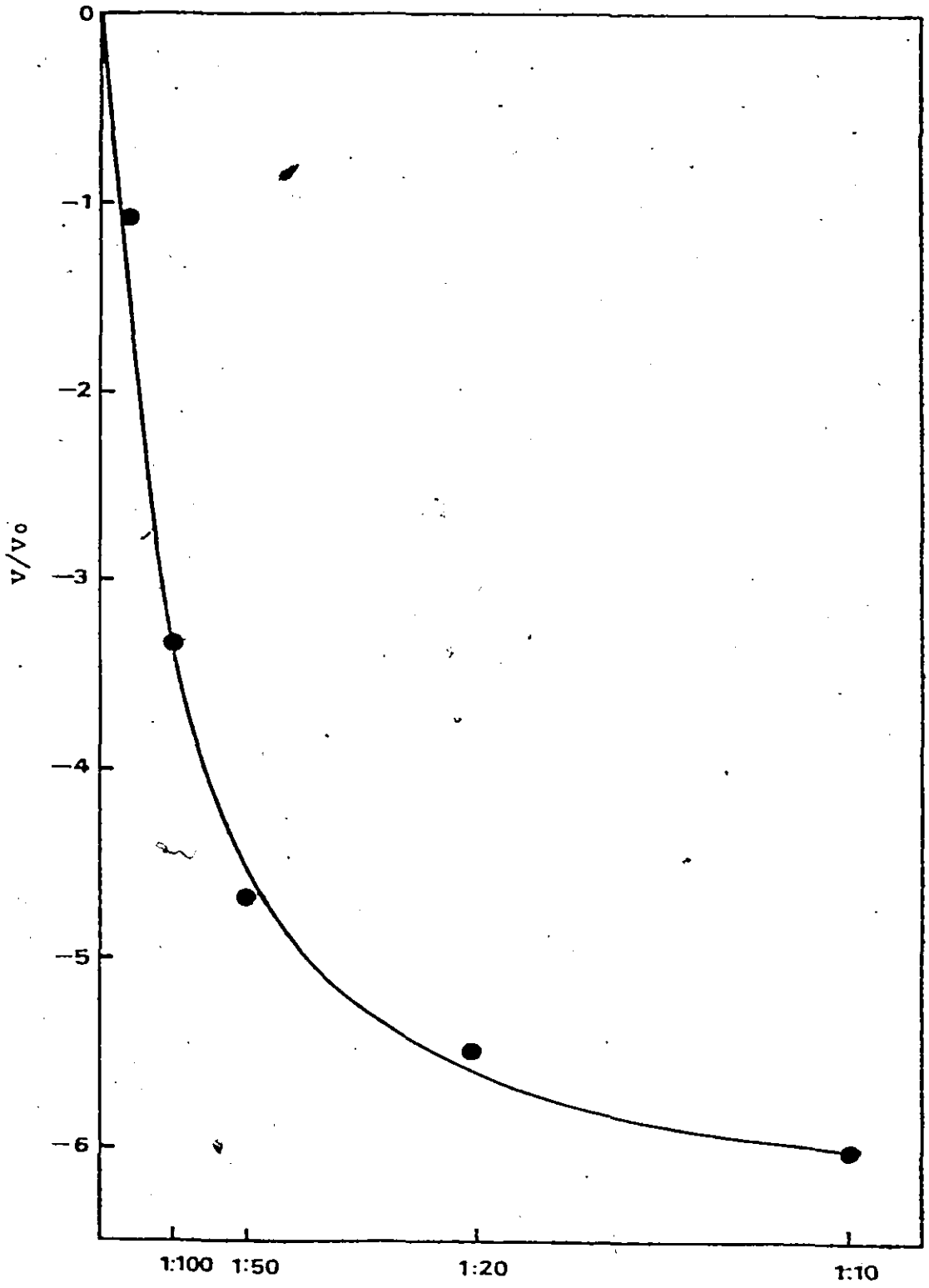
1. Virus Neutralizing Activity of Anti-VSV Serum

Growth of VSV in cells pre-infected with murine leukemia virus (MuLV) yields a certain proportion of progeny virus as VSV (MuLV) pseudotypes. As described in the Introduction, these particles can be separated from wild type VSV by selective neutralization with anti-VSV serum. To ensure that pseudotypes prepared in this manner are relatively free of wild type virus, it is first necessary to establish the percentage of normal VSV escaping neutralization by specific antiserum.

For this experiment, anti-VSV serum was prepared in rabbits as described in Methods. Virus-neutralizing activity was determined by adding a standard infectious titre of VSV to equal volumes of appropriately diluted anti-VSV serum. After incubating the mixtures for 30 minutes at 37°C, residual virus infectivity was determined by plaque assay on NIH/3T3 cells. As can be seen from the neutralization curve in Figure 1, a 1:10 dilution of antiserum reduced VSV

Figure 1: Virus neutralizing activity of anti-VSV serum

VSV grown in L cells was mixed with equal volumes of rabbit anti-VSV antiserum at the indicated dilutions. Neutralization was for 30 minutes at 37°C. Mixtures were then appropriately diluted and residual infectivity determined by plaque assay on NIH/3T3 cells. The log of the fraction of non-neutralized virus v/vo is plotted against antibody concentration.



Relative Ab. concentration

infectivity by 6 logarithmic units. This dilution was used to select VSV pseudotypes in all subsequent experiments.

2. VSV Pseudotype Production with Moloney Leukemia Virus and Temperature-Sensitive Mutant ts_3

A mutant of Moloney leukemia virus (MoLV), designated ts_3, was recently shown to have a temperature-sensitive step associated with the release of virus particles budding from the cell membrane (Wong and McCarter, 1974). In the following experiment, pseudotypes of VSV were prepared with ts_3 and wild type MoLV in an attempt to determine whether the temperature-sensitive defect affects VSV pseudotype formation.

Duplicate cultures of chronically infected TB cells were prepared in 16 oz Brockway bottles and incubated for at least 24 hours at 34°C (permissive) temperature) and 39°C (non-permissive temperature). To avoid possible leak of mutant virus, all incubations at the non-permissive temperature were carried out in a 39°C water bath. At near confluency, cultures were superinfected with VSV at an MOI of 10 PFU per cell and then maintained at the temperature of growth. VSV stock grown in TB cells was used as infecting virus (see Methods). After a total incubation of 18

hours, extracellular virus was collected and stored at -70°C. The level of VSV pseudotypes in each stock was determined by plaque titration on TB (sensitive) and TB-ts_3 (restrictive) cells both before and after neutralization with VSV antiserum. Virus appropriately diluted in PBS was used as a neutralization control.

The results are presented in Table 1. Stock VSV, grown in uninfected TB cells, was included in each assay as a control for virus-neutralizing activity. As indicated, VSV antiserum reduces the plaquing of control virus on TB cells to a greater extent (> 5 logs) than the plaquing of VSV grown in MoLV-infected cells (2.1 - 3.7 logs). This relative resistance reflects the presence of VSV pseudotypes, which, having acquired MoLV envelope antigens, are resistant to anti-VSV serum. In addition, these particles can be distinguished from wild type virus by their inability to plaque on murine leukemia virus infected cells (Zavada, 1972). This is shown in Table 1: prior to neutralization VSV stocks plaque equally well on both TB and TB-ts_3 cells, whereas after neutralization, pseudotypes in these stocks titre only on TB cells.

These results allow a comparison of VSV pseudotype production at 34°C and 39°C. As shown in Table 1, raising the temperature does not reduce the

Table 1

Assay of Moloney Leukemia Virus Pseudotypes of VSV Produced at 34°C and 39°C

Growth of VSV			Titration					
			TB cells			TB-ts_3 cells		
Host cell	Virus determining pseudotype	Temperature	Total PFU/ml	After anti-VSV	Log surviving fraction	Total PFU/ml	After anti-VSV	Log surviving fraction
TB-ts_3	ts_3	34°C	1.9×10^7	7.8×10^4	-2.39	2.1×10^7	2.0×10^2	-5.02
		39°C	2.2×10^7	4.0×10^3	-3.74	1.6×10^7	$<10^2$	<-5.20
Mol-TB	MoLV	34°C	1.7×10^7	1.3×10^5	-2.12	1.1×10^7	$<10^2$	<-5.04
		39°C	5.0×10^6	2.7×10^4	-2.27	6.5×10^6	$<10^2$	<-4.81
TB	-	37°C	1.8×10^7	$<10^2$	<-5.26	1.5×10^7	$<10^2$	<-5.18

TB cells, chronically infected with wild type MoLV or ts_3, were grown for 24-48 hours at 34°C or 39°C. At near confluency, cells were superinfected with VSV at an MOI of 10 and then maintained at the temperature of growth. After 18 hours, culture fluid was collected, cell and cell debris removed, and the supernatants frozen at -70°C. For neutralization, samples were thawed and 0.2 ml aliquots mixed with an equal volume of anti-VSV serum for 30 minutes at 37°C. Plaque assay was at 37°C. Briefly, plates were exposed to 0.1 ml of appropriately diluted virus for 45 minutes at 37°C, washed twice with PBS, and then overlayed with agar medium. Assay on TB and TB-ts_3 cells was done on the same day. Stock virus grown in uninfected TB cells was included as a control for VSV-neutralizing activity.

level of phenotypic mixing between VSV and wild type MoLV. In contrast, the level of VSV pseudotypes formed with ts_3 is reduced 20-fold at the higher temperature. VSV stocks grown in the presence of ts_3 at 34 °C and 39 °C contained 0.4% and 0.02% VSV pseudotypes respectively. This result indicates that phenotypic mixing between VSV and ts_3 is a temperature-dependent process.

3. Virus Production of ts_3 at the Permissive and Non-Permissive Temperature

In the following experiment, ts_3 virus production was examined at the permissive and non-permissive temperature. TB cells were infected with ts_3 at a multiplicity of approximately 20 PFU per cell. After 8 days, chronically infected cultures were prepared in 16 oz Brockway bottles and incubated at 34 °C and 39 °C. At time zero, culture fluid was removed and fresh growth medium added to the cells. After an additional 24 hours of incubation, samples were taken from near-confluent cultures and titred at 37 °C by XC plaque assay. FLV grown in NIH/3T3 cells at 39 °C was included as a control.

The results (Table 2) indicate that the titre of ts_3 is approximately 200-fold higher following 24 hours growth at the lower temperature. Similar results have been reported by Wong and McCarter (1974).

Table 2

Virus Production of ts_3 at 34°C and 39°C

Virus	Titre	
	34°C	39°C
ts_3	2.8×10^6	1.2×10^4
FLV	NA†	3.4×10^6

TB cells, infected with ts_3 at an MOI of 20 PFU per cell, were cultured for 8 days at 37°C. Cultures of 5×10^6 cells were then prepared and incubated at 34°C and 39°C. Culture fluid was removed after 1 day; fresh medium was added and incubated for 24 hours, then harvested and assayed at 37°C. Supernatant medium from NIH/3T3 cells chronically infected with FLV was harvested after 24 hours at 39°C.

† NA, not assayed.

Accordingly, it was reasoned that the temperature-dependence of VSV (ts_3) pseudotype production results from a temperature-sensitive step associated with ts_3 virus replication.

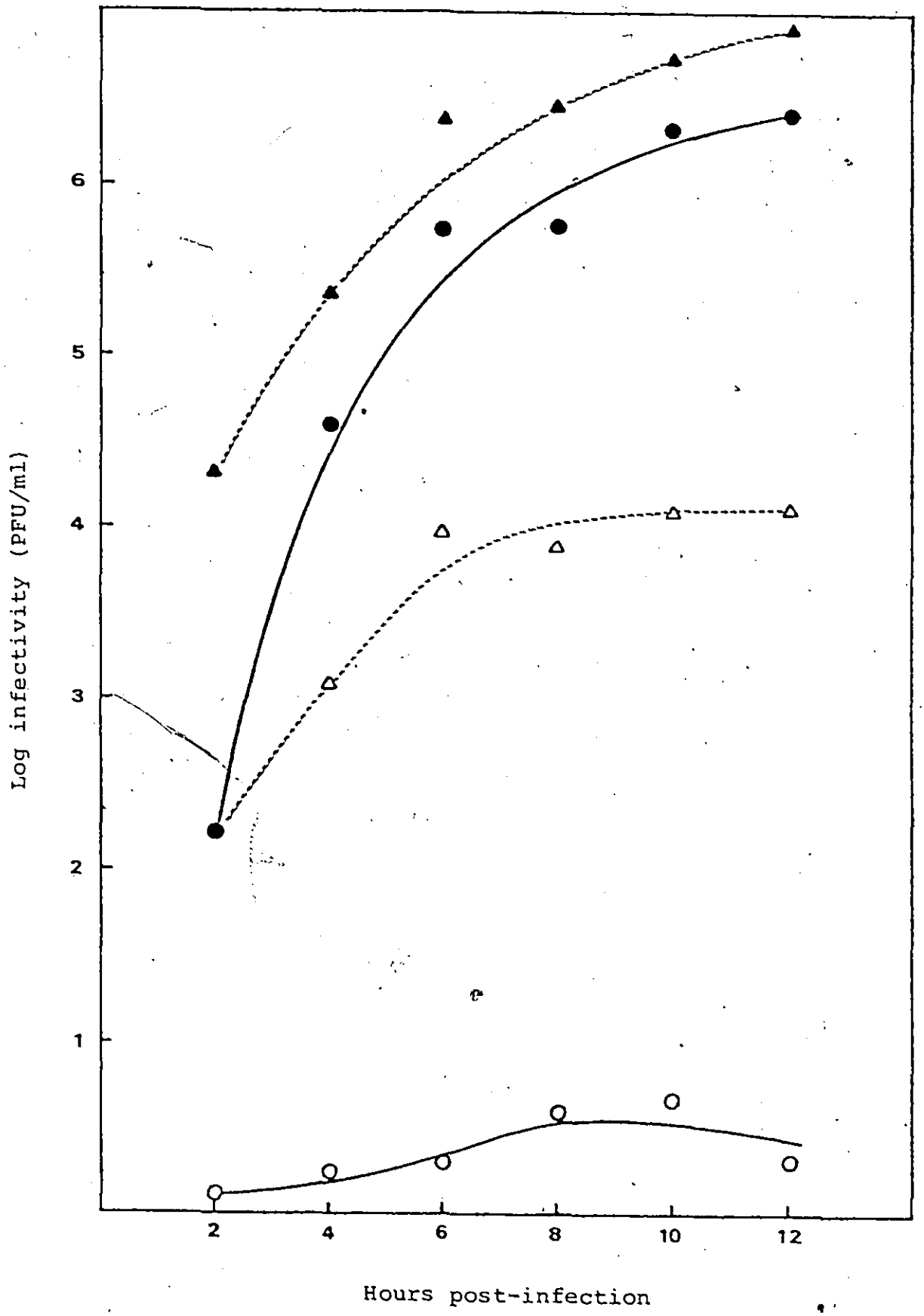
4. Kinetics of Pseudotype Formation Between VSV and ts_3

To study the kinetics of VSV (ts_3) pseudotype formation, a single-step growth curve of VSV was determined in TB cells chronically infected with ts_3 virus. Petri dish cultures, incubated for 24 hours at 34°C and 39°C, were superinfected with VSV at an MOI of 10 PFU per cell and then maintained at the temperature of growth. Samples were collected at 2 hour intervals post-VSV infection and VSV (ts_3) pseudotype levels determined by plaque assay on NIH/3T3 cells as previously described.

As shown in Figure 2, VSV growth at either temperature is marked by an exponential increase early in the replicative cycle. Release of infectious virus then declines, presumably as a consequence of cell death, and a plateau is reached at 10-12 hours post-infection. Although similar growth rates are observed, slightly higher yields are produced at the lower temperature. Neutralization of time samples produced at 34°C reveals a significant population of VSV (ts_3)

Figure 2: Kinetics of phenotypic mixing between VSV and ts_3 at the permissive and non-permissive temperature

Petri dish cultures of TB cells, pre-infected with ts_3, were incubated for 24 hours at 34°C and 39°C and then superinfected with VSV (MOI 10) and maintained at the temperature of growth. Samples were collected at 2 hour intervals post-infection and the level of VSV (ts_3) pseudotypes determined by plaque titration on NIH/3T3 cells both before and after neutralization with VSV antiserum. Infectivity before, 34°C (▲), 39°C (●) and after neutralization 34°C (Δ), 39°C (o).


Log infectivity (PFU/ml)
1
2
3
4
5
6
2
4
6
8
10
12
Hours post-infection

pseudotypes, whereas virus grown at 39°C contains only a minor component of non-neutralized virus, probably reflecting the limit of VSV-neutralizing activity. As indicated, the kinetics of pseudotype production closely corresponds to the growth of total virus. However, the appearance of a plateau as early as 6 hours post-infection as well as a decrease in the proportion of VSV (ts_3) pseudotypes in the total yield, suggests that either phenotypically mixed virions possess a reduced stability or that a slower rate of turnover exists for the envelope proteins of MoLV relative to VSV. An alternative explanation would be that at 6 hours post-VSV infection, a VSV-induced shut-off of further MoLV synthesis occurs.

5. Pseudotype Formation Between VSV and ts_3 Following Temperature Shift-Down

In order to determine the time of expression of the temperature-sensitive step, VSV (ts_3) pseudotype production was investigated following temperature shift from 39°C to 34°C. Cultures of TB-ts_3 cells were incubated for at least 24 hours at the two temperatures and then infected with VSV as previously described. As most phenotypic mixing between VSV and ts_3 occurs within 6 hours of VSV infection, 2-4

hours before the completion of VSV production (Figure 2), this time was selected for temperature shift. Accordingly, at 6 hours post-VSV infection, representative cultures were shifted from 39°C to 34°C and virus harvested after an additional 2-4 hours incubation.

The results are shown in Table 3 and Figure 3. Pseudotypes were determined by their acquired characteristics, resistance to anti-VSV serum and restricted host range. VSV grown in uninfected TB cells reflects the background level of wild type virus escaping neutralization. Consistent with earlier studies, the level of VSV (ts_3) pseudotypes produced at 39°C is significantly lower than at 34°C. In addition, no increase in this level occurs during the 4 hours following temperature shift. These results suggest that an irreversible temperature-sensitive step occurs before the process of phenotypic mixing, as VSV (ts_3) pseudotypes are not released upon temperature shift-down.

6. Virus Production of ts_3 Following Temperature Shift-Down

As an irreversible block to VSV (ts_3) pseudotype production was found to occur, an assay of virion-associated reverse transcriptase was used to study

Table 3

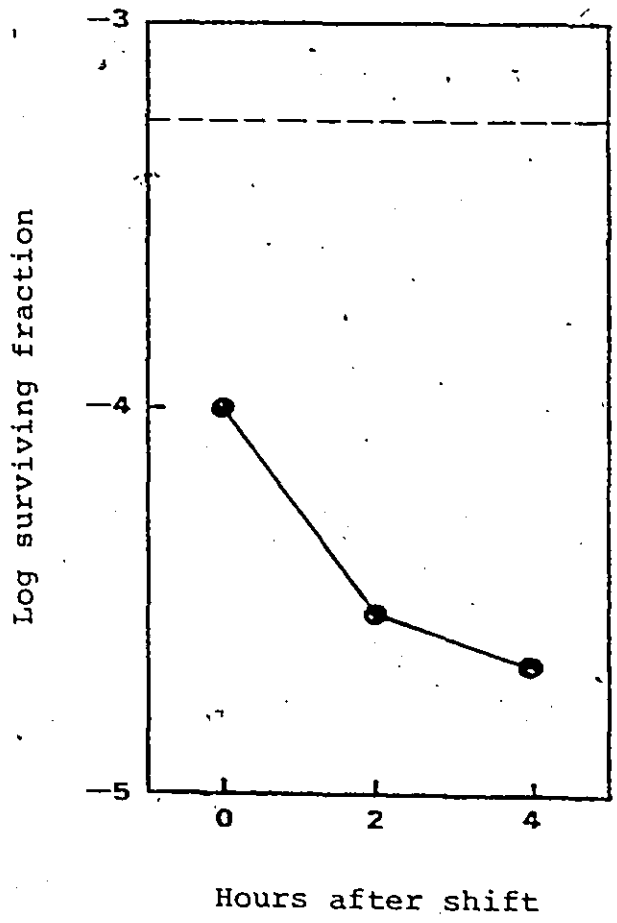
Pseudotype Formation Between VSV and ts_3 Following Temperature Shift-Down

Growth of VSV			Titration					
			TB cells			$TB\text{-}ts_3$ cells		
Host cell	Hours at 39°C	Hours at 34°C	Total PFU/ml	After anti-VSV	Log surviving fraction	Total PFU/ml	After anti-VSV	Log surviving fraction
$TB\text{-}ts_3$	-	6	1.1×10^8	6.2×10^4	-3.25	1.7×10^8	$<10^2$	<-6.23
	6	-	2.0×10^7	2.0×10^3	-4.00	2.1×10^7	$<10^2$	<-5.32
	6	2	5.2×10^7	1.5×10^3	-4.54	4.9×10^7	1.5×10^2	-5.51
	6	4	5.3×10^7	1.1×10^3	-4.68	5.4×10^7	$<10^2$	<-5.73
TB	(at 37°C)		2.5×10^8	2.2×10^3	-5.06	4.0×10^8	2.1×10^3	-5.28

Near confluent cultures of $TB\text{-}ts_3$ cells, pre-incubated for 24 hours at 39°C, were superinfected with VSV at an MOI of 10 PFU per cell. At 6 hours post-infection, cultures were shifted to 34°C and samples collected at the time of shift and at 2 and 4 hours after shift. A parallel culture, pre-incubated at 34°C was superinfected for 6 hours with VSV to indicate the control level of VSV (ts_3) pseudotype production at the permissive temperature. Stock virus grown in uninfected TB cells provides a control for VSV-neutralizing activity. Plaque assay was at 37°C.

Figure 3: Irreversible temperature-sensitive block to VSV (ts_3) pseudotype production

Pseudotype levels were determined as indicated in Table 3. Control level of VSV (ts_3) pseudotype production at the permissive temperature (---). VSV (ts_3) pseudotype production following temperature shift-down at 6 hours post-VSV infection (●).


−3
−4
−5
Log surviving fraction
0
2
4
Hours after shift

the production of ts_3 following a shift from the non-permissive to the permissive temperature. Chronically infected TB-ts_3 cells were prepared in 16 oz Brockway bottles and incubated for 24 hours at 39°C. The culture medium was then changed and the cells incubated for an additional 2 hours at 39°C. After this time, extracellular virus was harvested, fresh medium added, and the cells incubated for 2 hours at 34°C to allow collection of virus at the permissive temperature. To determine whether cycloheximide inhibits the production of virus following temperature shift-down, fresh medium containing cycloheximide (40 μg/ml) was added to a set of parallel cultures 30 minutes before transfer from 39°C to 34°C. Harvests were cleared of cellular material and virus pelleted by centrifugation in a SW27 rotor for 75 minutes at 24,000 rpm at 5°C. Virus was then resuspended in 0.2 ml of PBS and the reverse transcriptase activity determined using 10 μl samples as described in Methods.

The results of duplicate samples are presented in Table 4. As indicated, a large increase in virion-associated reverse transcriptase activity is observed within 2 hours of shift-down to the permissive temperature. Moreover, cycloheximide is shown to have very little effect at reducing this increase. These results agree with earlier studies by Wong and

Table 4

Release of Virion-Associated Reverse Transcriptase Activity Following Temperature Shift-Down

Time of incubation	Temperature	Cycloheximide †	Incorporation of ^{3}H-TTP (cpm)
2	39°C	–	7,528
	–	–	7,916
	34°C	–	68,062
		–	71,639
		+	37,914
		+	42,107

Cultures of chronically infected TB-ts$_3$ cells were incubated for 24 hours at 39°C. Culture fluid was then changed; fresh medium was added and incubated for an additional 2 hours at 39°C, then harvested and reverse transcriptase activity determined. Cultures were then transferred to 34°C, fresh medium added, and the release of reverse transcriptase activity determined after an additional 2 hours of incubation at the permissive temperature. The results of duplicate samples are presented.

† Cycloheximide (40 μg/ml) was added 30 minutes prior to shift-down.

McCarter (1974) who used a modification of the XC assay to show that TB cells, infected with ts_3 and incubated for 42 hours at 39°C, release large quantities of infectious virus following a transfer to 32°C. Cycloheximide was reported to have little effect on this release for the first hour after down-shift. Accordingly, the above results indicate that the temperature-sensitive defect occurs after protein synthesis. However, this finding does not exclude the possibility that the defect is a protein in a reversibly inactivated configuration (Wong and McCarter, 1974).

7. Effect of VSV on Virus Production of ts_3 Following Temperature Shift-Down

As previously shown (Table 3; Figure 3), ts_3-infected TB cells, pre-incubated for 24 hours at 39°C and superinfected with VSV, exhibit an irreversible block to VSV (ts_3) pseudotype production. After 6 hours of VSV infection, a temperature shift from 39°C to 34°C fails to restore VSV (ts_3) pseudotype production. In the following experiment, an assay of virion-associated reverse transcriptase activity was used to determine whether VSV-infected cells release ts_3 following temperature shift-down. Cultures of TB-ts_3

cells, pre-incubated for 24 hours at 39°C, were superinfected with VSV at an MOI of 10 PFU per cell. At 6 hours post-infection, the cultures were shifted back to 34°C and virus harvested after an additional 2-4 hours incubation. Parallel cultures, pre-incubated at 34°C, were superinfected with VSV at 34°C to provide a control for ts_3 production in the presence of VSV.

The results of two such experiments are presented in Table 5. As shown, no increase in reverse transcriptase activity is observed following the transfer of VSV-infected cells from the non-permissive to the permissive temperature. In addition, the results indicate that VSV does not prevent the release of ts_3 upon superinfection at the permissive temperature.

8. Heat Inactivation of Reverse Transcriptase Activity

It seemed possible that pre-incubated TB-ts_3 cells superinfected with VSV release ts_3 following a transfer from 39°C to 34°C, but that the virion-associated enzyme is subsequently inactivated. Accordingly, the following experiment was carried out in order to determine the thermal inactivation of reverse transcriptase activity following incubation of ts_3 at 34°C and 39°C. Duplicate sets of TB-ts_3 cultures were prepared in 16 oz Brockway bottles and incubated for

Table 5

Effect of VSV on the Release of Virion-Associated Reverse Transcriptase Activity Following Temperature Shift-Down

Hours at		Incorporation of ^{3}H-TTP (cpm)	
39 °C	34 °C	Experiment 1	Experiment 2
6	–	8,922	10,186
6	2	4,188	13,392
6	4	2,769	10,131
–	6	45,852	23,924

Near confluent cultures of TB-ts$_3$ cells, pre-incubated for 24 hours at 39 °C, were superinfected with VSV at an MOI of 10 PFU per cell. At 6 hours post-infection, cultures were shifted to 34°C. Culture medium was collected for determination of reverse transcriptase activity at the time of shift and at 2 and 4 hours after shift. A parallel culture, pre-incubated for 24 hours at 34°C, was included to provide a control for the release of virion-associated reverse transcriptase upon superinfection with VSV at the permissive temperature.

24 hours at 34°C. After this time, the medium was changed and one set superinfected with VSV at an MOI of 10 PFU per cell. At 6 hours post-infection, culture medium was harvested from both uninfected and VSV-infected cultures and the virus samples pelleted in an SW27 rotor for 75 minutes at 24,000 rpm at 5°C. Virus pellets were then resuspended in 0.8 ml of PBS and individual aliquots incubated for 2 and 4 hours at 34°C and 39°C. Control levels of reverse transcriptase activity were provided by incubation at 0°C.

The results of two separate assays (Table 6) indicate that no loss in reverse transcriptase activity occurs following 4 hours of incubation of ts_3 at 34°C. In contrast, a 3-4 fold reduction in activity occurs after 2 hours of incubation of ts_3 at 39°C. However, the assay procedure used here is too insensitive to detect any additional inactivation resulting from 4 hours of incubation at 39°C. Furthermore, Table 6 shows that ts_3 produced and incubated in the presence of VSV possesses the same kinetics of reverse transcriptase heat inactivation. It would, therefore, appear that pre-incubated cells, superinfected for 6 hours with VSV, fail to release ts_3 upon temperature shift-down.

Table 6

Heat Inactivation of Reverse Transcriptase Activity

Sample	Hours of Incubation	Temperature	Incorporation of ^{3}H-TTP (cpm)	
			Assay 1	Assay 2
ts_3	2	0°C	10,664	8,011
		34°C	13,289	8,902
		39°C	4,383	3,445
	4	0°C	14,753	11,565
		34°C	14,504	9,527
		39°C	3,146	1,935
ts_3 + VSV	2	0°C	40,144	21,551
		34°C	36,344	22,432
		39°C	9,969	5,357
	4	0°C	55,135	27,702
		34°C	35,529	21,658
		39°C	13,124	4,175

Duplicate sets of TB-ts_3 cultures were incubated for 24 hours at 34°C. The culture medium was then changed and one set superinfected with VSV at an MOI of 10 PFU per cell. At 6 hours post-infection, culture medium was harvested from both uninfected and VSV-infected cells and the virus samples pelleted in a SW27 rotor for 75 minutes at 24,000 rpm at 5°C. Virus pellets were resuspended in 0.8 ml of PBS pH 7.5 and individual aliquots incubated for 2 and 4 hours at 34°C and 39°C. Control levels of reverse transcriptase activity were provided by incubation at 0°C.

9. Pseudotype Formation Between VSV and ts_3 Following Temperature Shift-Down at Different Times Post-VSV Infection

To further define the temperature-sensitive step of pseudotype production, VSV (ts_3) pseudotype formation was examined by temperature shift-down at different times post-VSV infection. Cultures of TB-ts_3 cells, pre-incubated for 24 hours at 39°C, were superinfected with VSV at an MOI of 10 PFU per cell. A parallel culture, pre-incubated at 34°C, was superinfected with VSV at 34°C to indicate the control level of VSV (ts_3) pseudotype production occurring at the permissive temperature. At times 0, 2 and 4 hours post-infection, representative cultures were shifted from 39°C to 34°C. At 6 hours post-infection, all samples were collected and pseudotype levels determined.

The pseudotype levels are presented in Table 7. As illustrated in Figure 4, the level of VSV (ts_3) pseudotypes formed progressively increases with the length of time the infected cells are incubated at 34°C. However, using the control level as a reference, a partial block to VSV (ts_3) pseudotype formation can be detected even when VSV infection occurs entirely at 34°C (shift-down at time zero). It would, therefore, appear that the block to VSV (ts_3) pseudotype formation

Table 7

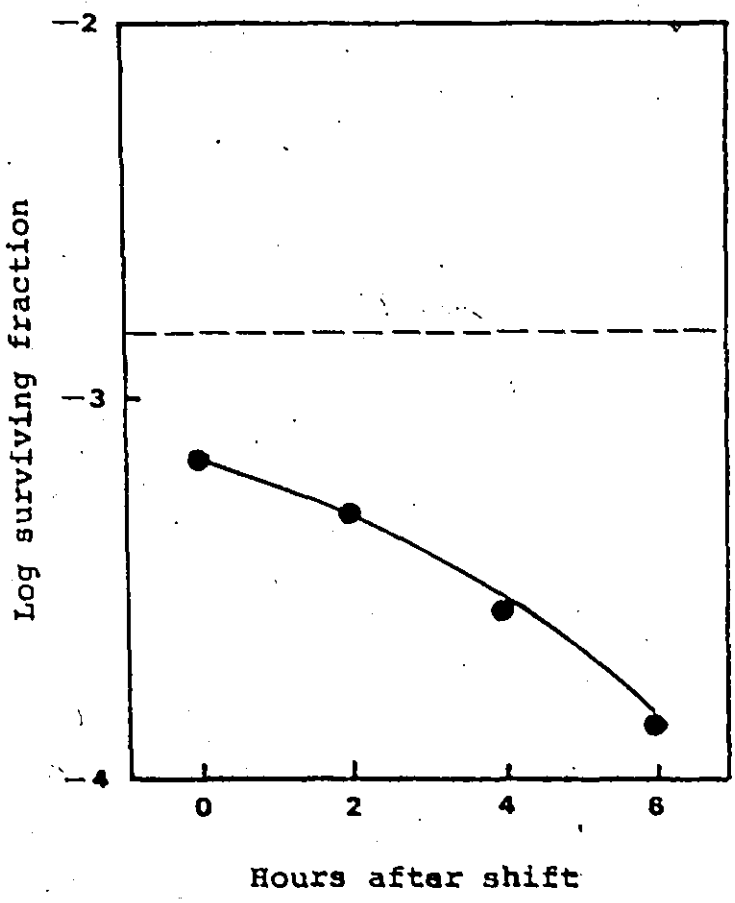
Pseudotype Formation Between VSV and ts_3 Following Temperature Shift-Down at Different Times Post-VSV Infection

Growth of VSV		Titration					
		TB cells			$TB-ts_3$ cells		
Host cell	Time of shift-down post-VSV infection (hrs)	Total PFU/ml	After anti-VSV	Log surviving fraction	Total PFU/ml	After anti-VSV	Log surviving fraction
$TB-ts_3$	0	5.6×10^7	4.1×10^4	-3.14	1.4×10^8	$<10^2$	<-6.15
	2	3.9×10^7	2.0×10^4	-3.29	7.3×10^7	$<10^2$	<-5.86
	4	1.6×10^7	4.5×10^3	-3.55	4.9×10^7	$<10^2$	<-5.69
	6	1.4×10^7	2.0×10^3	-3.85	1.7×10^7	$<10^2$	<-5.23
	(34° Control)	3.8×10^7	5.7×10^4	-2.82	1.1×10^8	$<10^2$	<-6.04
TB	(37° Control)	1.6×10^8	7.0×10^2	-5.35	2.8×10^8	1.2×10^3	-5.37

Near confluent cultures of $TB-ts_3$ cells, pre-incubated for 24 hours at 39°C, were superinfected with VSV at an MOI of 10 PFU per cell. At times 0, 2 and 4 hours post-infection, representative cultures were shifted from 39°C to 34°C. At 6 hours post-infection, all samples were collected and VSV (ts_3) pseudotype levels determined. A parallel culture, pre-incubated at 34°C, was superinfected with VSV for 6 hours to indicate the control level of VSV (ts_3) pseudotype production at the permissive temperature. Stock virus grown in uninfected TB cells was included as a control for VSV-neutralizing activity. Plaque assay was at 37°C.

Figure 4: Temperature shift down at different times post VSV-infection

Pseudotype levels were determined as indicated in Table 1. Control level of VSV (ts_3) pseudotype production at the permissive temperature (---). VSV (ts_3) pseudotype production following temperature shift-down at different times post-VSV infection (●).


−2
−3
−4
Log surviving fraction
0
2
4
6
Hours after shift

occurs during the 24 hours of pre-incubation at 39°C. Moreover, the above result suggests that the block can be overcome by a shift to the permissive temperature a few hours in advance of VSV infection.

10. Pseudotype Formation Between VSV and ts_3 Following Temperature Shift-Up/Shift-Down

The previous study suggests that pre-incubating the cultures for 24 hours at 39°C, introduces a temperature-sensitive block to VSV (ts_3) pseudotype formation. The effect of this block is still detected 6 hours after shift-down to the permissive temperature (Figure 4). In the following study, a temperature shift-up/shift-down experiment was carried out in order to determine the effect of a relatively brief incubation at the non-permissive temperature. Cultures of TB-ts_3 cells, preincubated for 24 hours at 34°C, were superinfected with VSV at a multiplicity of 10 PFU per cell and then transferred to 39°C. At 6 hours post-infection, the cultures were shifted back to 34°C and virus harvested after an additional 2-4 hours incubation. A control level of pseudotype production was provided by 6 hours of VSV infection without shift to the non-permissive temperature.

As can be observed (Table 8; Figure 5a), a 6 hour incubation at 39 °C, coincident with VSV infection, is sufficient to introduce a temperature-sensitive block to VSV (ts_3) pseudotype production. However, this block does not prevent the release of pseudotypes following shift-down to the permissive temperature. Within 2 hours of shift-down (Figure 5a), the pseudotype level returns to 70% of the control level. After 4 hours at 34 °C, the level is equivalent to that produced at the permissive temperature. This rapid release suggests that a brief incubation at 39 °C introduces a reversible temperature-sensitive block acting very late in the production of VSV (ts_3) pseudotypes.

Wong and McCarter (1974) have shown that cycloheximide has very little effect on the release of ts_3 during the first hour following a shift from the non-permissive to the permissive temperature. In order to determine whether protein synthesis is required for the release described above, cycloheximide (40 μg/ml) was added to a set of VSV-infected cultures 30 minutes prior to shift down. Galet (1973) has shown that the addition of cycloheximide (20 μg/ml) to L cells 3 hours post-VSV infection causes an immediate inhibition of further VSV-specific RNA transcriptase activity.

As indicated (Table 9; Figure 5b), protein synthesis is not required for the production of VSV (ts_3)

Table 8

Pseudotype Formation Between VSV and ts_3 Following Temperature Shift-Up/Shift-Down

Growth of VSV			Titration					
			TB cells			$TB\text{-}ts_3$ cells		
Host cell	Hours at 39°C	Hours at 34°C	Total PFU/ml	After anti-VSV	Log surviving fraction	Total PFU/ml	After anti-VSV	Log surviving fraction
$TB\text{-}ts_3$	6	-	1.9×10^7	2.5×10^3	-3.88	4.5×10^6	$<10^2$	<-4.65
	6	2	1.3×10^7	7.7×10^3	-3.22	6.1×10^6	$<10^2$	<-4.78
	6	4	1.5×10^7	1.3×10^4	-3.07	6.7×10^6	$<10^2$	<-4.83
	-	6	1.8×10^7	1.6×10^4	-3.06	8.5×10^6	$<10^2$	<-4.93
TB	(At 37°C)		1.6×10^8	1.5×10^3	-5.02	6.4×10^7	$<10^2$	<-5.81

Near confluent cultures of $TB\text{-}ts_3$ cells, pre-incubated for 24 hours at 34°C, were superinfected with VSV at an MOI of 10 PFU per cell and then transferred to 39°C. At 6 hours post-infection cultures were shifted back to 34°C and samples collected at the time of shift and at 2 and 4 hours after shift. A control level of VSV (ts_3) pseudotype production was provided by 6 hours of VSV infection without transfer to the non-permissive temperature. Stock virus grown in uninfected TB cells was included as a control for VSV-neutralizing activity. Plaque assay was at 37°C.

Table 9

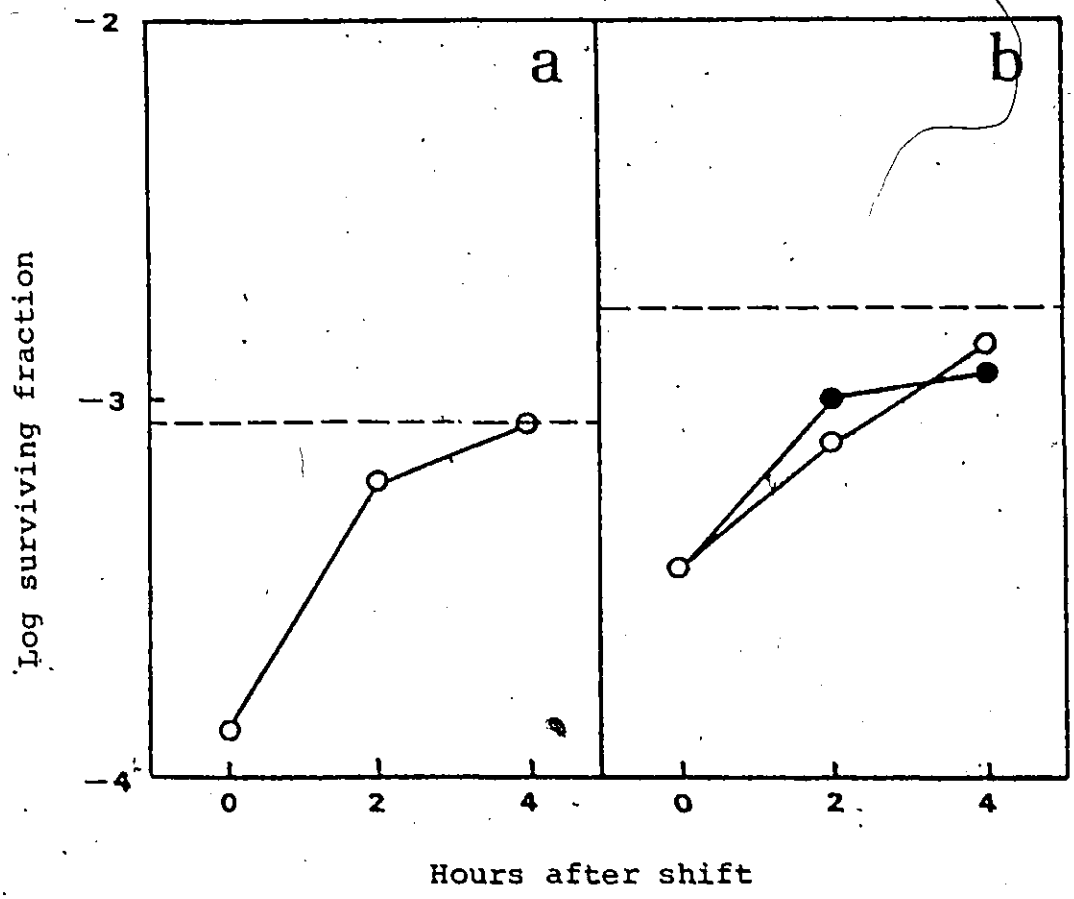
Effect of Cycloheximide on Pseudotype Formation Between VSV and ts_3 Following Temperature Shift-Up/Shift-Down

Host cell	Growth of VSV: Hours at 39°C	Hours at 34°C	CM†	Titration: TB cells: Total PFU/ml	After anti-VSV	Log surviving fraction	TB-ts_3 cells: Total PFU/ml	After anti-VSV	Log surviving fraction
TB-ts_3	6	-	-	3.8×10^7	1.4×10^4	-3.45	6.6×10^7	2.0×10^2	-5.52
	6	2	-	5.0×10^7	3.9×10^4	-3.11	7.5×10^7	1.0×10^2	-5.88
	6	2	+	3.2×10^7	3.2×10^4	-3.00	4.4×10^7	$<10^2$	<-5.64
	6	4	-	5.4×10^7	7.2×10^4	-2.87	8.3×10^7	1.5×10^2	-5.74
	6	4	+	5.7×10^7	6.8×10^4	-2.93	1.1×10^8	1.5×10^2	-5.86
	-	6	-	5.0×10^7	8.7×10^4	-2.76	9.8×10^7	3.0×10^2	-5.51
TB	(AT 37°C)		-	1.8×10^8	4.0×10^2	-5.66	3.2×10^8	1.5×10^2	-6.33

† Except for the addition of cycloheximide (40 μg/ml) 30 minutes prior to temperature shift-down, the experimental procedure is identical to that of Table 8.

Figure 5: Reversible temperature-sensitive block to VSV (ts_3) pseudotype production

Pseudotype levels were determined as indicated in Tables 8 and 9. Control levels of VSV (ts_3) pseudotype production at 34°C (-¬-). VSV (ts_3) pseudotype production following temperature shift-down in the presence (●) and absence (o) of cycloheximide (40 μg/ml).


a
b
−2
−3
−4
Log surviving fraction
0
2
4
0
2
4
Hours after shift

pseudotypes for at least the first 2 hours following shift-down. It would, therefore, appear that the temperature-sensitive defect resides in a pre-existing envelope component of ts_3. This mutation prevents the release of budding particles from the cell membranes (Wong and McCarter, 1974; Wong and MacLeod, 1975).

11. Effect of VSV on Virus Production of ts_3 at the Non-Permissive Temperature

As both VSV and ts_3 are enveloped RNA viruses which mature at the cell membrane, it seemed possible that a function of VSV replication might rescue ts_3 at the non-permissive temperature. Furthermore, since VSV (ts_3) pseudotypes have a temperature-sensitive step associated with the release of pre-formed particles (Figure 5), it was reasoned that the temperature-sensitive defect of ts_3 resides in the viral envelope. This finding suggested that VSV envelope components might rescue budding particles of ts_3 as ts_3 (VSV) pseudotypes.

To check this, duplicate sets of TB-ts_3 cultures were prepared in 16 oz Brockway bottles and incubated for 24 hours at 39°C. The medium was then changed and one set of cultures superinfected with VSV at an MOI of 10 or 100 PFU per cell. At 2 or 6

hours post-infection, medium was harvested from both sets of cultures. Fresh medium was then added to the uninfected cells and the cultures incubated for an additional 2 or 6 hours at 34°C. Virus harvested from these cells provided a control for the production of ts_3 at the permissive temperature. Virus samples were then pelleted and the release of ts_3 determined by assay of virion-associated reverse transcriptase activity.

The results of three such experiments (Table 10) indicate that superinfection with VSV does not release or rescue ts_3 from TB cells pre-incubated for 24 hours at the non-permissive temperature.

Table 10

Effect of VSV on the Release of Virion-Associated Reverse Transcriptase Activity at the Non-Permissive Temperature

Hours of incubation	Temperature	VSV-infected (MOI 10)	Incorporation of ^{3}H-TTP (cpm)		
			Expt. 1	Expt. 2	Expt. 3
2	39°C	-	5,666	9,268	7,528
		+	2,344	3,550†	6,827
	34°C	-	64,702	65,600	68,062
6	39°C	-	3,000		
		+	1,804	ND*	ND
	34°C	-	44,594		

Duplicate sets of TB-ts$_3$ cultures were incubated for 24 hours at 39°C. The medium was then changed and one set of cultures superinfected with VSV. After 2 or 6 hours post-infection, medium was harvested from both sets of cultures and the level of virion-associated reverse transcriptase activity determined. Fresh medium was then added to the uninfected cells and reverse transcriptase activity determined after an additional 2 or 6 hours incubation at 34°C.

† VSV-infected at MOI 100.

* ND, not done.

DISCUSSION

The studies presented in this thesis indicate that phenotypic mixing between VSV and ts_3 is a temperature-dependent process. Whereas similar levels of phenotypic mixing occur between VSV and wild type MoLV at both 39°C and 34°C, the level of pseudotypes formed between VSV and ts_3 is considerably lower at 39°C (non-permissive temperature) than at 34°C (permissive temperature). The results of temperature-shift experiments indicate that two separate blocks to VSV (ts_3) pseudotype production may occur depending on the length of time ts_3-infected cells are incubated at the non-permissive temperature.

Pre-incubation of ts_3-infected cells for 24 hours at 39°C, prior to superinfection with VSV, introduces an irreversible block to VSV (ts_3) pseudotype formation. Hence, after 6 hours of VSV infection, a temperature shift from 39°C to 34°C fails to restore VSV (ts_3) pseudotype production. Moreover, the temperature-sensitive block appears to form during the 24 hours of pre-incubation at 39°C: slightly depressed yields of pseudotypes are produced even when superinfection with VSV occurs entirely at the permissive temperature.

The most plausible explanation for this block would be that during the 24 hour pre-incubation at 39°C,

ts_3- specific membrane-associated protein normally available for phenotypic mixing with VSV is sequestered into budding virus particles which accumulate at, and fail to be released from, the cell membrane. This explanation would seem reasonable in light of recent scanning electron microscope studies of the ts_3 budding process. Wong and MacLeod (1975) have reported that the number of ts_3 virus particles observed at the cell membrane progressively increases from 8 to 48 hours after infection at 39°C. After 48 hours, when a maximum density of membrane-associated virus occurs, it was calculated that as much as 10% of the total cell surface is occupied by budding virus particles. As chronically infected cells have been used in the present study, it is probable that a maximum density of membrane-associated virus occurs within the 24 hours of pre-incubation at 39°C. Furthermore, the failure to observe a release of VSV (ts_3) pseudotypes following temperature shift-down implies that after 24 hours of pre-incubation at 39°C, almost all ts_3 envelope protein present on the cell membrane is associated with budding virus particles. According to the observation of Wong and MacLeod (1975), this would suggest that approximately 10% of the cell membrane is modified by ts_3-directed membrane-associated protein. This allows the speculation that it is the amount of ts_3 envelope protein present on the cell

membrane which limits the number of budding particles that accumulate at the cell surface.

Assuming that the irreversible block does represent a sequestering of ts_3 envelope protein, ts_3- infected cells, pre-incubated for 24 hours at 39°C, should gradually become competent for VSV (ts_3) pseudotype formation following a shift to the permissive temperature. This would express itself through the rapid release of membrane-associated virus and the gradual appearance of 'available' ts_3 envelope protein at the cell membrane.

In this regard, an assay of virion-associated reverse transcriptase indicates that large quantities of ts_3 are released from chronically infected cells within 2 hours of a transfer to the permissive temperature. The finding that cycloheximide has very little effect at blocking this release agrees with the earlier report (Wong and McCarter, 1974) that cycloheximide does not significantly reduce the level of infectious virus released during the first hour after down-shift.

Following pre-incubation, the gradual appearance of 'available' ts_3 envelope protein is detected by VSV (ts_3) pseudotype production following temperature shift-down at different times post-VSV infection. These results indicate that the amount of ts_3-directed membrane-associated protein available for VSV (ts_3) pseudotype formation progressively increases after a transfer to

the permissive temperature. Presumably, this new ts_3 envelope protein appears at the cell membrane in advance of assembled virus cores, otherwise an accumulation of viral cores might once again be expected to block VSV (ts_3) pseudotype production. In this regard, new ts_3 envelope protein probably begins to form on the cell membrane immediately following the release of membrane-associated virus as the level of VSV (ts_3) pseudotype production is observed to increase as early as 2 hours after temperature shift-down. Moreover, by a comparison of the levels of VSV (ts_3) pseudotype production, it appears that within 6 hours of a transfer to the permissive temperature, ts_3-infected cells, pre-incubated for 24 hours at 39°C, contain almost as much membrane-associated ts_3 envelope protein as do control cells chronically infected with ts_3 at the permissive temperature.

Of particular interest is the finding that ts_3-infected cells, pre-incubated for 24 hours at 39°C, release high levels of reverse transcriptase following temperature shift-down, whereas pre-incubated cells superinfected for 6 hours with VSV do not. At present, an explanation for this result is lacking. However, several possibilities have been examined. VSV, at the multiplicity used in the present study (MOI 10), does not inhibit the release of ts_3 upon superinfection at the permissive temperature. In addition, the possibility

that ts_3 is released but that the virion-associated enzyme is subsequently inactivated has been ruled out by thermal inactivation studies. However, this does not exclude the possibility that the virion-associated enzyme is inactivated prior to virus release. Heat inactivation studies indicate that a 3-4 fold reduction in reverse transcriptase activity occurs within 2 hours of incubation at 39°C. Accordingly, it may well be possible that a cytopathic effect induced by VSV renders the membrane-associated virus susceptible to heat inactivation prior to temperature shift-down and subsequent virus release. Further studies involving the use of radiolabels should be able to distinguish between these two possibilities.

In contrast to the irreversible block, a relatively brief incubation of ts_3- infected cells at 39°C introduces a reversible block to VSV (ts_3) pseudotype production: a 6 hour incubation at 39°C coincident with VSV infection blocks VSV (ts_3) pseudotype production. A subsequent transfer to 34°C results in the rapid release of large quantities of VSV (ts_3) pseudotypes. An attempt to block this release with cycloheximide indicates that protein synthesis is not required for VSV (ts_3) pseudotype production for at least the first 2 hours following down-shift. Accordingly, prior to temperature shift down, large amounts of ts_3 envelope protein are available for

VSV (ts_3) pseudotype formation. However, the finding that VSV (ts_3) pseudotypes fail to be released at 39°C suggests that an envelope component of ts_3, presumably present in a reversibly inactivated configuration, is defective for the release of associated virus. This finding is particularly interesting because it indicates that an envelope component of a murine leukemia virus is capable of triggering the release of a structurally dissimilar virus. Moreover, it would appear that the temperature-sensitive defect is expressed only during virus release: ts_3 is capable of adsorbing to and penetrating susceptible cells at the non-permissive temperature (Wong *et al.*, 1973).

At present, the identity of the defective component remains unclear. Pseudotypes of VSV have been shown to acquire the neutralization and viral interference specificities of a coinfecting murine leukemia virus (Zavada, 1972). Since murine leukemia virus (MuLV) envelope glycoprotein gp 69/71 has the capacity to interfere with MuLV infection (Hunsmann *et al.*, 1974) and to induce neutralizing antibody (Strand *et al.*, 1974), it would seem probable that VSV (MuLV) pseudotypes contain at least MuLV glycoproteins gp 69/71. Whether or not VSV (MuLV) pseudotypes contain additional MuLV proteins remains to be determined.

As the temperature-sensitive defect of ts_3 appears to reside in the viral envelope, an attempt was made to

see whether VSV complements ts_3 through ts_3 (VSV) pseudotype production at the non-permissive temperature. Failure to observe complementation is not surprising: ts_3 -infected cells were pre-incubated for 24 hours at 39° C prior to superinfection with VSV. As previously discussed, pre-incubation results in the accumulation of a maximum number of budding virus particles at the cell membrane. Since these particles have already begun the viral budding process, it seems highly unlikely that the particles should subsequently associate with VSV envelope protein present at the cell membrane.

The present studies have demonstrated the effectiveness of using VSV pseudotype production as a method for studying the properties of MuLV envelope components. In addition to acquiring the neutralization, host range and interference specificities of a coinfecting MuLV, it has been shown that VSV (MuLV) pseudotypes acquire an envelope component required for the release of virus particles budding at the cell membrane. This component has been shown by Wong and McCarter (1974) to be required for the rescue of a murine sarcoma virus (MuSV) from a non-producer MuSV-infected cell line.

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